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HAEMATOLOGIA

is an international quarterly publishing original papers on haematology. It also provides the reader with complex and up-to-date information on both research and clinical practice. A General Survey, an Open Forum, Book Reviews, Abstracts of more important papers from other periodicals and a Documentation of the well-known and the less accessible journals are to serve this purpose.

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HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

EDITOR-IN-CHIEF:
S. R. HOLLÁN

EDITOR:
I. BERNÁT

A special issue in honour of Susan R. Hollán upon the occasion of the 25th Anniversary of the National Institute of Haematology and Blood Transfusion

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Contents

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

IRON DEFICIENCY

IMMUNOLOGY

ABO, Rh and HL-A system

Humoral and cellular immune response

HAEMOTHERAPY

HEPATITIS B ASSOCIATED ANTIGEN

HAEMOSTASIS AND BLOOD COAGULATION

HAEMO-LYMPHOCYTOPOIESIS

MYELO- AND LYMPHOPROLIFERATIVE DISEASES

VARIA

An Appreciation

This issue of Haematologia is published in honour of Dr. Susan R. Hollán, Editor-in-Chief of the journal. She had launched Haematologia Hungarica, the predecessor of this journal, 15 years ago when she became the director of the Hungarian Blood Transfusion Service, which celebrates this year the 25th anniversary of its foundation.

Within these last 15 years Susan played a leading part in the development of haematology and blood transfusion in Hungary. It is given to few people what she undoubtedly possesses, to be an excellent clinician, an outstanding research worker, a remarkable teacher, and an attractive, charming and gentle human being to whom patients, associates and students are equally devoted. All who know Susan realize that she has a peculiar magic, a magic by which she has attracted most able coworkers, and stimulated, inspired, catalyzed and guided their work to build up the internationally recognized National Institute of Haematology and Blood Transfusion and also to fashion this journal to a publication of high reputation.

The contributions of many distinguished experts in haematology, immunology and blood transfusion from all parts of the world and the warmth of their replies to the invitation to contribute mark their affection and admiration for Susan, the result of which is this triple-expanded tributary volume. It is a pleasure to join my colleagues from Hungary and abroad in wishing Susan, my gifted and beloved student, good health, happiness and further success in her work.

17th September 1974

Dr. István Rusznyák
Professor of Medicine
President Emeritus of the
Hungarian Academy of Sciences

Short Biography of Susan R. Hollán

Born on October 26, 1920, Budapest, Hungary; married, two children.

Attended the Medical University of Pécs (Hungary), qualified in medicine with Sub Laurea Amae Matris; received M. D. in 1945 at Budapest University Medical School.

Intern at the Second Department of Medicine, Rókus Hospital, Budapest, 1945 to 1950.

Research Assistant at the First Department of Medicine, University Medical School, Budapest, 1950 to 1954.

Scientific associate since 1954 at the Institute for Experimental Medical Research, Hungarian Academy of Sciences.

Awarded degree in medical sciences on a thesis: Neurohumoral regulation of haemopoiesis, 1956.

Received D. Sc. in 1971, on a thesis: Haemoglobins and haemoglobinopathies.

Since 1959, Director of the National Institute of Haematology and Blood Transfusion, and head of the Clinical Department of Haematology.

Professor of Haematology, Postgraduate Medical School, 1970. Elected corresponding member of the Hungarian Academy of Sciences, 1973.

Main fields of research: Anaemia and trophic disturbances following nerve resection; infection and tumour growth; haemoglobin and haemoglobinopathies; structure and function of erythrocyte membrane; clinical haematology and blood transfusion.

Publications: 130 papers and several books.

Editor-in-Chief of the quarterly Haematologia since 1967 and of the Medical Encyclopaedia, published in 1967–1973.

Editorial Boards: Blut (München), Blood Cells (Berlin – Heidelberg), Folia Haematologica (Leipzig), Haematologica (Tortona), International Archives of Allergy and Applied Immunology (Basel), Orvosképzés (Budapest), Problemi Hematologii i Transfusii Krvi (Moscow), Revue Française de Transfusion (Paris), Transfusio (Budapest), Vox Sanguinis (Basel) .

Vice President of the International Society of Blood Transfusion, Counsellor of the International Society of Haematology, Vice President of MOTESZ (Federation of Hungarian Medical Societies), Honorary Member of the Purkinje Society, Chairman of the joint committee of the Hungarian Academy of Sciences for Human Genetics. Vice President of the Hungarian Society of Haematology, Honorary President of the Hungarian Society of Genetics.

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Contributed chapters to numerous handbooks on haematology and immunology.

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Les très longues rémissions complètes des leucémies aiguës

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Institut de Recherches sur les Maladies du Sang, Hôpital Saint-Louis, Paris, France

(Reçu le 5 octobre 1973)

Étude de 216 observations personnelles de leucémie aiguë dont la durée a dépassé 4 ans. Analyse du rôle de différents facteurs: âge, sexe, type cellulaire, thérapeutique initiale, durée de la thérapeutique.

La première rémission complète de leucémie aiguë a été obtenue en 1947 [5]. La première très longue rémission complète a été signalée dès 1950: l'évolution de la leucémie aiguë d'un danseur de l'Opéra de Paris, traité par d'abondantes transfusions, se prolongea 3 ans et 7 mois [7]. Pendant longtemps de tels documents demeurèrent exceptionnels. L'étude systématique de ces très longues rémissions a été entreprise après 1961 avec la description des leucémies aiguës "au long cours" [1] et la première enquête internationale [6].

Le présent travail est fondé sur l'analyse de 216 leucémies aiguës dont la durée dépasse 4 ans. Il est conduit avec l'espoir de tirer de cette analyse d'utiles leçons.

Caractères généraux

Nous avons retenu les observations répondant aux critères suivants:

Le diagnostic de leucémie aiguë et de sa variété cellulaire a été dans tous les cas porté ou confirmé dans notre laboratoire.

La durée de la rémission complète a dépassé 4 ans. Ces 216 cas se répartissent de la façon suivante:

Age	enfants	189
	adultes	27
Type cellulaire	granulocytaire	22
	lymphoblastique	194

Evolution

— leucémies aiguës granuleuses

morts entre la 4e et la 8e année après le diagnostic	6
vivants après une rechute	3
toujours en 1ère rémission complète	13

— leucémies aiguës lymphoblastiques
morts entre la 4e et la 8e année après le diagnostic 53
vivants après une ou plusieurs rechutes 36
toujours en 1ère rémission complète 105
dont anciens protocoles: 28
protocoles récents: 27

Facteurs influençant la durée de l'évolution

Ces facteurs peuvent être classés sous trois chefs:

Le malade

Le rôle de l'âge est capital. Il n'apparaît pas nettement pour les leucémies granuleuses en raison du petit nombre de longues rémissions. Il est évident pour les leucémies lymphoblastiques. Dans l'ensemble de notre série de longues rémissions, on compte 9 fois plus d'enfants que d'adultes alors qu'au départ la proportion d'enfants est de 60%. Le nombre élevé d'enfants en rémission après 4 ans est une satisfaction. La rareté persistante des longues rémissions d'adultes même pour les leucémies lymphoblastiques est très décevante.

Si le taux des premières rémissions est plus élevé dans le sexe féminin, la durée de la rémission, le nombre des longues rémissions sont les mêmes dans les deux sexes.

La maladie

1° *D'emblée, type cellulaire.* La fréquence des longues rémissions complètes dépend pour une large part du type cellulaire. Les longues rémissions sont très rares dans les leucémies aiguës granuleuses. Nous comptons 22 longues rémissions de leucémies granuleuses pour 194 longues rémissions de leucémies aiguës lymphoblastiques. Parmi les formes granuleuses, les leucémies promyélocytaires tiennent une place particulière en raison de leur remarquable sensibilité à la daunorubicine et des longues rémissions qui en sont les conséquences. Les longues rémissions sont donc relativement fréquentes dans les leucémies lymphoblastiques. Nous n'avons pu jusqu' à présent préciser la fréquence respective de ces longues rémissions dans les diverses sous-variétés éventuelles de ces formes lymphoblastiques.

Nombre de cellules. Les longues rémissions sont très rares dans les leucémies hyperleucocytaires très leucémiques au sens strict avec forte hypertrophie de la rate, du foie, des ganglions. Elles sont plus fréquentes dans les formes leucopéniques et lorsque les organes hématopoïétiques sont peu augmentés de volume.

2° *Pendant l'évolution; moment de la rechute.* Les meilleurs cas sont les cas sans rechute. Pour les cas avec rechute, le moment de la rechute est important. Les rechutes tardives sont en règle graves, soit fatales, soit précédant une série de rechutes de plus en plus rapprochées et finalement rebelles. Les rechutes pré-

coces surviennent pendant la première année chez des malades dont le traitement initial n'avait pas été satisfaisant, dont le traitement ultérieur est correct. Ces rechutes précoces peuvent être suivies d'une très longue rémission avec une bonne chance de longue survie (10 cas dans notre série).

Méningites leucémiques. 44 des 194 longues rémissions de leucémie aiguë lymphoblastique ont été compliquées de méningites leucémiques. Les 44 méningites leucémiques ont eu l'évolution suivante: 17 morts, 8 rechutes hématologiques avec survie, 19 rémissions sans rechute (délai variant de quelques mois à 5 1/2 ans). La méningite leucémique a pu être la première manifestation de la rechute après 7 ans de rémission complète.

Très remarquable est un groupe de malades avec méningite leucémique précoce (pendant les deux premières années) et longue survie ultérieure (de 4 à 7 ans) sans incident. Aucun cas comparable n'est relevé lorsque la localisation méningée survient après la 3^{e} année; la méningite leucémique est alors presque toujours très grave avec une évolution fatale à brève échéance. Les longues survies après méningite précoce sont d'autant plus dignes d'intérêt (10 survies sur 13 patients atteints de méningites dans la première année de la rémission).

Le traitement

Deux grandes classes thérapeutiques peuvent être distinguées:

1° Dans une première classe peuvent être rangés les traitements très divers, souvent modérés, de durée variable, notés dans les observations anciennes.

2° A la deuxième classe appartiennent les thérapeutiques vigoureuses, prolongées, comportant essentiellement des réinductions systématiques que nous avons proposées les premiers en 1964 [9].

Les longues rémissions complètes de la première classe ont les caractères suivants:

1 – Elles ne paraissent pas directement liées au mode de traitement: le traitement a induit la rémission. Il est moins sûr qu'il soit responsable de la durée de la rémission.

2 – Elles sont très rares. Leur fréquence, qu'il n'est pas très facile d'apprécier, est estimée selon les documents assemblés par Burchenal ou Pierce à 0.8 à 1% à 5 ans [6].

3 – Elles sont rarement compliquées de rechute.

28 malades de notre série personnelle de leucémie aiguë lymphoblastique sont de cette sorte. La durée de la rémission complète a dépassé 8 ans pour 20 malades, 10 ans pour 12 malades. 8 1/2 ans après le diagnostic, 80% des patients sont vivants, en bon état et la courbe évolutive est horizontale. Une fille atteinte de leucémie lymphoblastique et traitée en 1956 a donné naissance 13 ans plus tard à un enfant normal. Une autre jeune fille a battu un important record de natation après 4 ans d'évolution et a gagné un concours hippique * la 7^{e} année. Un seul malade de ce groupe souffrit d'une méningite leucémique après 3 1/2 ans

de rémission. La rémission complète se poursuit actuellement 6 1/2 ans après l'atteinte méningée.

Les très longues rémissions complètes de la deuxième classe ont des caractères différents [8–11].

1° Elles paraissent bien les conséquences de ces traitements, leur nombre et leur durée augmentant avec l'amélioration des méthodes thérapeutiques (réinduction systématique, etc.).

2° Elles sont relativement fréquentes. Sur 296 malades traités par ces nouveaux protocoles, 75 ont survécu plus de 4 ans (25%) [4].

3° Elles sont moins stables que celles de la première classe. Les courbes de survie dans les différents protocoles de cette classe ne sont pas horizontales mais descendent lentement. Il paraît difficile chez des malades qui demeurent longtemps menacés par des rechutes, d'interrompre précocement le traitement. Plus précisément, il est probable que sont confondus dans cette classe deux sortes de patients: ceux pour lesquels la poursuite prolongée du traitement est nécessaire, ceux pour lesquels on pourrait après quelques années envisager l'arrêt du traitement. C'est un objet des études en cours de tenter de distinguer ces deux catégories.

Ainsi l'étude de ces très longues rémissions a fait connaître les faits suivants:

1° Elle établit l'existence de très longues rémissions maintenues pendant très longtemps, sans rechute. Certaines de ces très longues rémissions ne sont pas différentes d'une guérison. Le médecin qui entreprend le traitement d'une leucémie aiguë peut concevoir l'espoir d'une guérison. C'est là un évènement considérable, même si le taux des guérisons est encore très petit.

2° Elle assure le rôle important de deux facteurs: la forme cellulaire et la méthode thérapeutique [2].

3° Elle a confirmé le rôle essentiel de l'âge, mais n'a pu aller plus loin dans l'interprétation des facteurs personnels. Le rôle de l'âge est lui-même ambigu et l'on ne sait si n'intervient pas la fréquence particulière de formes cellulaires favorables ou par d'autres mécanismes.

Il est raisonnable de supposer à l'origine des très longues rémissions l'intervention associée de quatre facteurs: l'âge, la forme cellulaire, la méthode thérapeutique, un état particulier des défenses individuelles constitutionnel ou acquis. Il n'a pas été possible jusqu'à présent de définir cet état. Les études immunologiques poursuivies par Cannat et Séligmann [3] chez nos malades en longue rémission sont restées vaines.

Les recherches dont ces longues rémissions font actuellement l'objet s'orientent dans les deux directions suivantes:

1° Travaux de cinétique cellulaire dont l'objet est double: a) obtenir une meilleure coordination, voire une synchronisation des médicaments administrés tant à la période initiale que pendant la rémission, b) mieux comprendre la physiologie des cellules leucémiques pendant la rémission (cellules quiescentes?).

2° Travaux d'immunologie ou plus généralement d'écologie cellulaire, tentant de reconnaître les facteurs qui pendant la rémission influencent les cellules leucémiques, les éventuels agents étiologiques.

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Very Long Complete Remissions in Acute Leukaemia

A report of 216 own cases of acute leukaemia, all showing remissions of more than 4 years is presented. The influence of age, sex, cellular variety, the beginning of treatment and its duration are discussed.

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Chromosome Changes in the Blastic Transformation Stage of Chronic Granulocytic Leukaemia*

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A study of the bone marrow chromosomes of patients in the blastic transformation stage of chronic granulocytic leukaemia is reported, with particular reference to the appearance of chromosome abnormalities additional to the Philadelphia chromosome (Ph^1). Thirty patients were studied, of whom 28 yielded satisfactory chromosome preparations. In all 28 patients, chromosome abnormalities in addition to the Ph^1 were demonstrated, the most common being an increase in the number of chromosomes, usually due to either an extra C or extra Ph^1. It is considered that the appearance of additional abnormalities is a reliable indication of transformation. Clinical and haematological remission was induced in 11 patients and was asscciated with the disappearance of the additional chromosome abnormalities.

Introduction

Until fairly recently it was considered that once a patient with chronic granulocytic leukaemia manifested symptoms and signs of the blastic transformation stage (also known as metamorphosis) [1], the prognosis was extremely poor [14]. However, with the use of combination anti-leukaemic therapy remission is now not uncommon [2]. For this reason the early diagnosis of transformation may be of importance, as it is possible that earlier commencement of therapy may produce better results. Because the appearance of chromosome abnormalities additional to the Philadelphia chromosome (Ph^1) in the stage of blastic transformation has been described by a number of investigators [4, 5, 8, 11, 12, 16, 18] it was thought that cytogenetic studies of the marrow might provide diagnostic evidence of the development of this phase. This study was undertaken to investigate the incidence and nature of additional chromosome abnormalities together with changes occurring following treatment.

Patients and Methods

Over a period of 5 years, 30 consecutive patients in blastic transformation were studied; 19 were male and 11 female. The Philadelphia chromosome was present in the bone marrow cells of all patients (Ph^1 positive chronic granulocytic

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leukaemia). Twenty-six of the patients had been previously diagnosed in the chronic phase of the disorder, and in 8 cytogenetic studies had been performed in this phase. The majority had been treated with busulphan for varying periods of time; several had also received radiotherapy to the spleen. The average length of time in the chronic phase was 2.9 years with a range from 4 months to 7.5 years. Four patients presented for the first time in blastic transformation, 3 being thought at diagnosis to have acute myeloblastic leukaemia. Patients were referred from a number of different clinicians and in those treated for blastic transformation there was no uniform method of treatment.

Bone marrow chromosome preparations were made by a modification [7] of the direct method of Kiossoglou et al. [10]. Giemsa banding procedures were performed by the method of Sumner et al. [17].

Results

Of the 30 patients studied satisfactory chromosome preparations were obtained in twenty-eight. In the other 2 patients repeated marrow aspirates yielded preparations with many cells but no mitoses. All 28 patients were found to have additional chromosome abnormalities, 19 hyperdiploidy, 7 hypodiploidy and 2 pseudodiploidy (Table 1). The hyperdiploidy, with one exception, ranged from 47 to 53 chromosomes per cell, the majority containing 47; the exception was a markedly hyperdiploid increase with a mode varying from 75 to 85. In the hyperdiploid group the commonest additional chromosome was a member of the C group; the next most common was an extra Ph^1 chromosome (Table 2). In the hypodiploid group all cells contained 45 chromosomes and the missing chromosome was most often a C chromosome.

Table 1

Ploidy of bone marrow cells

	Model number of chromosomes per cell										Total number of patients
	45	46*	47	48	49	50	51	52	53	75–85	
Number of patients	7	2	13	1	1	—	2	—	1	1	28

* Pseudodiploid

In all of the 5 patients (Cases 25, 26, 27, 29, 30) in whom Giemsa banding was performed, the Ph^1 chromosome was shown to have been produced by a translocation between the long arms of a 22 chromosome and the long arms of a 9 chromosome as demonstrated by Rowley [13] (Fig. 1).

Table 2
Chromosome studies

Patient No.	Cell line in B.T.*	Cell line in remission	Length of remission (months)	Cell line in relapse
1	48,XX,Ph¹,C+,G+	—	—	—
2 +	47,XY,Ph¹,C+	—	—	—
3	47,XX,Ph¹,A+/48,XX,Ph¹,Ph¹,A+	46,XX,Ph¹	6	48,XX,Ph¹,Ph¹,A+
4	45,XX,Ph¹,C−	—	—	—
5 +	45,XY,Ph¹,Ph¹,C+,D− − −	—	—	—
6	47,XY,Ph¹,Ph¹	—	—	—
7 +	47,XX,Ph¹,C+	46,XX,Ph¹	7	47,XX,Ph¹,C+/77Ph¹Ph¹
8 +	51,XX,Ph¹,Ph¹,C++,D+,G+	46,XX,Ph¹	4	51,XX,Ph¹,Ph¹,C++,D+,G+
9	45,XY,Ph¹,G−	—	—	—
10	47,XX,Ph¹,C+	—	—	—
11	47,XY,Ph¹,Ph¹	—	—	—
12	47,XY,Ph¹,C++,E−	—	—	—
13	45,XY,Ph¹,C−/45,XY,Ph¹,D−	46,XY/46,XY,Ph¹	7	46,XY,Ph¹,Ph¹C−/45,XY,Ph¹,D−
14	47,XX,Ph¹,G+,C+	—	—	—
15	75−85,XY,Ph¹,Ph¹	46,XY/46,XY,Ph¹	5	75−85,XY,Ph¹,Ph¹,Ph¹
16	45,XY,Ph¹,C−	46,XY/46,XY,Ph¹	22	45,XY,Ph¹,C−
17	47,XY,Ph¹,E+	—	—	—
18	45,XY,Ph¹,Ph¹,C+,B−,D−,F−	—	—	—
19	45,XY,Ph¹,C−	—	—	—
20	47,XY,Ph¹,C+	46,XY,Ph¹	4	48,XY,Ph¹,C++
21	49,XY,Ph¹,Ph¹,C+,G+	—	—	—
22	47,XX,Ph¹,E++,C−	—	—	—
23 +	N.M.	N.M.	3	N.M.
24	47,XY,Ph¹,Ph¹	—	—	—
25 ++	47,XX,Ph¹,C++,E−	46,XX,Ph¹	6	—
26 ++	53,XY,Ph¹,Ph¹,Ph¹,C++++,F+	46,XY,Ph¹/45,XO, Ph¹	11	—
27 +	51,XY,Ph¹,Ph¹,C+,D++,E+	—	—	—
28 +	N.M.	—	—	—
29	46,XY,Ph¹,Ph¹,C−	46,XY,Ph¹	3	47,XY,Ph¹,Ph¹
30 +	46,XX,Ph¹,C+,E−	—	—	—

* B.T. = Blastic transformation
\+ Chromosome studies performed in chronic phase of C.G.L.
++ Alive

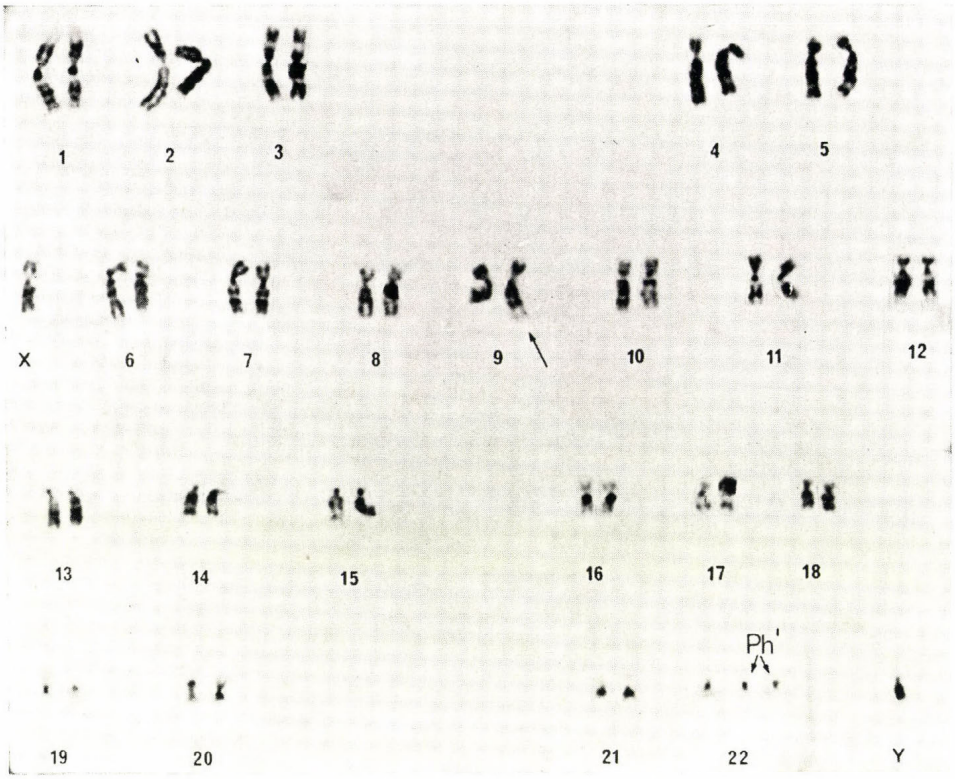

Fig. 1. Case 29. Giemsa banding of bone marrow chromosomes showing the translocation between chromosomes 22 and 9 to produce the Ph^1 chromosome

Patients in remission

In 11 patients clinical and haematological remission of the blastic transformation occurred following the use of combination anti-leukaemic therapy; these included the 4 who first presented in transformation. The remission times varied from 3 to 22 months with an average of 6.5 months. In addition, 2 patients are still in remission, Case 25 after 6 months and Case 26 after 11 months. The onset of transformation was rapid in all 11 patients responding; however, 12 patients with rapid onset did not respond. The 7 patients with slow onset of transformation did not respond to therapy. Of those responding, the chromosome findings at presentation were hyperdiploidy in 7, hypodiploidy in 2, pseudo-diploidy in 1 and in 1 no mitoses were demonstrated; details of the abnormal karyotypes are shown in Table 2. The patient with the longest remission (Case 16) showed a 45,XY, Ph^1,C− cell line, as did the patient described by Canellos and colleagues in whom remission lasted 28 months [3].

In all patients who remitted the abnormal cell lines disappeared, and all reverted to a 46 Ph^1 positive line. When further relapse occurred in 9 patients the same abnormal cell lines present at the time of initial transformation recurred; 5 of the 9 showed clonal evolution of the blastic transformation cell line (Cases 3, 7, 13, 20, 29).

Three patients developed Ph^1 negative cells in the early stage of remission before the reappearance of Ph^1 positive cells (Cases 13, 15, 16). Case 26 showed two cell lines in remission; one 46,XY,Ph^1 and the other 45,XO,Ph^1 [7].

Discussion

In the present study it was found that blastic transformation of Ph^1 positive chronic granulocytic leukaemia was accompanied by the appearance of further chromosome abnormalities in all but 2 of the 30 patients. This would seem to support the view [1] that the presence of the Ph^1 chromosome predisposes the leukaemic cell to further chromosome aberrations, thereby implying that the Ph^1 chromosome has a major role in the pathogenesis of the disease [1, 14]. The fact that the incidence of chromosome abnormalities is higher than in acute leukaemia in which abnormalities have been demonstrated in only 35% to 50% of cases [9, 15] also supports this view. In addition, the type of abnormality differs; in acute myeloblastic leukaemia hypodiploidy is usual whereas in the majority of cases in this study (21 of 28) hyperdiploidy was found.

No explanation can be offered for the fact that in two patients repeated examination failed to yield any mitoses.

It is now standard practice in cases diagnosed as chronic granulocytic leukaemia to perform bone marrow chromosome studies to establish the presence of the Ph^1 chromosome. Apart from the recognized cytogenetic variant 45,XO,Ph^1 [7], any change in the basic Ph^1 cell line suggests the possibility of blast cell transformation. The regular occurrence in this study of additional abnormalities suggests that bone marrow chromosome examination supplies valuable confirmatory evidence of the transformation. Thus a case can be made for the performance of serial marrow studies to determine whether these chromosome changes may antedate clinical and haematological features of blastic transformation and thereby give warning of its impending onset.

It appears that the reversion to the 46 Ph^1 cell line with disappearance of other abnormalities is a good indication of remission. Furthermore, chromosome studies may be of value in assessing the likelihood of remission, in that signs of chromosome reversion usually occurred within two to three weeks from the commencement of therapy. Since the study was performed over a period of 5 years and since the patients were referred by a number of different clinicians there was variation in the method of treatment and no conclusion about the efficacy of particular types of treatment can be made; however, the remission rate of about 30% is similar to that reported by Canellos et al. [2].

A point of interest is that in 3 patients (Cases 13, 15, 16), 2 of whom achieved long remissions, Ph^1 negative cells appeared before the Ph^1 positive cells. The explanation for these Ph^1 negative cells is not known; it may be that the marrow had been rendered quite aplastic (as is often the case when combination therapy is used in these patients) and that the cells obtained in mitosis from the relatively aplastic marrow were lymphoid in origin and thus did not carry the Ph^1 abnormality. However, in the 2 patients with long remissions, the Ph^1 negative cells persisted for 6 to 16 months while the marrow was quite normocellular. This may indicate that there is always a Ph^1 negative myeloid clone of cells, suppressed or overgrown by the Ph^1 positive cells, even in the chronic phase [6]. It has been suggested [1] that therapy would perhaps be more efficacious if directed at producing Ph^1 negative cells in the marrow in the chronic phase but that this remains a hazardous procedure not lightly undertaken.

*

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Virus–Zell-Wechselbeziehungen in der viralen Leukämogenese*

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Der in vorausgehenden Untersuchungen *in vivo* beobachtete Zytotropismus des Gr.-Leukämie-Virus zur spezifischen Targetzelle wurde im *in vitro*-System ebenfalls nachgewiesen. Mittels Milz- bzw. Thymuszellen gebildete Milzkolonien wurden *in vitro* gezüchtet, mit Virus aus myeloischer bzw. lymphatischer Gr.-Leukämie inkubiert und das Nährmedium neonatalen syngenen Mäusen appliziert. Dabei zeigte sich, daß die Nährmedien der Kombination Milzzellen + Virus aus myeloischen Leukämien bzw. Thymuszellen + Virus aus lymphatischen Leukämien relativ hohe Leukämiewerte induzierten, wogegen im Kreuzversuch nur ein geringes Leukämieauftreten zu verzeichnen war.

Die leukämogenen C-Typ-Viren sind im Hinblick auf die induzierte Leukämieform monovalent, d. h. sie rufen unter den üblichen Übertragungsbedingungen im syngenen System einen bestimmten Leukämietyp hervor. Das Gross- und Moloney-Virus induzieren ausschließlich lymphatische, das Friend- und Rauscher-Virus ausschließlich Erythroblastenleukämien und das Virus der myeloischen Leukämie der Maus [5], im folgenden als Gr.-Virus bezeichnet, primär myeloische Leukämien. Durch schnell aufeinanderfolgende syngene Passagierung konnte eine wesentliche Aktivitätssteigerung des Gr.-Virus erzielt werden. Überraschenderweise wurden nunmehr vom gleichen Virus hämatologisch unterschiedliche Leukämietypen induziert. Damit zeigt das aktivierte Gr.-Virus im Hinblick auf die Leukämieinduktion als einziges der bekannten murinen leukämogenen Viren eine polyvalente Wirkung [3, 6]. Dadurch war die Möglichkeit gegeben, mit einem definierten Virus in einem identischen System den Fragenkomplex der Targetzellen der verschiedenen Leukämietypen zu untersuchen als auch das Vorliegen eines Zytotropismus des leukämogenen Virus zur Targetzelle zu verifizieren.

Für uns stellte sich nun die Frage, ob bei einer direkten Infektion der Targetzellen, die *in vitro* vorgenommen werden muß, dieser Mechanismus ebenfalls nachzuweisen ist.

* Zu Ehren des 25jährigen Jubiläums des Nationalen Institutes für Hämatologie und Bluttransfusion, Budapest, Frau Professor S. Hollan gewidmet.

Material und Methoden

a) *Mäuse*

Für die Versuche wurden ausschließlich Mäuse des in Koloniezucht weitergeführten Inzuchtstammes XVII/Bln verwendet; das Alter der Tiere zur Zeit der Bestrahlung betrug 6 bis 8 Wochen.

Zur Bereitung von Zellsuspensionen wurden die Milzen und Thymi 10 Tage alter XVII/Bln-Mäuse verwendet.

Die für den Biotest verwendeten Neugeborenen waren im Durchschnitt 1–2 Tage alt.

b) *Virus*

Ausgangsmaterial für die Viruspräparationen waren primäre selektierte myeloische und lymphatische Gr.-Virus-Leukämien des Stammes XVII/Bln. Die Herstellung des Virus enthaltenden zellfreien Filtrats wurde in früheren Arbeiten beschrieben [3]. Die neugeborenen Mäuse erhielten das Filtrat in Dosen von 0.1 ml und blieben bis zur Geschlechtsreife mit den Müttern zusammen. Die Tiere wurden in 14tägigen Abständen auf das Vorliegen einer Leukämie untersucht. Bei Manifestwerden der Leukämie wurden, nach Anfertigung von Blutausstrichen, die Tiere abgetötet und die hämatopoetischen Organe für histochemische und histologische Zwecke präpariert. Die Diagnosestellung erfolgte durch Auswertung der Blutausstriche und Kriterien, die sich aus zytochemischen Befunden ergaben.

c) *Bestrahlung*

Die Bestrahlung wurde in der Abt. Klinische Strahlenbiologie (Leiter Dr. Magdon) unseres Zentralinstitutes mit einer Telekobalt-Einheit vorgenommen. Daten: 24.43 r/min, 34.59 min t, 52 cm Tubus, gesamt 850 r.

d) *Milzkolonie-Erzeugung*

Ein Tag nach der Bestrahlung bekamen die Mäuse Milz- und Thymuszellsuspensionen von 8–10 Mill. Zellen intravenös verabreicht.

e) *Kultur in vitro*

Die nach 9 Tagen gebildeten Milzkolonien wurden unter dem Cytoplast steril herauspräpariert und anschließend auf Kollagen-Unterlage in Bucher-Kulturflaschen angesetzt. Einzelheiten der Züchtungsmethodik wurden früher beschrieben [8]. Die Nährlösung wurde einmal wöchentlich gewechselt und nach zellfreier Filtration mittels G_4-Filter in Dosen von 0.1 ml neugeborenen Mäusen appliziert.

Versuche und Ergebnisse

In der ersten Versuchsanordnung wurden letal bestrahlte XVII-Mäuse 24 Stunden nach der Bestrahlung mit Milz- oder Thymuszellsuspensionen behandelt. Diese Zellen wurden vorher eine Stunde *in vitro* mit Virus aus einer myeloischen Leukämie bei Zimmertemperatur inkubiert. Nach der Inkubation wurden die Zellen 2mal sorgfältig ausgewaschen und in Ringerlösung intravenös appliziert. Von den 26 mit Milzzellen behandelten Mäusen entwickelten 19 Leukämien, wogegen von 17 mit Thymuszellen behandelten Tieren 8 Leukämien bekamen. In der ersten Versuchsgruppe traten fast ausschließlich myeloische Leukämien auf, während bei der mit Thymuszellen substituierten Gruppe eine breite hämatologische Aufsplitterung zu beobachten war.

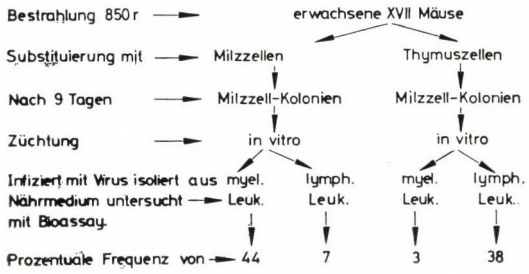

Abb. 1. Gr.-Virus Zytotropismus *in vitro*

In der zweiten Versuchsanordnung erhielten junge erwachsene XVII-Mäuse eine Ganzkörper-Letalbestrahlung und wurden einen Tag später mit Zellsuspensionen behandelt, die aus Milzen und Thymi syngener Mäuse hergestellt wurden. Nach 9 Tagen wurden die Milzen der Tiere entnommen und die gebildeten Milzkolonien herauspräpariert. Die Milzkolonien stellen bekanntlich Zellklone dar, deren Entstehung auf eine CFU (colony-forming-unit) zurückgeht. Diese Zellkolonien aus Milz- bzw. Thymuszellen wurden 4 bis 6 Tage in der Kultur gezüchtet und danach 2 Std. bei Zimmertemperatur mit Virus aus myeloischer bzw. lymphatischer Leukämie inkubiert. Nach der Inkubation wurde das Virus sorgfältig ausgewaschen. Zehn bzw. 20 Tage nach der Virusinkubation wurde die Nährlösung neugeborenen syngenen Mäusen injiziert.

Nährlösung aus Milzzell-Kolonien, die mit Virus aus myeloischer Leukämie behandelt wurden, induzierten nach einer relativ langen Latenzzeit von 11 Monaten 16 vorwiegend myeloische Leukämien von 36 auswertbaren Tieren. Nährlösung aus Thymuszell-Kolonien, die mit Virus aus lymphatischer Leukämie behandelt wurden, induzierten nach einer Latenzzeit von 13 Monaten von 37 auswertbaren Tieren 14 vorwiegend lymphatische und Mischform-Leukämien. Die Nährlösung aus Milzzell-Kolonien, die im Kreuzversuch mit Virus aus lymphatischer Leukämie, und Thymuszell-Kolonien, die mit Virus aus myeloischer Leukämie inkubiert

wurden, induzierten nach Latenzzeiten von 10—11 Monaten 2 Leukämien von 28 bzw. 1 Leukämie von 32 auswertbaren Tieren. Zur besseren Veranschaulichung der Versuchsanordnung ist diese in Abb. 1 zusammenfassend dargestellt.

Besprechung

Wir konnten in vorangehenden Versuchen nachweisen, daß das Gr.-Virus in Abhängigkeit vom Replikationsort in unterschiedlichem Grade die Ausgangs-Leukämieform induziert. Einen ausgeprägten Zytotropismus zeigt das Virus aus reifzelligen Leukosen, da dieses in hohem Maße nur die spezifischen Targetzellen infiziert und transformiert und damit die identische Leukämieform reproduziert [4]. Durch Selektion reifzelliger Leukosen konnten reine myeloische und lymphatische Gr.-Leukämielinien erzeugt werden. Splenektomie reduziert die Gr.-Virus-induzierte myeloische [3], Thymektomie die lymphatische Leukämogenese [9]. Aus diesen Untersuchungen geht hervor, daß nach Entfernen des Targetorgans und damit der Targetzellen, das betreffende leukämogene Virus nur in sehr geringem Maße befähigt ist, andere Zellen zu transformieren. Hier wird also wiederum ein Tropismus des Virus zur Targetzelle ersichtlich. Durch Reisolierungsversuche ließ sich nachweisen, daß das primär in den Targetzellen replizierte Virus infolge eines Zytotropismus nach Übertragung nur wieder die spezifischen Targetzellen infiziert.

Die in der ersten Versuchsanordnung *in vitro* erfolgte Infektion von Milz- und Thymuszellen, die nachfolgend letal bestrahlten Mäusen verabreicht wurden, ergibt zwar einen Hinweis für das Vorliegen eines Zytotropismus auch in diesem System, das Ergebnis von 72% induzierten Leukämien nach Milzzell-Applikation gegenüber 46% nach Thymuszell-Verabreichung ist jedoch nicht signifikant. Möglicherweise haftet das Virus stärker an den Zelloberflächen und läßt sich auch nicht durch mehrmaliges Auswaschen entfernen. Diese mögliche Unsicherheit wurde in der zweiten Versuchsanordnung vermieden, indem nicht die virusinfizierten Zellen direkt, sondern das von ihnen produzierte Virus im Biotest Aufschluß über einen vorliegenden Zytotropismus geben. Wie zu erkennen ist, treten in den Kombinationen Milzzellen + Virus aus myeloischer Leukämie und Thymuszellen + Virus aus lymphatischer Leukämie im gleichen, relativ hohem Prozentsatz Leukämien auf, wogegen der Kreuzversuch Milzzellen + Virus aus lymphatischen Leukämien und Thymuszellen + myeloische Leukämien nur eine geringe leukämogene Wirkung aufzeigt. Hieraus läßt sich ableiten, daß das Virus aus der myeloischen Leukämielinie bzw. das Virus aus der lymphatischen Leukämielinie auch *in vitro* im Sinne eines Zytotropismus vorzugsweise ihre spezifische Targetzellen infizieren und in diesem repliziert werden.

Auch die vorwiegende Induktion der identischen Leukämieform, und damit eine geringe Aufsplitterung in hämatologisch unterschiedliche Typen, spricht für das Vorliegen eines Zytotropismus. Im Kreuzversuch sind die für das Virus spezifischen Targetzellen nicht vorhanden und infolgedessen kommt es nur zu einer geringen Virusinfektion und -replikation.

Es erhebt sich nun die Frage, welcher Mechanismus dem Zytotropismus muriner Onkornaviren zugrunde liegen könnte. Aus den angeführten Befunden wurde deutlich, daß die Beschaffenheit der Oberfläche der Leukämiezellen, in denen das Virus gebildet wird, in Beziehung zu dem beobachteten Zytotropismus stehen könnte. Wir haben nun diskutiert, daß das Virion, das in Zellen eines bestimmten hämatologischen Typs als Targetzelle reproduziert wird, während des *budding*-Prozesses bei der Bildung des Virion-Envelopes durch die Zelloberfläche spezifische Anteile der Mutterzelle inkorporiert. Dadurch kann das Virus befähigt werden, aufgrund von Enzym- oder Rezeptorwirkung, evtl. über einen Derepressionsmechanismus, bevorzugt einen bestimmten Zelltyp als Targetzelle zu infizieren und zu transformieren. Je unreifer die Zelle ist, in der das Virus repliziert wird, desto weniger distinkt ist der spezifische Charakter der Zelle ausgeprägt, und um so geringer ist auch die Inkorporation spezifischer Zellkomponente in das Envelope des Virions. Hieraus resultiert dann auch ein gering ausgeprägter Zytotropismus, der zu der beobachteten Aufsplitterung in hämatologisch differente Leukämietypen führt.

Durch eine Vielzahl von Arbeiten ist inzwischen der Einbau zelleigenen Materials in das Virus-Envelope nachgewiesen worden [1, 7].

Aufgabe zukünftiger Arbeiten auf diesem Gebiet wird es sein, den Mechanismus, der dem beobachteten Zytotropismus zugrunde liegt, weiter aufzuklären, um zusätzliche Möglichkeiten eines gezielten Eingriffes in die Leukämogenese in die Hand zu bekommen.

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Virus Cell Change Relationships in Viral Leukaemogenesis

The previously observed *in vivo* cytotropism of Gr.-leukaemia virus to specific target cells has been demonstrated *in vitro*. Spleen colonies of spleen or thymus cells were cultivated *in vitro* and incubated with virus from myeloid or lymphoid Gr.-leukaemia. The culture media were administered to newborn syngeneic mice. The culture media of the combination of spleen cells + virus from myeloid leukaemia, and thymus cells + virus from lymphoid leukaemia were found to induce leukaemia with high frequency, whereas with the crossed combinations the incidence of leukaemia was low.

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Neue biochemische Befunde bei Leukämie*

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Es wird gezeigt, daß in Leukämiezellen eine DNA-Polymerase nachgewiesen werden kann, die die Template Charakteristika einer reversen Transcriptase aufweist und die imstande ist, eine Information von einer heteropolymeren natürlichen RNA abzulesen und in eine DNA Sequenz umzuwandeln. Sie konnte von den DNA-codierten DNA-Polymerasen der Leukämiezellen abgetrennt werden. Die reversen Transcriptasen bei myeloischer und lymphatischer Leukämie unterscheiden sich durch ihre Molekülgröße. Sie werden durch Rifamycinderivate gehemmt.

Die Diagnose der Leukämie erfolgt heute aus dem klinischen Bild sowie aus Morphologie und Cytologie der Zellen, wobei die cytochemisch nachweisbaren Veränderungen nicht Ursache sondern Folge der leukämischen Zellveränderungen sind. Es fehlt bisher der biochemische Nachweis ätiologisch bedeutsamer Veränderungen in den Zellen. Es ist nun schon seit längerer Zeit bekannt, daß bei verschiedenen Tierarten, wie Vögel, Mäuse, Ratten, Hamster, Katzen und Affen Leukämien durch RNA-Tumorviren, die Oncornaviren, ausgelöst werden können [12]. Es ergibt sich nun die Frage, ob derartige Viren auch bei der Entstehung menschlicher Leukämien eine Rolle spielen. In den letzten Jahren haben die Arbeitsgruppen um Huebner, Spiegelman, Todaro, Temin, Gallo, sowie Moser Hinweise für eine mögliche Bedeutung von RNA-Viren für die Leukämiegenese geliefert [3—5, 11, 12, 15].

In den menschlichen Zellen erfolgt der zur Zellfunktion erforderliche Informationsfluß von der Desoxyribonukleinsäure des Kernes, in der die genetische Information gespeichert ist, über die mRNA, die an der DNA des Kernes als Matrize von einer DNA-abhängigen RNA-Polymerase gebildet wird (Transcription) zum Ribosom, wo die Proteinsynthese erfolgt. Werden Zellen von DNA-Viren befallen, so kann die virale Information in die vorhandenen Desoxyribonukleinsäuren des Kernes eingebaut werden, wodurch die Zelle gezwungen werden kann, die vom Virus stammende Information für ihre Synthesevorgänge zu verwerten.

Wird die Zelle jedoch von einem oncogenen RNA-Virus befallen, so kann die virale Information mit dem in der Zelle vorhandenen Rüstzeug nicht verwertet

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werden. Das RNA-Virus bringt jedoch ein Ferment mit, mit dessen Hilfe die Zelle eine der Virusribonukleinsäure komplementäre Desoxyribonukleinsäure aufzubauen imstande ist. Bei diesem Ferment handelt es sich um eine RNA-codierte DNA-Polymerase. Da hier ein entgegengesetzter Informationsfluß ausgelöst wird, bezeichnet man dieses Ferment auch als reverse Transcriptase. Temin [17] postulierte bereits 1964, daß eine Informationsübertragung von einer RNA auf eine DNA möglich sein könnte, und konnte gemeinsam mit Mizutani [20] 1970 in Rous Sarcoma Virus eine reverse Transcriptase nachweisen. Ihr Vorkommen ist von mehreren Arbeitsgruppen [12, 15] bestätigt worden. Damit ist die Informationsübertragung der viralen RNA auf die DNA mit anschließender Integration in die Wirts-DNA auch biochemisch möglich. Seither wurde in nahezu allen RNA-Tumorviren, die für Oncogenität oder prospektive Oncogenität verantwortlich sind, auch eine reverse Transcriptase nachgewiesen.

Nach der Oncogenhypothese von Huebner und Todaro [7, 8] wird die Ribonukleinsäure des Genoms des oncogenen RNA-Virus (das Virogen), das die für die maligne Transformation entscheidende Information (das Oncogen) enthält, durch die reverse Transcriptase in eine DNA transcribiert, die in die DNA der Wirtszelle eingebaut wird. Die von der Virus-RNA transcribierte DNA, die in das Genom der Zelle integriert wird, kann nun folgerichtig als DNA-Provirus bezeichnet werden, da es die Information der viralen RNA trägt. Die maligne Transformation braucht aber nicht sogleich zu erfolgen, sondern die virogene Information könnte durch einen Repressor blockiert und auf die Nachkommen vertikal übertragen werden [2]. Durch chemische, toxische oder andere Agenzien kann eine Derepression erfolgen. Wird nur das Oncogen dereprimiert, so werden "transforming proteins" gebildet und die Zelle erleidet eine maligne Degeneration. Wird jedoch das ganze Virogen dereprimiert, so erfolgt eine Virusbildung in der Zelle. Während für die Prägung der Zelle die reverse Transcriptase erforderlich ist, muß sie zum Zeitpunkt der Derepression theoretisch nicht mehr vorhanden sein und ist auch für die maligne Umwandlung nicht erforderlich [15].

Nach der Teminschen Protovirustheorie [18] soll die maligne Degeneration einer Zelle wie folgt entstehen: Die Information einer in ihrer Zusammensetzung veränderten RNA, die von außen in die Zelle eingebracht wird, die aber auch durch intrazelluläre Mechanismen in der Zelle selbst entstehen könnte, wird mit Hilfe einer RNA-abhängigen DNA-Polymerase (reverse Transcriptase) auf die DNA übertragen und in die wirtseigene DNA inkorporiert. Diese pathologische Information könnte dann zur malignen Entartung der Zelle führen. Für das Wirksamwerden dieser Hypothese ist also eine Informationsübertragung von der RNA auf die DNA notwendig (reverse Transcriptase). Die reverse Transcriptase ist für die Genamplifikation und zelluläre Differenzierung weiterhin erforderlich.

Aus den Arbeiten der letzten zwei Jahre von Baxt und Spiegelman [1], Sarngadharan, Sarin, Reitz und Gallo [16], wie unserer eigenen Arbeitsgruppe [10–14], wird es wahrscheinlich, daß auch in Blutzellen bei Leukämie ein Ferment mit den Charakteristika der reversen Transcriptase gefunden werden kann. Um eine RNA-abhängige DNA-Polymerase als reverse Transcriptase anzuerken-

nen, sind strenge Kriterien anzulegen. So muß sie vor allem von den eukaryoten DNA-abhängigen DNA-Polymerasen (Polymerase I und II) abgetrennt werden. In manchen Zellen wie Hühnerembryonen und Placenta finden sich außerdem sogar RNA-abhängige DNA-Polymerasen [11, 13].

Die Reinigung der reversen Transcriptase wurde an anderer Stelle ausführlich beschrieben [11]. In der allerletzten Zeit wurde eine Verbesserung dieser Reinigung durch Affinitätschromatographie an Oligo-dT-Zellulose nach dem Vorschlag von Gerwin und Milstein [6] erzielt. Obwohl eine besondere Affinität der viralen DNA-Polymerase zu Oligo-dT-Zellulose besteht, darf diese nicht als Kriterium für das Vorliegen einer reversen Transcriptase angesehen werden [12, 15].

Wird ein an DEAE-Zellulose vorgereinigter Extrakt aus leukämischen Zellen verschiedener Leukämieform über Phosphozellulose weiter gereinigt, so zeigt sich, daß 2 DNA-abhängige DNA-Polymerasen gefunden werden können [11]. Ihr Elutionsmuster und ihr Verhalten an Sephadex G 200 ist bei akuten und chronischen Leukämien unterschiedlich [11]. Außerdem findet man eine RNA-abhängige DNA-Polymeraseaktivität [11]. Die Molekulargröße der reversen Transcriptase ist bei akuten lymphatischen und myeloischen Leukämien unterschiedlich [11].

Zur Charakterisierung der Polymerasen wird das Vermögen des Fermentes geprüft, markiertes Thymidintriphosphat in eine DNA einzubauen, wobei verschiedene synthetische und natürliche Nukleinsäuren als Matrize (Template) dienen [10]. Es zeigt sich, daß sich die reverse Transcriptase aus Leukämiezellen und aus Affen-Myeloblastosevirus weitgehend analog, die DNA-abhängigen DNA-Polymerasen aus *E. coli* bzw. Kalbsthymus (Kornberg-Enzyme) abweichend verhalten (Tabelle 1). Bei Verwendung der homopolymeren synthetischen Matrize Poly rA · dT zeigt die reverse Transcriptase eine höhere ^{14}C-TTP-Inkorporation als die Kornberg-Enzyme, während bei Poly dA · dT als Matrize die DNA-abhängigen Polymerasen eine höhere TTP-Inkorporation bewirken. Bei Verwendung von Poly rA · rU ist der Unterschied noch deutlicher. Diese Befunde besagen aber nur,

Tabelle 1

Template (Primär) Charakteristika verschiedener DNA-Polymerasen (TTP-Inkorporation in pMol/h/μg Eiweiß)

Template	RNA-abhängige DNA-Polymerase		DNA-abhängige DNA-Polymerase	
	leukämische Zellen	AMV[1]	E. coli	Kalbsthymus
dA · dT	48	28	96	107
rA · dT	56	81	21	30
rA · rU	30	27	4.4	15
DNA nativ	2	1.4	2.9	0.9
Q_{beta} RNA	8.1	0.4	—	—

[1] Avian Myeloblastose Virus.

daß auch das Kornberg-Enzym Poly rA · dT abzulesen vermag, so daß diese Fähigkeit keine echte Unterscheidungsmöglichkeit zwischen DNA- und RNA-codierten DNA-Polymerasen bietet. Erst bei der Verwendung der natürlichen heteropolymeren einsträngigen Phagennukleinsäure Q_{beta} RNA erkennt man, daß diese nur von der reversen Transcriptase abgelesen werden kann. Vergleicht man Polymerase I und II und das von uns als reverse Transcriptase bezeichnete Ferment aus leukämischen Zellen hinsichtlich ihres Verhaltens gegenüber verschiedenen Matrizen, so ergibt sich ein analoger Befund (Tabelle 2). Auch hier bewirkt die als reverse Transcriptase angesehene Fraktion mit Poly rA · dT_{10} eine bessere

Tabelle 2

Template (Primär) Charakteristika von DNA-Polymerase Aktivitäten bei Leukämie (TTP-Inkorporation in pMol/h/μg Protein). Die in der Sephadexchromatographie bezeichneten Fraktionen Polymerase I, Polymerase II und reverse Transcriptase wurden gesammelt, eingeengt und mit den verschiedensten Templates auf DNA-Polymerase-Aktivität getestet. (Aus [11])

Template		DNA-Polymerase		
		I	II	reverse
dT_{10} · rA	Mg^{++}	12	0	361.00
dT_{10} · rA	Mn^{++} (— Mg)	23	10	472.00
dT_{10} · dA	Mg^{++}	97	5400	320.00
Poly rA · dT	Mg^{++}	0	20	98.00
Poly rA · dT	Mn^{++} (— Mg)	56	40	125.00
Poly dA · dT	Mg^{++}	48	2570	38.30
DNA nativ	Mg^{++}	2	670	13.00
DNA denat.	Mg^{++}	3	2060	24.00
Poly rA · rU	Mg^{++}	3	0	280.00
Poly rA · rU	Mn^{++} (— Mg)	9	0	321.00
t-RNA $^{Phe}_{E.coli}$	Mg^{++}	32	120	384.00
Q_{beta}-RNA+dT ^{14}C-TTP	Mg^{++}	10	50	325.00
Q_{beta}-RNA+dT ^{3}H-dGTP	Mg^{++}	0	0	287.00

Inkorporation als mit Poly dA · dT_{10}. Der Unterschied gegenüber Polymerase I und II ist noch deutlicher bei Verwendung von Poly rA · rU als Template sowie bei Verwendung der natürlichen einsträngigen heteropolymeren RNA. Das als reverse Transcriptase angesprochene Enzym vermag bei Verwendung einer einsträngigen natürlich vorkommenden RNA als Template (z. B. Q_{beta} RNA) sowohl markiertes TTP, als auch dGTP in ihr Endprodukt einzubauen, womit gezeigt ist, daß es verschiedene Nukleotide zum Einbau zu verwenden vermag und eine echte heteropolymere DNA aufzubauen imstande ist.

Wird dem Testansatz Ribonuklease zugesetzt, die die RNA der Matrize zerstört, so verschwindet die Aktivität, womit gezeigt ist, daß das Enzym tatsächlich RNA-abhängig ist. Das Endprodukt der Wirkung aller 3 Enzyme ist eine Desoxyribonukleinsäure. Sie wird durch eine Desoxyribonuklease zerstört (Tabelle 3) [10].

Die Fermente unterscheiden sich weiter in ihrem pH Optimum, das für Polymerase I bei 8.6, für Polymerase II bei 8.2 und für die RNA-codierte DNA-Polymerase bei 7.8 liegt. Für die Wirkung der reversen Transcriptase ist Mg^{++} unbedingt erforderlich, kann aber durch Mn^{++} ersetzt werden [10].

Die Anforderungen zur Erkennung eines Enzyms als reverse Transcriptase seien kurz zusammengefaßt [12, 15]:

1. Das Enzym muß imstande sein, eine einsträngige natürliche heteropolymere RNA in eine homologe DNA Sequenz zu übersetzen.

Tabelle 3

Identifizierung des Endproduktes der DNA-Polymerasen (Angaben in pMolTTP/h/μg Protein). Nach Durchführung eines Standardpolymeraseassays wurde dieser 30 min mit DNAse, RNAse und Trypsin inkubiert. (Aus [10])

Template	Polymerase		
	I	II	reverse
Ohne Zusatz	97	5400	361
+ DNAse	0	0	0
+ RNAse	126	6200	348
+ Trypsin	89	4800	369

Tabelle 4

Template (Primär) Charakteristika einer reversen Transcriptase aus dem Plasma eines Patienten mit Pancytopenie als Vorstufe einer akuten Leukämie. Inkorporation von Trinukleotiden in mMol/h/μg Eiweiß. Das Enzym wurde vorgereinigt an DEAE Zellulose, Phosphozellulose, Sephadex G 200 und durch Affinitätschromatographie an Oligo dT Zellulose. Testung der Polymerase-Aktivität in 50 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 60 mM NaCl, 5 mM NaF, 5 mM DTT, 80 μM TTP, dATP, dCTP und dGTP und 10 μM von ^{14}C TTP (^{3}H-dGTP). Die Menge der säurefällbaren Aktivität wurde in einem Liquid Scintillation Counter bestimmt

Template	Reverse Transcriptase
$rA \cdot dT_{10}$	17.2
$dA \cdot dT_{10}$	5.3
$rA \cdot rU$	7.2
Q_{beta} RNA+dT_{12-18} ^{14}C-TTP	0.043
Q_{beta} RNA+dT_{12-18} ^{3}H-dGTP	0.01

2. Das Enzym muß alle 4 Nukleotide zum Einbau verwenden können.
3. Es soll eine rA · dT_{10} besser ablesen können als dA · dT_{10}.
4. Es bedarf bivalenter Kationen zu seiner Wirkung (Magnesium, Mangan).
5. Nachweis der RNase-Sensibilität der Matrize und der DNase-Sensibilität des Endproduktes.
6. Endprodukt und Matrize hybridisieren [3].
7. Es besteht eine Kreuzreaktion mit Antikörpern gegen reverse Transcriptase von Mäuse- und Affenleukämievirus [21].

Bisher konnte eine reverse Transcriptase von Gallo und Mitarb. [5] bei allen untersuchten Fällen von akuten Leukosen, von Baxt und Mitarb. [1] bei 94% und von Rainer und Mitarb. [12] bei 95% der untersuchten Patienten mit verschiedenen Leukämieformen nachgewiesen werden. Wie Rainer zeigen konnte, weist die reverse Transcriptase bei den verschiedenen Leukämieformen eine Heterogenität auf und unterscheidet sich im Molekulargewicht, das bei der reversen Transcriptase der myeloischen Leukämie wesentlich größer ist als bei der lymphatischen Leukämie, ein Befund, der bisher von Todaro und Gallo bestätigt werden konnte [11, 15]. Eine derartige Transcriptase konnte bisher weder in normalen noch in PHA-stimulierten Leukozyten nachgewiesen werden. Hingegen konnten Rainer, Piller, Deutsch und Moser [14] bei einer Patientin mit Pancytopenie und Glutathionreduktasemangel zu einem Zeitpunkt, zu dem auch durch sorgfältigste klinische und histochemische Untersuchung im peripheren Blut und im Knochenmark keine Leukämie nachgewiesen werden konnte, eine RNA-abhängige DNA-Polymerase mit den typischen Template Charakteristika einer reversen Transcriptase finden. Kurze Zeit darauf entwickelte die Patientin eine akute peroxydasepositive Leukämie, so daß hier erstmals ein biochemischer Nachweis eines präleukämischen Stadiums gelungen zu sein scheint.

Die Isolierung und Charakterisierung der reversen Transcriptase ist eine mühsame Aufgabe, die nur schwer auf breiter Basis durchgeführt werden kann. Vielleicht wird es möglich sein, die Tatsache der bereits erwähnten Kreuzreaktion der Transcriptase menschlicher Leukämiezellen mit Antikörpern gegen Transcriptase der Affenleukämie zu einer einfachen Reaktion auszubauen. Eine solche Reaktion könnte beträchtlichen diagnostischen Wert zur Früherkennung von Leukämien oder z. B. zum Nachweis kompletter Remissionen und Heilung erlangen.

Eine Hemmung der reversen Transcriptase konnte mit Rifamycin- und Distamycinderivaten *in vitro* nachgewiesen werden. Hierbei zeigte sich, daß die Rifamycinderivate C 11, C 22 und C 27 die DNA-abhängigen DNA-Polymerasen aktivieren und nur die RNA-abhängigen DNA-Polymerasen hemmen, was für eine eventuelle therapeutische Anwendung von Vorteil wäre. Das Distamycinderivat C XII hingegen hemmt alle geprüften Polymerasen in gleicher Weise [11]. Ob sich aus diesem Befund tatsächlich therapeutische Konsequenzen werden ableiten lassen, muß einstweilen dahingestellt bleiben.

Die hier wiedergegebenen Befunde stellen einen Anfang in einer noch langwierigen Entwicklung dar. Es wird noch endgültig zu beweisen sein, daß die bei Leukämien gefundene RNA-abhängige DNA-Polymerase tatsächlich mit der reversen Transcriptase aus Oncornaviren identisch ist, daß das Enzym nur bei manifest leukämischen und präleukämischen Patienten gefunden werden kann, daß es tatsächlich mit der Auslösung und Aufrechterhaltung des krankhaften Zustandes zu tun hat. Sollte sich dies als richtig herausstellen, so wird sich vielleicht aus dem Nachweis der reversen Transcriptase eine Hilfe bei der Diagnose präleukämischer Zustände sowie bei der Feststellung echter Remissionen oder gar Heilung der Leukämie ergeben.

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Recent Biochemical Findings in Leukaemia

A DNA-polymerase with the template characteristics of a reverse transcriptase has been demonstrated in leukaemia cells. It can use the information from a heteropolymeric natural RNA and transform it into a DNA sequence. It could be separated from the DNA-dependent DNA-polymerase of the leukaemia cells. The reverse transcriptases in myeloid and lymphoid leukaemia differ in molecular sizes. They are inhibited by rifamycin derivatives.

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Beeinflussung der T-Zellaktivität bei chronischer Lymphadenose durch verschiedene Therapieformen

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Die vor, während und nach 21 Therapieserien an insgesamt 16 Patienten gefundenen Veränderungen der T-Zellaktivität in den PHA-Kulturen ergaben eine deutliche Besserung nach extrakorporaler Blutbestrahlung (ECIB) in Fällen mit langsamer Progredienz und ohne ausgeprägte Organomegalie. Nach Milzbestrahlung und nach zytostatischer Therapie fand sich in allen Fällen eine Steigerung der PHA-Reaktion. Während der Therapieserien wurde eine Verstärkung der PHA-Depression durch ECIB, Zytostase und ACTH deutlich, während Milzbestrahlung die geringste depressorische Wirkung zeigte. Der positive Effekt auf die T-Zellaktivität läßt sich im wesentlichen durch verbesserte Zirkulations- und Regenerations-Bedingungen der T-Zellfraktion erklären. Dem Ziel der Untersuchung entsprechend, leiten sich unter gleichzeitiger Berücksichtigung der CLL-Kinetik die praktisch therapeutischen Konsequenzen ab: Langsam akkumulative Fälle depletorischer Therapie (ECIB oder Leukophorese) zu unterziehen, bei splenomegalen Fällen die Milzbestrahlung einzusetzen, und bei klinischen Zeichen rascheren Zellumsatzes Zytostatika zu verwenden [46, 48]. Für Alkylanzien wie für das kompensationserhaltende ACTH folgt im besonderen Maße, nicht durch Dauertherapie den Immundefekt der CLL zu verstärken, sondern durch effektive Kurzzeittherapie einen Anstieg der zellständigen Immunaktivität zu induzieren.

Das klinische Immundefektsyndrom bei Patienten mit chronisch lymphatischer Leukämie (CLL) bestimmt in den meisten Fällen den Krankheitsverlauf entscheidend [4, 7]. Dies konnte im wesentlichen auf einen Defekt der CLL-Lymphozyten zur adäquaten Zelltransformation und zellulären Reaktion auf antigene Reize zurückgeführt werden [29, 33, 34, 41], wie sie durch das pflanzliche Protein Phytohämagglutinin (PHA) stimuliert wird [11]. Das Ausmaß dieses Defektes korreliert mit der Lymphozytose nicht streng [49]. Im Vordergrund klinischer Fragestellungen steht das Problem, welche Therapiemethoden geeignet sind, neben den übrigen hämatologischen Parametern auch den zellulären Immundefekt und damit die Prognose des Patienten in diesem kritischen Punkte zu verbessern [46].

In der vorliegenden Arbeit wurde versucht, in der CLL-Langzeitbehandlung mit verschiedenen therapeutischen Methoden diese Frage über erste Ergebnisse nach extrakorporaler Blutbestrahlung (ECIB) hinaus [47, 49] vergleichend zu verfolgen und die Veränderungen in der PHA-Reaktivität dem Verlauf zu korrelieren.

Material und Methoden

Zwischen Oktober 1970 und August 1973 wurden insgesamt 16 Patienten mit typischer chronischer Lymphadenose in verschiedenen Phasen ihrer Erkrankung entsprechenden unterschiedlichen therapeutischen Verfahren unterzogen. Vor, während und nach der Therapie wurde möglichst in 4- bis 6wöchigen Intervallen die Lymphozytentransformierbarkeit überprüft. Auf Hauttests wurde wegen ihrer bekannten Ambivalenz [6, 13] und der Schwierigkeit bei ambulanter Behandlung verzichtet. Die klinischen Daten sind dem Ergebnisteil zu entnehmen. Zur *in-vitro*-Testung der Lymphozyten wandten wir die von Flad und Mitarb. [16] modifizierte Methode von Greaves und Mitarb. [19] an: dabei wird das Leukozytenkonzentrat aus heparinisiertem Patientenserum in einem Kulturmedium aus 4 Teilen MEM-Eagle und 1 Teil inaktiviertem fetalem Kälberserum mit dem Zusatz von 1% 200 mM L-Glutamin und 1% Penicillin-Streptomycin resuspendiert. Die Abtrennung von Granulozyten und Monozyten läßt sich mittels Passage durch Leukopak-Nylonfasern in einer Glassäule erreichen. Hierbei werden fast keine Lymphozyten, und wenn dann nur einige aus der B-Zellpopulation, retiniert [19, 41]. Die Lymphozyten werden so angereichert, daß 1 ml des Kulturmediums 1 Million Zellen enthält. Zu jedem Ansatz werden 0.025 ml PHA (Wellcome) zugesetzt. Ernten erfolgen in 24stündigen Abständen nach PHA-Zusatz bis zum 8. Tag. Zwei Std. zuvor wird 1 μCi/ml ^{3}H-Cytidin bzw. ^{3}H-Thymidin zugegeben. In mehreren Waschvorgängen beseitigen wir die ungebundene Aktivität. Die Bestimmung des inkorporierten Nukleosids erfolgt nach Zusatz thixotropen Gels im Flüssigkeitsszintillationszähler. Jeder Ansatz wird 3fach durchgeführt. Die folgenden Werte stellen Mittelwerte der durchschnittlich $\pm 7\%$ streuenden Einzelbestimmungen dar. Die Streuung der individuellen Werte bei Normalpersonen ist bekanntermaßen breit [13]. Sie lag bei uns für die Thymidin-Inkorporation zwischen 6000 und 10 000 cpm und war für die Cytidin-Inkorporation noch weiteren Schwankungen zwischen 20 000 und 40 000 cpm unterworfen. Daher werden im folgenden lediglich die Veränderungen im Verlaufe der Behandlung eines Patienten relativ zum Ausgangswert als Quotient ausgedrückt (vergleiche Tabelle 1). Dabei ist das Schwergewicht auf die weniger labilen, aussagekräftigeren Thymidin-Werte [11] gelegt. Wir verglichen im Laufe der Therapie eines Patienten jeweils den Maximalwert, der ohne Konstanz am 3. bis 5. Tag, meist am 4. auftrat. Deutliche Zweigipfligkeit war [34], wie auch bei anderen Autoren [30, 35], selten zu verzeichnen.

Ergebnisse

Verläufe bei extrakorporaler Blutbestrahlung

Die Methodik der extrakorporalen Blutbestrahlung wurde anderenorts detailliert beschrieben [4]. Während der initialen Serie wurden fast täglich 4 Blutvolumenäquivalente durch eine ^{90}Co-Quelle (Dosisleistung 25 000 R/min) mit einer Geschwindigkeit geleitet, die eine mittlere Transitdosis von 180 rad ergab.

Tabelle 1

1.	2.	3.	4.	5.	6.	7.	8.	9.
ECIB								
HH	A	1			15		137	—
	B			10	130	8.6	11	
HI	A	1			51		72	—
	B		10		15	0.3	46	
	C			15	50	1	13	
	D		31		22	0.4	18	
	E		47		30	0.6	3	
	F			132	65	1.3	20	
	G			171	15	0.3	35	
SM	A	36			60		68	—
	B		16		15	0.25	11	
	C			4	10	0.17	8	
	D			75	81	1.3	22	
	E			130	32	0.5	21	1
NJ	A	15			30		190	—
	B		164		15	0.5	25	
	C		210		8	0.28	44	
	D			34	48	1.62	30	
	E			128	164	5.5	45	
SA	A	1			15		158	—
	B			10	25	1.7	14	
	C			224	15	1	96	
BF	A	1			10		52	10
	B		5		5	0.5	38	15
	C			5	11	1	6	16
	D			30	9	0.9	5	15
	E			240	4	0.4	7	17
HoH	A	5			8		120	24
	B		4		5	0.63	70	22
	C			90	5	0.64	15	25
DH	A	15			15		45	8
	B		9		5	0.3	32	10
	C			7	8	0.53	20	12
	D			180	7	0.47	110	15

Tabelle 1 (Forts.)

1.	2.	3.	4.	5.	6.	7.	8.	9.
MG	A	1			10		100	5
	B		10		7	0.7	52	6
	C			3	6	0.6	20	6
	D			30	11	1.1	90	10
Milz-bestrahlung								
RA	B	38			10		81	6
	C			8	25	2.5	4	2
	D	8			15		80	3
	E		33		10	0.7	13	2
	F			69	40	2.7	18	1
KF	A	1			11		22	7
	B			1	80	8	3	3
	C			480	40	4	1	3
MM	A	3			8		80	10
	B			19	77	9.3	8	3
GE	A	1			10		20	21
	B			1	21	2	4	12
	C			27	15	1.5	4	11
	D	13			6.6		20	21
	E		21		8.5	1.3	8	20
	F			4	10	1.5	1	21
	G			46	10	1.5	2	22
SA	C	54			15		96	2
	D			4	25	1.7	13	1
HH	C	30			19		106	13
	D			93	58	3	17	10
Zytostatika								
SA	E	153			11		48	2
	F			43	35	3.5	4	1
	G			56	66	6.6	3	1
	H			176	60	6	11	1
SJ	A	1			10		27	—
	B		132		8	0.8	28	
	C			46	49	4.9	45	

Tabelle 1 (Forts.)

1.	2.	3.	4.	5.	6.	7.	8.	9.
DE	D	1			25		125	18
	E			53	65	2.6	49	7
ACTH								
PA	A	1			34		34	8
	B			30	9	0.26	58	6
	C		60		15	0.4	7	10
	D			60	13	0.4	30	12
SM	G	5			10		56	3
	H		17		15	1.5	13	1.5
	I			12	20	2	10	2
	K			46	29	3	6	3
	L	8			25		8	1
	M		27		8	0.3	5	—
	N		61		13	0.5	7	—
	O		90		8	0.3	6	—
RA	F	7			35		13	1
	G		360		23	0.6	27	—

Die Tabelle führt sämtliche im Ergebnisteil besprochenen Kulturen nach Patient und Therapie gegliedert auf.

1. Die erste Kolumne enthält Patientensignum.
2. Die zweite Kolumne enthält Kultursignum.
3.—5. Kolumne: Zeitangabe des Kulturansatzes vor, während oder nach der Therapie.
6. Kolumne: Maximale ^{3}H-Thymidin-Inkorporation in gemittelten Werten und um 10^2 gekürzt.
7. Kolumne: Quotient aus Thymidin-Wert zum jeweiligen Zeitpunkt zu letztem Wert vor der jeweiligen Therapieserie.
8. Kolumne: Lymphozytenwert/μl $\times 10^3$ zum jeweiligen Zeitpunkt.
9. Kolumne: Klinische Milzgröße zum jeweiligen Zeitpunkt in Zentimetern unter dem Rippenbogen.

Die einzelnen Termine sind den Graphiken zu entnehmen. Die akkumulative Gesamtdosis ist bei der Fallbeschreibung jeweils angegeben.

Pat. *H. H.*, weiblich, 55 Jahre (Abb. 1). Anamnesedauer 2 Jahre. Keine Vorbehandlung; innerhalb des Jahres vor Aufnahme steiler Lymphozytenanstieg ohne Organomegalie. Serie extrakorporaler Blutbestrahlung mit insgesamt 10 800 rad in 12 Sitzungen. Beendigung wegen Shunt-Thrombose.

Minimale PHA-Stimulierbarkeit vor ECIB (A). Zehn Tage nach letzter ECIB-Sitzung Steigerung bis in den oberen Normbereich festgestellt (B).

Pat. *H. I.*, weiblich, 52 Jahre (Abb. 2). Anamnesedauer 1 Jahr, keine Vorbehandlung, keine Organomegalie. Vor ECIB guter Nukleosideinbau, knapp

unterhalb des Normbereichs (A). Während der 1. Serie totale Depression (B). Erholung 15 Tage nach ECIB mit 8000 rad festgestellt (C). Unter 2. Bestrahlungsserie von insgesamt 17 000 rad wieder Depression, jedoch nicht so ausgeprägt wie zuvor. Erholung und Steigerung der Thymidin-Inkorporation in den unteren Normbereich nach 132 Tagen festgestellt; nach weiteren 40 Tagen trotz relativer Konstanz der Leukozytenzahlen wieder zunehmende Depression.

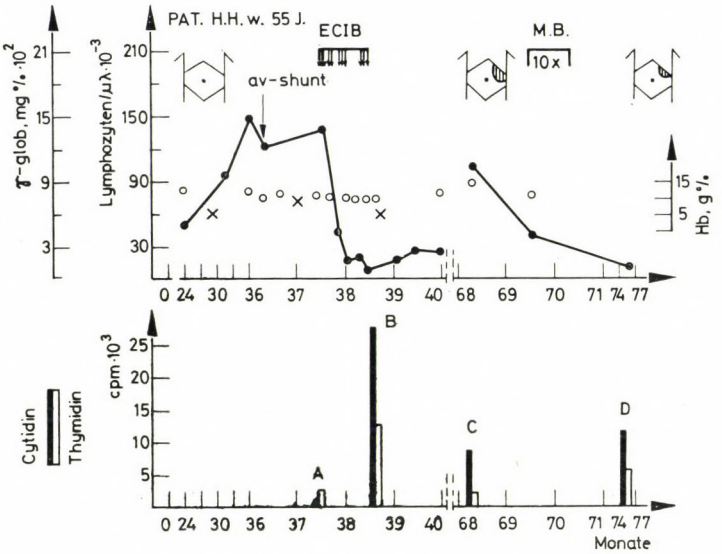

Abb. 1. Die obere Hälfte der Abbildungen zeigt den Verlauf der absoluten Lymphozytenwerte (●), Hämoglobin (○) und absoluten Gamma-Globulin-Werte im Serum (×) während der Erkrankung über der Abszisse mit dem Zeitverlauf seit Diagnosestellung in Monaten. Zu beachten sind Raffungen des Zeitparameters in ambulanten Therapiephasen und Unterbrechungen (‖) in größeren beobachtungsfreien Intervallen. An Therapieformen sind eingetragen: ECIB = extrakorporale Blutbestrahlung; MB = Milzbestrahlung (mit Angabe der Anzahl von Sitzungen); Z = Zytostatische Therapie; ACTH = Tetracosactid.

Auf der unteren Hälfte der Abbildungen ist über der gleichen Zeitachse der ^{3}H-Cytidin- und Thymidin-Einbau in jeweils 10^6 Zellen mit dem jeweiligen Maximalwert (am 3. bis 5. Tag der PHA-Kultur) aufgetragen. Weitere Angaben s. Material und Methoden

Pat. *S. M.*, männlich, 61 Jahre (Abb. 3). Anamnesedauer 4 Jahre, keine Vorbehandlung, keine Organomegalie. Vor Therapie Nukleosideinbau im unteren Normbereich. Totale Depression während ECIB (11 000 rad) und 4 Tage danach. Deutliche Steigerung des Cytidin- und Thymidin-Einbaus in den unteren Normbereich nach 75 Tagen. Nach 130 Tagen wieder deutliche Depression bei gleichzeitigem Lymphozytenanstieg.

Pat. *N. J.*, weiblich, 61 Jahre (Abb. 4). Anamnesedauer 2 Jahre, keine Vorbehandlung, keine Organomegalie. Starker PHA-Defekt vor Therapie. Weitere

deutliche Depression unter intermittierender ECIB mit insgesamt 14 000 rad. Deutlicher Anstieg erst 34 (D) und weiter 128 (E) Tage nach letzter Bestrahlung bis auf das 5fache des Ausgangswertes.

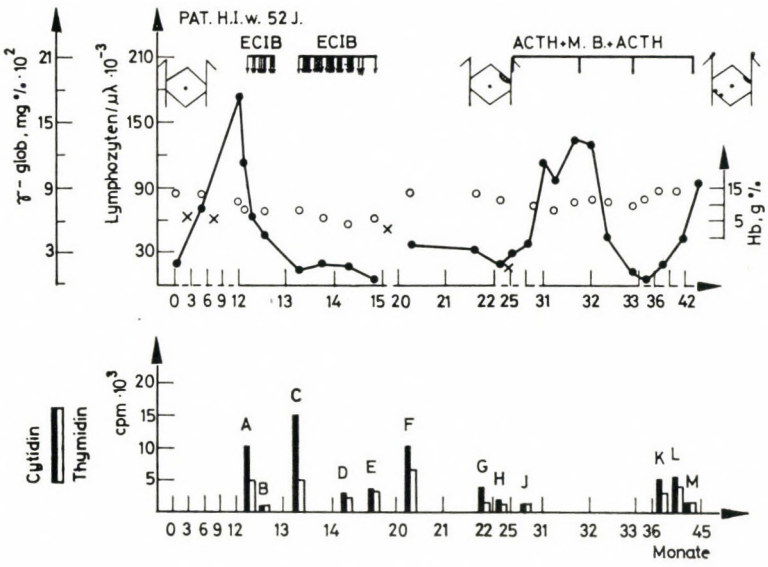

Abb. 2

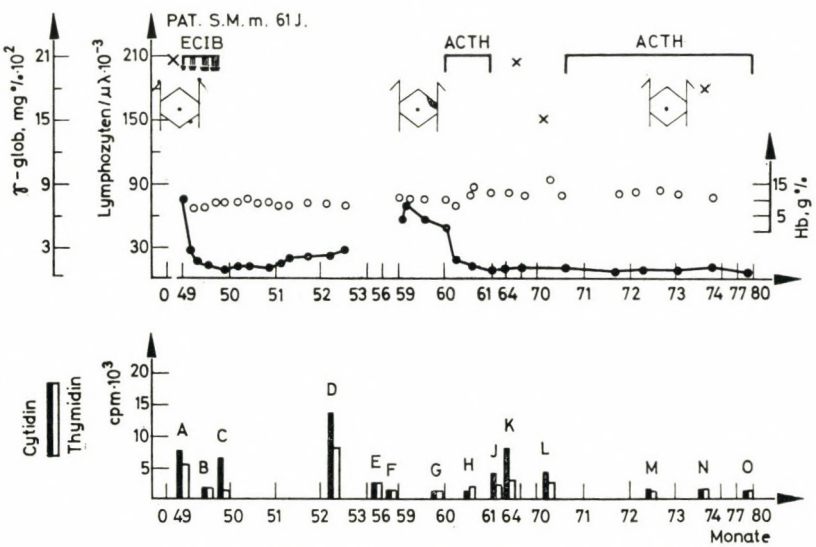

Abb. 3

Pat. *S. A.*, weiblich, 75 Jahre (Abb. 5). Anamnesedauer 1/2 Jahr, keine Milzvergrößerung. Stark erniedrigte Ausgangstransformation. Nach initialer Lymphozytendepletion durch die ersten 18 Bestrahlungen zeigt sich eine deutliche Steige-

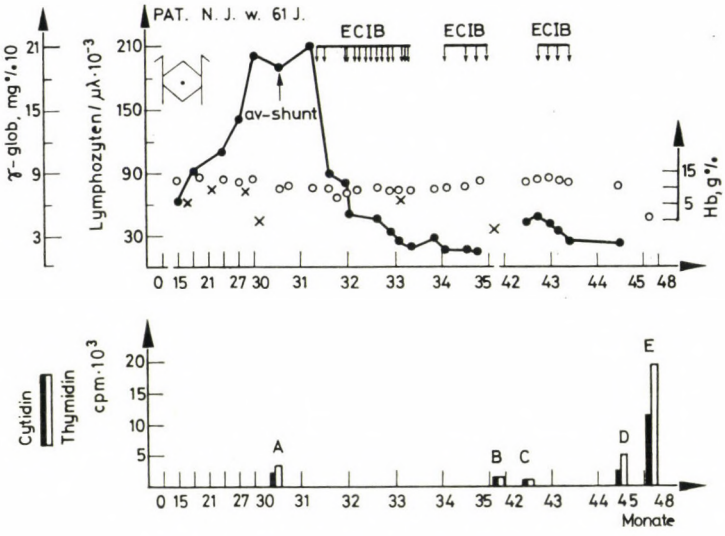

Abb. 4

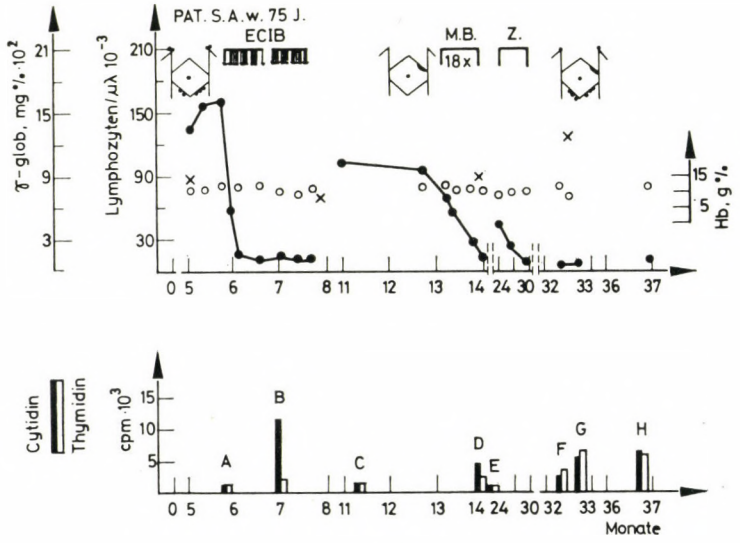

Abb. 5

rung der Nukleosidinkorporation, insbesondere im Cytidin-Wert. Fünf Monate nach Ende der ECIB-Serie wieder stark erniedrigte Werte bei gleichzeitigem Lymphozytenanstieg.

Pat. *B. F.* weiblich, 70 Jahre. Anamnesedauer 4 Jahre, rasch zunehmende Splenomegalie auf 10–15 cm unter dem Rippenbogen. Minimale Ausgangswerte der Nukleosidinkorporation. Keine Reaktion nach 11 ECIB (8000 rad). Weiterer Verlauf durch unbeeinflußbare Hyperspleniesymptomatik ohne Lymphozytenwiederanstieg bestimmt. 240 Tage nach letzter Bestrahlung fortschreitende Erniedrigung der PHA-Transformationswerte.

Pat. *Ho. H.*, männlich, 62 Jahre. Anamnesedauer 7 Jahre. Keine Therapie im Laufe des letzten Jahres. Leukozytenanstieg bei gleichzeitiger Entwicklung eines Splenogigantismus 24 cm unter dem Rippenbogen. Maximale PHA-Depression vor Therapie, die sich während ECIB-Bestrahlungsserie mit insgesamt 11 500 rad noch verstärkt ausgeprägt zeigte. Keine Beeinflussung des Milzwachstums. Rascher Lymphozytenneuanstieg mit einer Verdoppelungszeit von etwa 2 Monaten.

Pat. *D. H.*, männlich, 55 Jahre. Anamnesedauer 4 Jahre. Zwei Jahre zuvor extrakorporale Blutbestrahlung. Starke Leukozytose und Splenomegalie. Deutlicher Transformationsdefekt vor ECIB, der sich während der Bestrahlung deutlich verstärkte. Sieben und 180 Tage nach einer Bestrahlungsserie mit insgesamt 980 rad weiterbestehende starke Depression. Keine Beeinflussung des Milzwachstums. Rascher Leukozytenwiederanstieg mit einer Verdoppelungszeit von etwa 2 Monaten.

Pat. *M. G.*, weiblich, 60 Jahre. Anamnesedauer 2 Jahre. Keine Vorbehandlung. Rascher Leukozytenanstieg im Halbjahr vor Therapie. Starke Depression der PHA-Reaktion vor Therapie, die während und 3 Tage danach noch deutlich ausgeprägter wurde. Dreißig Tage nach ECIB war der niedrige Wert der Ausgangsreaktion erreicht. Die Milz hatte an Wachstum zugenommen. Rascher Anstieg der Lymphozyten mit einer Verdoppelung in etwa 14 Tagen.

Zusammenfassung der Ergebnisse bei extrakorporaler Blutbestrahlung

Für die detailliert analysierbaren 9 ECIB-Fälle lassen sich folgende generelle Feststellungen hinsichtlich der T-Zellaktivität, gemessen am Wert der ^{3}H-Thymidin-Inkorporation machen: Während der Bestrahlungsserie, 24 Std. nach der letzten Bestrahlung untersuchte Zellen zeigten in allen Fällen eine Erniedrigung auf ein Drittel bis die Hälfte des Ausgangswertes.

Nach Ende der Bestrahlungsserie war in 4 Fällen eine Steigerung auf das 1.3- bis 5.5fache, in einem Fall sogar auf das über 8fache zu verzeichnen. Derartige Steigerungen der Thymidin-Inkorporation wurden frühestens nach 10 und spätestens nach 130 Tagen nachgewiesen.

In 4 weiteren Fällen wurde nach der Bestrahlungsdepression der Ausgangswert weder erreicht noch überstiegen.

Klinisch unterschieden sich letztere Fälle von der ersten Gruppe durch massive Splenomegalie und/oder raschen Neuanstieg der Lymphozytenwerte.

Verläufe bei Milzbestrahlung

Die Milzbestrahlung erfolgte mit dem Gammatron, täglich einmal mit 10–20 rad. Die Dauer der Bestrahlung ist den Graphiken zu entnehmen.

Pat. *R. A.*, männlich, 77 Jahre (Abb. 6). Anamnesedauer 1 Jahr. Starker PHA-Defekt vor Therapie (Kultur B). Acht Tage nach Milzbestrahlung bereits deutliche Steigerung von Cytidin- und Thymidin-Inkorporation. Während der nächsten 10 Monate ohne Therapie wieder deutliche Ausprägung des Defektes (D), der sich während der zweiten Milzbestrahlungsserie noch gering verstärkte. 69 Tage nach Ende der Bestrahlungsserie jedoch deutliche Steigerung der Thymidin-Inkorporation (F).

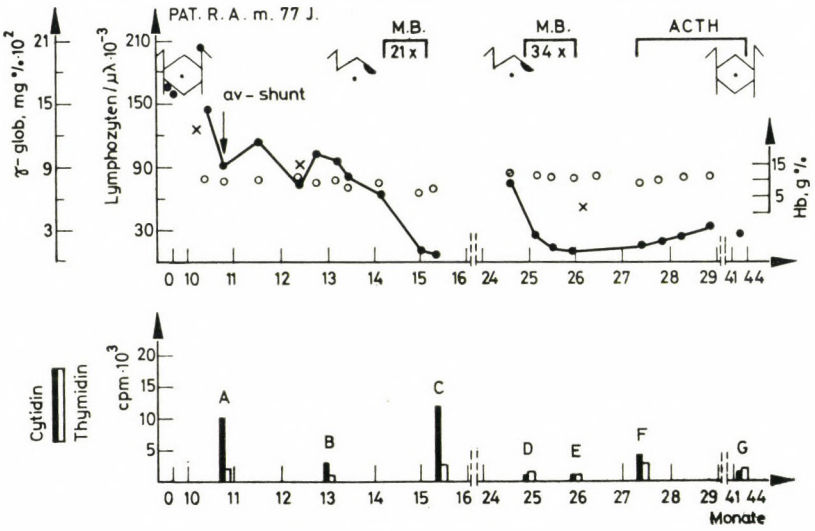

Abb. 6

Pat. *K. F.*, männlich, 62 Jahre. Anamnesedauer 1 Jahr, keine Vorbehandlung. Starker Transformationsdefekt vor Therapie. Bereits einen Tag nach Ende der Milzbestrahlungsserie wurde eine massive Steigerung der Thymidin-Inkorporation bis in den Normbereich festgestellt. Noch nach 480 Tagen auf das 4fache des Ausgangswertes gesteigerte Transformationsfähigkeit ohne weitere Therapie bei niedrig bleibenden Lymphozytenwerten.

Pat. *M. M.*, männlich, 52 Jahre. Anamnesedauer etwa 1 Jahr. Keine Vorbehandlung. Starker initialer Defekt. Neunzehn Tage nach Milzbestrahlung ist Steigerung der Thymidin-Inkorporation in den Normbereich auf das 9fache des Ausgangswertes zu verzeichnen.

Pat. *G. E.*, männlich, 77 Jahre. Anamnesedauer 2 Jahre. Keine Vorbehandlung. Splenogigantismus. Stark ausgeprägter Defekt vor Therapie. Geringe Ver-

besserung bei Ende der Milzbestrahlungsserie, die bereits nach einem Monat wieder abgeklungen war. Nach einem weiteren halben Jahr erneut maximale Defektsituation. Während der zweiten Milzbestrahlungsserie nach 12 Sitzungen mäßige Besserung der Nukleosidinkorporation, die sich auch 4 Tage und 6 Wochen nach beendeter Serie nicht verstärkt. Die Milzgröße blieb ebenfalls unbeeinflusst.

Pat. *S. A.*, weiblich, 75 Jahre (vergleiche Abb. 5). Deutliche Verbesserung besonders der Thymidin-Inkorporation 4 Tage nach Ende der Milzbestrahlungsserie festgestellt (C → D).

Pat. *H. H.*, weiblich, 55 Jahre (vergleiche Abb. 1). Mittelgradiger Defekt (C) vor Milzbestrahlung (200 rad). Deutliche Steigerung in den unteren Normbereich 93 Tage nach Milzbestrahlungsserie festgestellt (D).

Zusammenfassung der Befunde bei Milzbestrahlung

Während der Bestrahlungsserie bestimmte Werte zeigen keine oder nur geringfügige Erniedrigung gegenüber den Ausgangswerten. Nach Ende der Therapieserie wurde in allen Fällen schon nach 1 bis 8 Tagen eine deutliche Steigerung des ^{3}H-Thymidin-Einbaus auf das 2- bis 8fache des Ausgangswertes gemessen. Bei extremer und schwer beeinflußbarer Splenomegalie (GE) war diese Steigerung am geringsten ausgeprägt. Diese Verbesserung der Transformierbarkeit war noch über längere Zeiträume (70, 93, 480 Tage) feststellbar.

Verläufe bei zytostatischer Behandlung

Pat. *S. A.*, weiblich, 75 Jahre (vergleiche Abb. 5). Maximaler Defekt vor Zytostatikserie (E). Über 6 Monate auswärts Einnahme von 8 mg Chlorambucil pro Tag. 43 Tage nach Absetzen der Alkylanz schon Steigerung auf das 3.5fache, die am 56. Tag sich bis in den Normbereich weiterentwickelt hatte (G). Etwa gleichbleibende volle PHA-Reaktion noch 176 Tage nach zytostatischer Therapie.

Pat. *S. J.*, männlich, 66 Jahre. Frisch diagnostiziert. Bei 30 000 Lymphozyten/μl wegen belastender zervikaler Lymphome Therapie mit 10 mg Vinblastin pro 14 Tage. Starker PHA-Defekt vor Therapie. Zehn Tage nach der 10. derartigen Injektion gleiche Depression wie anfänglich. 46 Tage nach Ende der zytostatischen Behandlung 5fache Steigerung des Nukleosideinbaus.

Pat. *D. E.*, männlich, 54 Jahre. Anamnesedauer 4 Jahre. Bei strahlenrefraktärer Splenoorganomegalie und Lymphozytenwerten um 100 000 Einsatz von 5 mg Vinblastin pro Woche und 10 mg Chlorambucil pro Tag. Vor Zytostatikastoß deutliche Erniedrigung, 53 Tage danach Steigerung des Thymidin-Einbaus auf das 2.6fache mit Erreichen unterer Normwerte.

Zusammenfassung der Befunde bei zytostatischer Therapie

In jedem der 3 analysierbaren Fälle wurde etwa 2 – 3 Monate nach Therapie eine Steigerung der ^{3}H-Thymidin-Inkorporation auf das 2.6- bis 6.6fache in der PHA-Kultur nachgewiesen.

Verläufe bei ACTH-Behandlung

Wir verwendeten synthetisches Tetracosactid mit ACTH-Wirkung [22],* wobei 1 ml 1.42 mg Corticotrophin-Hexaacetat in Depotform an Zinkchlorid enthielt. Die maximale Steigerung des Serumcortisolspiegels nach der Injektion von 1 ml wird nach 8 Std. verzeichnet, jedoch liegt sie noch nach 24 Std. über dem Normbereich [27]. Initial verabfolgten wir über 5 Tage täglich 1 ml, um dann auf 2mal wöchentlich 1/2 ml überzugehen. Das zur PHA-Kultur vorgesehene Blut wurde jeweils 3 Tage nach einer Injektion abgenommen. Zu diesem Zeitpunkt war bei Kontrolle des Serumcortisols kein über den Normbereich hinaus gesteigerter Wert nachweisbar.

Pat. *P. A.*, männlich, 71 Jahre. Anamnesedauer 2 Jahre. Bei Lymphozytenwerten um 30 000/μl relativ gute Stimulationswerte. Initialer Einsatz von Tetracosactid 0.5 ml pro Tag 10 Tage lang. Noch 30 Tage nach dieser Serie starke Depression auf 1/3 der Ausgangswerte, die sich auch unter wöchentlich einmal 1 ml in den nächsten 2 Monaten nicht erholte und noch nach 60 Tagen ausgeprägt nachweisbar war. Zunehmende Hyperspleniesymptomatik.

Pat. *S. M.*, männlich, 61 Jahre (vergleiche Abb. 3). Vor erster intensiver ACTH-Serie mit 1/2 ml 2tägig über 4 Wochen maximale Depression (Kultur G), die sich während der Serie, die zu steilem Lymphozytenabfall führte, nicht deutlich erholte und erst 4 Wochen nach letzter Injektion deutliche Verbesserung über den Ausgangswert hinaus zeigte. Nach 46 Tagen war eine Verdreifachung des Thymidin-Einbaus festzustellen. Dieser noch nach 1 1/2 Jahren relativ gute Wert (L) wurde durch eine eingesetzte intermittierende Therapie mit 2mal wöchentlich 1 bis 0.5 ml stark deprimiert und erholte sich trotz konstant niedriger Lymphozytenwerte im Laufe des folgenden Halbjahres nicht mehr (NO).

Pat. *R. A.*, männlich, 77 Jahre (vergleiche Abb. 6). Vor ACTH-Therapie mit 1 ml pro Woche gute Ausgangswerte. Nach einjähriger Dauer dieser Therapie unter kompensiert gehaltenen Lymphozytenwerten deutliche Erniedrigung der Nukleosidinkorporation.

Zusammenfassung der Ergebnisse bei ACTH-Therapie

In allen Fällen fand sich eine Depression bis auf ein Drittel des anfangs gemessenen Thymidin-Einbaus während des Verabfolgungszeitraums mit intermittierenden Injektionen. In einem nach Absetzen untersuchten Fall folgte nach 12 und 46 Tagen eine Steigerung auf das 2- bis 3fache bei anhaltend niedrigen Lymphozytenwerten.

Diskussion

Die PHA-Stimulierbarkeit der Lymphozyten, gemessen an ihrer ^{3}H-Thymidin-Inkorporation *in vitro* läßt sich als direktes Maß der T-Zellaktivierung deuten [25]. Die stark erniedrigte und meist verzögert einsetzende Reaktion bei CLL-

* Cortrophin-S-Depot®

Patienten erklärt sich dadurch, daß die überwiegende Mehrheit der Lymphozyten bei CLL B-Zellcharakteristika trägt [1, 51]; die noch erhaltene PHA-Reaktion läßt sich auf eine Restpopulation normaler T-Zellen zurückführen, wie sie Pentycross [31] forderte und Wybran und Chantler [52] isolieren konnten. Dies entspricht den Befunden normaler Kinetik der CLL-Blasten *in vitro* [30]. Verschiedene Untersuchungen [12, 52] weisen darauf hin, daß kein absoluter quantitativer T-Zellmangel vorliegt, sondern daß der T-Zelldefekt auch bei übernormal hoher absoluter Menge auf eine starke Verdünnung ihrer Konzentration durch B-Zellen zurückzuführen ist.

Die in unseren Befunden gezeigten Veränderungen der PHA-Reaktivität während und nach verschiedenen Therapien erlauben folgende Schlüsse, die am ausführlichsten am Modell der ECIB diskutiert werden sollen.

Die *während ECIB* beobachtete Reduktion der PHA-Reaktion erklärt sich naheliegenderweise dadurch, daß noch therapiegeschädigte Zellen zirkulieren. Die Bestrahlung erfolgte während der Therapieserie durchschnittlich ca. 20 Std. vor Abnahme des Blutes und dürfte den größten Teil des zirkulierenden Pools jeweils erfasst haben. Evans und Norman [15] zeigten nach 24 Std. Absterben bestrahlter normaler Lymphozyten *in vitro* zu 50%. Auch die Untersuchungen von Bremer und Mitarb. [10] weisen darauf hin, daß nach einer Transitdosis von 400 rad, die über das Doppelte der von uns gewählten betrug, nur 34 bis 70% der Lymphozyten akut aus der Zirkulation ausscheiden. Zumindest 20 Std. nach Bestrahlung ist also mit einem hohen Anteil strahlengeschädigter Lymphozyten in der Zirkulation zu rechnen. Da aber weder Ausmaß der Strahlenschädigung der T-Zellen noch ihr Anteil an der Gesamt-CLL-Population kalkulierbar ist, läßt sich keine Aussage über den Grad der Strahlensensibilität der erhaltenen T-Zellfraktion machen.

Zehn Tage nach Bestrahlung ist eine weitgehende Elimination strahlengeschädigter Zellen anzunehmen [45], so daß der Anstieg der PHA-Reaktion in diesem Zeitraum nach ECIB (H. H., S. A.) einer relativen Zunahme funktionstüchtiger T-Zellen entspräche. Schon Schrek und Stefani [39, 44] zeigten gesteigerte Strahlenresistenz PHA- bzw. tuberkulinsensibler Zellen und als solche wären ja die T-Zellen gegenüber den übrigen Zellelementen der CLL aufzufassen. Die hohe Strahlensensibilität der Masse der CLL-Lymphozyten gegenüber einer Normalpopulation (mit also nahezu 70% T-Anteil) ist bekannt [23, 24].

Daneben haben die T-Zellen zirkulationskinetische Vorteile gegenüber den CLL-B-Zellen: Binet und Mitarb. [5] und Bremer und Mitarb. [9] konnten bei CLL eine starke Störung der Rezirkulation markierter, transfundierter Lymphozyten über den *Ductus thoracicus* zeigen, was den Befunden von Hersey [20] entspricht, wonach die intravasale Masse der CLL-Lymphozyten in schlechtem Austausch mit extravasalen Speichern steht. Flad und Mitarb. [17] präzisierten diese Befunde dahingehend, daß die Zellen, die noch zur Rezirkulation über den *Ductus thoracicus* bei CLL fähig sind, sich vorwiegend wie normale T-Zellen verhalten, während die Rezirkulationshemmung sich nach Huber [21] auf die CLL-B-Fraktion beschränkt. Somit könnten sich die T-Zellen selektiv durch Austritt aus der Blutbahn und den weiteren Rezirkulationsprozeß einer Blutbestrahlung entziehen.

Wie groß der Anteil der möglichen Mechanismen (Strahlenresistenz, etwa durch verbesserte DNS-Reparation [28], oder zirkulatorisches Entkommen) an einer relativen Zunahme des T-Zellanteils nach Bestrahlung ist, muß offenbleiben. Das Argument der Strahlenselektion durch zirkulatorischen Vorteil gewinnt jedoch an Gewicht, seit Curtis und Mitarb. [12] und Stacher und Mitarb. [42] bei Depletion mittels Leukophorese, also ohne die Möglichkeit von kerngebundener Strahlenselektion, starken Anstieg der PHA-Antwort bis in den unteren Normbereich fanden.

Für die *zu späteren Zeitpunkten nach ECIB* (75, 128, 132 Tage) beobachteten Steigerungen der PHA-Reaktion gegenüber Ausgangswerten, insbesondere aber für den leider zu selten festgehaltenen Anstieg zu späteren gegenüber früheren Zeitpunkten (N. I.: D–E; S. M.: C–D; vergleichende Tabelle) muß als weiterer Faktor eine raschere Regeneration der T-Zellfraktion gegenüber den pathologischen CLL-Zellen erwogen werden, wie es Kagan und Johnson [26] bei Ganzkörperbestrahlung und Astaldi und Mitarb. [2] nach Röntgenteilbestrahlung annahmen. Die langen Umsatzzeiten der Lymphozyten bei CLL insgesamt sind öfters beschrieben worden [50]. Bei den Patienten S. M. und R. A., die nach ECIB und Milzbestrahlung guten PHA-Anstieg boten, erbrachten kinetische Untersuchungen [50] eine durchschnittliche Lymphozytenumsatzzeit von 270 bzw. 420 Tagen, wobei für ca. 95% aller Zellen noch deutlich längere und für 5% eine deutlich kürzere (etwa 20tägige Umsatzzeit) zu veranschlagen war. Da auch Rieke und Schwarz [32] und Stayner und Schwarz [43] im Tierexperiment die stärkste Stimulierbarkeit in der kurzlebigsten Fraktion fanden, könnte raschere Regenerationsfähigkeit der erhaltenen und sich gegenüber den entarteten CLL-B-Zellen wohl selbst regulierenden T-Population in den gefundenen Zeiträumen einen meßbaren Anstieg des T-B-Quotienten und damit der PHA-Reaktivität bringen, bis dieser Effekt in individuell unterschiedlichen Zeiträumen [38] durch die zahlenmäßig mindestens zehnmal so große Produktion der langsam regenerierenden CLL-B-Zellen [37,50] verdünnt wird. Diese zellkinetischen Bewegungen können Anstieg und Abfall der PHA-Reaktion nach Depletion bestimmen. Damit stimmt überein, daß die relativ splenomegalen Fälle (M. G., Ho. H., D. H.), in denen der Anstieg der T-Zellaktivität nach ECIB ausblieb, raschen Wiederanstieg der Lymphozytenwerte boten als Zeichen einer deutlich anderen Kinetik als der für S. M. und R. A. exemplarisch gezeigten.

Fröhlich und Bock [18] haben mit unseren Befunden übereinstimmend in 2 langsam progredierenden Fällen ohne stärkere Splenomegalie 2 und 5 Wochen nach ECIB eine verbesserte PHA-Reaktion gefunden.

Die im Zusammenhang mit ECIB diskutierten Möglichkeiten zur Erklärung einer gesteigerten T-Zellaktivität nach Behandlung (unterschiedliche Strahlensensibilität, zirkulationskinetische oder proliferationskinetische Unterschiede) lassen sich auf die übrigen therapeutischen Verfahren anwenden:

Im Gegensatz zur ECIB war *während der Milzbestrahlungsserien* keine Verminderung der PHA-Reaktion festzustellen, was sich aus der weit geringeren Menge von Lymphozyten erklärt, die mit niedrigen Dosen pro Behandlung

getroffen werden. Hinsichtlich der rasch eintretenden starken Steigerung schon 1 bis 8 Tage *nach der letzten Milzbestrahlung* läßt sich nicht entscheiden, ob Strahlenselektion oder Poolverschiebungen [36] sie bedingen. Unsere Befunde entsprechen den Ergebnissen von Astaldi und Mitarb. [3], die bei 4 Patienten schon wenige Tage nach jeder relativ hoch dosierten Milzbestrahlung deutliche Anstiege des PHA-Blastenindex fanden.

Die konstant *nach Chemotherapie* nachweisbaren Anstiege lassen sich kaum durch Zytostatikaresistenz der T-Fraktion erklären, für die es keine experimentellen Hinweise gibt [40]. Vielmehr wurden die Verbesserungen wie bei Bouroncle et al. [8] zu Zeitpunkten festgestellt, zu denen sich eine T-Zellpopulation in den oben angegebenen raschen Umsatzzeiten gegenüber einer langsameren B-Population expandiert haben könnte, d. h. ein kinetischer Vorteil der T-Fraktion käme auch hier zutage, wo die Regenerationsfähigkeit der Population nach Zerstörung der Produktionsstätten besonders Gewicht hat.

Die Depression unter Dauertherapie mit ACTH-Präparaten entspricht dem bekannten immunosuppressiven Effekt der Nebennierenrinden-Steroide ganz allgemein und weist auf die spezielle Sensibilität der T-Zellpopulation gegenüber den Stimulationsprodukten des ACTH hin, wie sie schon die Befunde über Thymusinvolution unter ACTH und Prednisolon [14] zeigten. Nach Absetzen manifestiert sich hier offenbar eine funktionelle Entblockung oder ein kinetischer Vorteil der T-Zellpopulation (Patient S. M.), so lange die B-Zellpopulation die typisch langsamen Umsatzzeiten zeigt.

*

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Influence of Different Kinds of Therapy on T-Cell Activity in Chronic Lymphadenoses

The changes of T-cell activity in PHA-culture of 16 patients before, during and after 21 therapeutic series of extracorporeal irradiation of blood (ECIB) were significantly advantageous in cases of slow progress and without definite organomegaly. After spleen irradiation and after cytostatic therapy the PHA reaction was enhanced in all cases. During the therapeutic series, PHA depression caused by ECIB, cytostasis and ACTH was considerably enhanced; the weakest effect was recorded from spleen irradiation. The positive effect on T-cell activity is ascribed to the significant improvement of circulation and the regeneration of the T-cell fraction. The simultaneous follow-up of CLL kinetics allowed some therapeutical conclusions. Depletion therapy (ECIB or leucophoresis) should be applied in slow accumulative cases, while spleen irradiation is indicated in the presence of splenomegaly, and cytostatics should be applied when clinical signs of rapid cell proliferation manifest themselves [46, 48]. Alkylating agents and ACTH administered for compensation should be applied in the frame of an effective short therapy to stimulate cellular immunity. The immune defect of CLL should not be enhanced by prolonged therapy.

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Genetic Control of Haemoglobin Alpha-chain Synthesis

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Another field in haematology to which Dr. Hollán has made an important contribution is the elucidation of the genetic control of haemoglobin synthesis, in which her main concern was with the possible duplication of the locus controlling the synthesis of the α-chains. It therefore seems appropriate to review this subject in this issue of Haematologia.

There are three distinct stages in human development and each of these has its own types of haemoglobin [16]. The embryonic haemoglobins, Hb-Gower 2 ($\alpha_2\varepsilon_2$), Hb-Gower 1 ($?\xi_2\varepsilon_2$) and Hb-Portland ($\xi_2\gamma_2$) are followed at about eight weeks' gestation by foetal haemoglobin ($\alpha_2\gamma_2$) and this is replaced after birth by the adult haemoglobin Hb-A ($\alpha_2\beta_2$) and Hb-A_2 ($\alpha_2\delta_2$). Thus α-chains are produced at all stages of development while there are specific non-α-chains (ε, γ, β and δ) for each stage in development. The synthesis of each of these haemoglobin polypeptide chains is controlled by its own structural locus and is outlined in Figure 1. Recent work [28] has shown that there are two types of γ-chains which only differ by the replacement of a glycine residue by alanine at position 136 of the amino acid sequence. As all individuals have these two types of γ-chains there must be (at least) two loci controlling the synthesis of γ-chains, the γ-Gly locus and the γ-Ala locus. Their presence is confirmed by the finding that abnormal foetal haemoglobins have only either γ^{136Gly}- or γ^{136Ala}-chains and this explains the finding that γ-chain variants have never exceeded 25% of total γ-chain production. If it is assumed that the four genes (at the two γ-loci) lead to the production of approximately equal amounts of gene product (i.e. γ-chains), then one mutant gene would lead to the production of ca. 25% abnormal haemoglobin. These γ-loci are probably linked to the β-locus. At the present time there is no classical genetic evidence for this but it is strongly suggested by the close linkage between the "High Foetal Gene" and the β-locus as well as by the occurrence of a "cross over" haemoglobin between the γ and β-chains, namely Hb-Kenya [8, 17].

The locus controlling the synthesis of β-chains is known to be single because the homozygous (or double heterozygous) occurrence of β-chain abnormal haemoglobins leads to the complete absence of normal β^A-chain synthesis. However, an apparent duplication of the β-chain locus has occurred as a result of the "Anti-Lepore" cross over giving rise to Hb-P-Nilotic [3]. In one family [10, 22] one

person carried Hb-A, Hb-S, Hb-P and Hb-A_2, and was therefore synthesizing β^A-, β^S-, and δ-chains as well as the $\beta\delta$-chain of Hb-P. Family studies showed that the $\beta\delta$-chain was linked to the β^A-chain.

The locus controlling the synthesis of the δ-chains can of course be considered a duplication of the β-locus leading to the formation of the minor adult component Hb-A_2. This locus is closely linked to that for the β-chains [27] and the corresponding "cross over" haemoglobins $\delta\beta$- (Lepore) type and $\beta\delta$- (anti Lepore) type have been described.

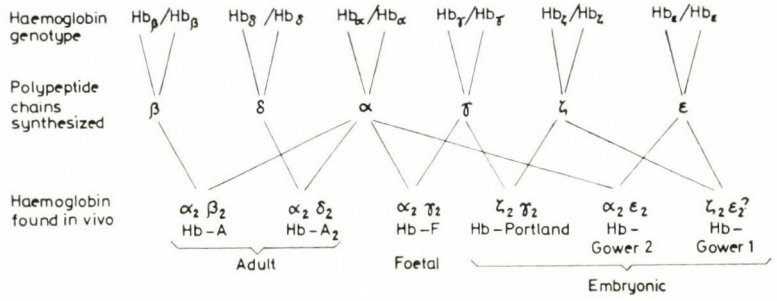

Fig. 1. The genetic control of the synthesis of normal adult haemoglobins, foetal haemoglobin, and the embryonic haemoglobins. There is good evidence that the Hb_γ-locus is duplicated (see text); the Hb_α-locus may also be duplicated (see text). The structure of Hb-Gower 1 is not yet established

α-chains are produced at all stages of development and combine with the non-α-chains to form Hb-Gower 2 ($\alpha_2\varepsilon_2$), Hb-F ($\alpha_2\gamma_2$) as well as Hb-A ($\alpha_2\beta_2$) and Hb-A_2 ($\alpha_2\delta_2$). Classical genetic studies have provided evidence both for and against the possible occurrence of a duplication of the α-chain locus. The suggestion for the existence of a duplicated α-locus arose out of the finding that some α-chain variants occurred in low proportion in the red cell and its possible occurrence has been used to explain the variability of α-thalassaemia. In this paper the data relevant to these three areas will be reviewed.

Genetic studies

There are several genetic studies relevant to the problem of duplication of the α-chain locus. Some of these indicate the presence of more than one α-locus, while others indicate that there is only one such locus.

(a) *Hungarian Family*

The family described by Hollán and her coworkers [15] (Fig. 2) contains three brothers who each carry three haemoglobins, Hb-A ($\alpha^A\beta_2^A$), Hb-Buda ($\alpha_2^B\beta_2^A$) and Hb-Pest ($\alpha_2^P\beta_2^A$). As these individuals are synthesizing three α-chains

(α^A-, α^B- and α^P-chains) they must have three genes controlling α-chain synthesis, i.e. two genetic loci: α^A/α^B, α^A/α^P, one gene at each locus being mutated. The proportions of the three haemoglobins, Hb-A (54%), Hb-Buda (23%) and Hb-Pest

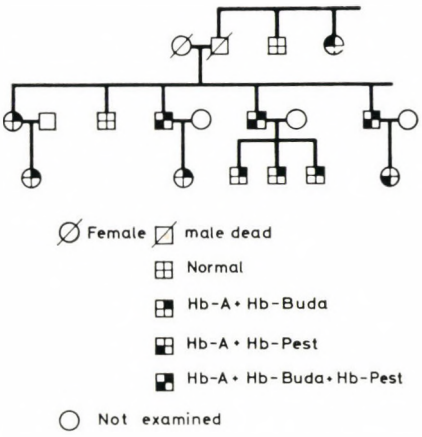

Fig. 2. Part pedigree of the Hungarian family with two haemoglobins with abnormal α-chains as well as Hb-A in some individuals

(21%), with twice as much Hb-A as each of the α-chain variants, are consistent with this interpretation. The family studies show no cross over in five opportunities and this suggests that the two α-chain loci present in this family may be linked.

(b) *Haemoglobin Constant Spring*

This abnormal haemoglobin forms about 1% of total haemoglobin in heterozygotes and consists of a normal α-chain to which 31 amino acids are added at the C-terminal end [9, 26]. This abnormal haemoglobin is commonly found in areas where α-thalassaemia is also common and is present in a proportion of patients with Hb-H disease. Studies [12] show that Hb-Athens, Hb-Thai, Hb-X-Malaysia, Hb-Hong Kong are identical with Hb-Constant Spring and the description of Hb-Koya Dora (Hb-KD) [6] strongly suggests that it is a further example of Hb-Constant Spring.

Haemoglobin Constant Spring has been described in the heterozygous state, homozygous state, in association with the α-thal-1 gene and together with an α-chain abnormal haemoglobin.

The *simple heterozygote* has no clinical abnormality, there is mild microcytosis, but no anaemia. Hb-CS amounts to ca. 1% of total haemoglobin.

The *homozygous state for Hb-CS* has only been described once [23]. In this individual 2–3% Hb-γ_4 together with Hb-F and Hb-A was present at birth;

later 6% Hb-CS was found as well as Hb-A and Hb-A_2, but no Hb-H; the patient had the clinical and haematological features of mild thalassaemia.

The *doubly heterozygous condition* Hb-CS/α-thal-1 leads to a form of Hb-H disease. These patients show mainly Hb-A, some Hb-β_4 (Hb-H) and ca. 2–3% Hb-CS (small amounts of Hb-A_2 and traces of Hb-γ_4 are also found).

The occurrence of Hb-CS with an *α-chain abnormal haemoglobin* (Hb-KD/Hb-Rampa) has also been described [6]. These individuals had Hb-A, Hb-Rampa and ca. 2% Hb-CS (Hb-KD).

In the last three genetic situations the results indicate that the chromosome responsible for the synthesis of the α-chains of Hb-CS also directs the synthesis of the α-chains of some Hb-A. In the homozygous state both Hb-A and Hb-CS, i.e. α^A- and α^{CS}-chains, are produced by each chromosome. In the double heterozygote Hb-CS/α-thal-1, the gene product, i.e. α-chain production, of one chromosome is completely suppressed by the α-thal-1 gene, thus both the α-chains of Hb-A and Hb-CS are gene products of the other chromosome. In the interaction of Hb-A, Hb-Rampa, Hb-CS three α-chains are produced (presumably Hb-A and Hb-CS by one chromosome and Hb-Rampa by the other). These findings can be explained in several ways. The simplest explanation is that the α-chain locus on the CS affected chromosome is duplicated but that there is no difference in the amino acid sequence of two genes (which is not surprising when it is remembered that the two γ-loci can only be distinguished by a single amino acid difference). One of the chain terminating codons (UAA)* of one of these loci is then mutated to one for GLN (CAA).* The small amount of Hb-CS found suggests that this second α-chain locus is produced by a minor locus (like the δ-locus of Hb-A_2) or it may be that the absence of a chain terminating codon slows up synthesis.

The production of α^A-chains and elongated α^A-chains (i.e. α^{CS}-chains) can also be explained by a mutation to a minor (or normally unused) Gln codon and the presence of a suppressor tRNA which reads this triplet for chain termination. (Alternatively, the mutation could be to an unused or minor chain terminating codon with a suppressor tRNA which reads this for Gln.) This kind of mutation has not yet been described in mammalian systems and is therefore purely speculative.

(c) *Haemoglobin-J Tongariki*

Haemoglobin-J Tongariki is an α-chain variant ($\alpha_2^J\beta_2^A$) which occurs with a measurable frequency in several Melanesian islands [4, 7, 14]. It has been found in both the heterozygous and homozygous form. The heterozygotes for Hb-J Tongariki have about 40–45% abnormal haemoglobin and appear clinically and haematologically normal. To date five homozygotes have been found

* The codons involved can be identified from the sequence of the frame shift mutant Hb-Wayne [25].

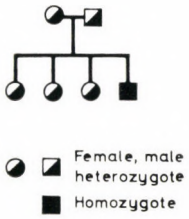

Fig. 3. Distribution of the proportions of α-chain and β-chain variants in heterozygotes as a percentage of total haemoglobin (Data from de Jong [19] and the literature)

[1, 5] one of whom has been proved by family studies (Fig. 3). Haematological studies of these individuals were normal and more specifically did not show any evidence of thalassaemia minor. On electrophoresis only Hb-J Tongariki ($\alpha_2^J\beta_2$) and Hb-J_2 ($\alpha_2^J\delta_2$) were found, Hb-A and Hb-A_2 being absent. Thus these individuals are only making α^J-chains and carry only one α-locus.

(d) *Haemoglobin-G Ibadan*

Hb-G Ibadan is another α-chain variant and was found in a mother and her daughter [30]. Both individuals were haematologically normal. The daughter had Hb-A + G, with 45% abnormal haemoglobin. She is presumably heterozygous for the Hb-G_α trait. The mother carried only Hb-G ($\alpha_2^G\beta_2$) and Hb-G_2 ($\alpha_2^G\delta_2$), no Hb-A or Hb-A_2 being found. This can be explained by the interaction of the Hb-G_α-trait with the α-thal-1 trait (see later). Another, more likely explanation is that she is homozygous for the Hb-G_α-trait and as she is only synthesizing α^G-chains she carried only one α-chain locus.

(e) *Hb-Q-H disease (Hb-Q/a thal-1)*

In individuals with this condition Hb-Q ($\alpha_2^Q\beta_2$) and Hb-H (Hb-β_4) as well as Hb-Q_2 ($\alpha_2^Q\delta_2$) are found, Hb-A and Hb-A_2 (i.e. α^A-chain synthesis) being completely absent. One parent has Hb-A + Q trait whilst the other has α-thal-1 trait [11, 24, 31]. The α-thal-1 gene completely suppresses α-chain synthesis due to the affected chromosomes and homozygotes synthesize no α-chains [33]. Thus in the Hb-Q-H situation α-chain synthesis due to one chromosome is completely suppressed while the other chromosome directs only the synthesis of α^Q-chains (α^A-chains being absent). There is therefore only one α-locus on the chromosome carrying the Hb-Q mutation.

The proportion of abnormal haemoglobin in heterozygotes

It has been suggested that the relatively low proportions of α-chain variants compared to β-chain variants found in heterozygotes is due to the presence of two separate α-chain loci [21]. On this hypothesis one of the four genes (two

at each locus) coding of α-chain is mutated leading to a mutant gene product of 25% (or less) of the total.

In Fig. 4 the proportion of abnormal haemoglobin in α- and β-chain variants is compared. It can be seen that both have a wide distribution but that the average for α-chain variants is distinctly lower than that for β-chain variants. However,

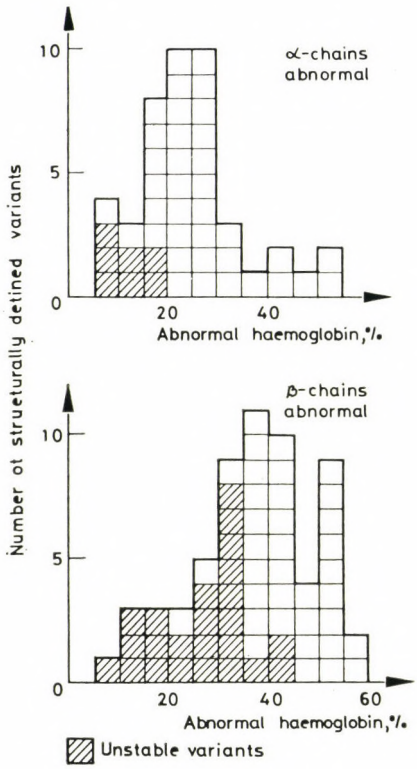

Fig. 4. Part pedigree of family with Hb-J-Tongariki showing a homozygote (from Beaven et al. [4])

a significant number of α-chain variants occur in a proportion higher than 25%. On the two loci hypothesis this requires explanation and it has been proposed that any α-chain mutants which have a higher proportion of abnormal haemoglobin in their cells will have the postulated linked second α^A-locus suppressed by an α-thalassaemia gene [22, 32]. Thus one might expect the proportions of α-chain mutants in heterozygotes to have a biphasic distribution but that does not appear to be the case (Fig. 4). The finding of only Hb-J Tongariki or Hb-G Ibadan in the homozygotes is similarly explained.

To support this idea French and Lehmann [13] have examined carriers of Hb-G Philadelphia ($\alpha_2^G\beta_2^A$), which forms more than 40% of total haemoglobin

in these individuals, and suggest that mild haematological changes of α-thalassaemia are present. However, no haematological abnormality was found in the heterozygotes or homozygotes (on the two loci hypothesis two α-thal-2 genes would be involved) for Hb-G_α Ibadan [30] or Hb-J_α Tongariki [1,5]. Perhaps one should remember that $1 = 2 - 1$ and that for practical purposes the deletion or absence of a second locus is the same if no disease is found in the relevant homozygote. Studies of the relative amounts of normal and abnormal α-chains and of β-chains synthesized in the various reported syndromes would help to resolve this problem (see below).

On the one locus hypothesis the lower average proportion of α-chain variants found in heterozygotes compared with the proportion found in β-chain variants requires explanation. There is evidence that the α- and β-chains are released from the ribosomes independently and that α-chain synthesis is somewhat in excess of β-chains, any α-chains not combining with β-chains being rapidly destroyed in the cell [18]. The lower proportion of α-chain mutants can then be explained by assuming that the available β^A-chains combine with normal α^A-chains in preference to abnormal α-chains. Thus Hb-A would be formed in preference to mutant haemoglobin.

Further work to solve this problem lies in studying haemoglobin synthesis in α-chain variants forming less than 25% of total haemoglobin to determine whether the cells make only 25% mutant α-chains or 50% mutant α-chains. Such experiments must of course take in account the rapid intracellular destruction of the normal excess of α-chain synthesized.

Discussion

Examination of the genetic evidence regarding the number of α-chain loci is confusing. On the one hand there is the Hungarian family with clear evidence of duplication, while on the other the Hb-J Tongariki and Hb-G_α homozygotes argue equally forcefully against there being such a duplication. At the present time it is not possible to say which of these is the exception or which is the more common situation and description of further families in different ethnic groups is needed. The occurrence of Hb-CS which is always associated with a clinical picture of mild α-thalassaemia argues that α-chain duplication is the rule in the affected population provided that it can be shown that Hb-CS is not due to a mutation at the haemoglobin locus associated with a suppressor. The segregation of Hb-CS in families is of course important in the investigation of this problem. Haemoglobin synthesis studies of Hb-CS using tRNA from different sources would also help to resolve this problem. However, on the present evidence the occurrence of Hb-CS is best interpreted as a duplication of the α-chain locus in the affected population. Against this idea is the data from the families with Hb-Q-H disease which occur in some of the same populations. In these families the chromosome carrying the Hb-Q mutation only carries a single α-chain locus.

These confusing observations suggest that there may be considerable heterogeneity in the number of α-chain loci. In the Hungarian family there is an extra major locus; in the Melanesian population there is only a single α-chain locus. In the areas where α-thalassaemia and Hb-CS are common, both duplicated and unduplicated α-loci may occur.

Can we get any further information from the proportions of α-chain mutant haemoglobins? Unfortunately, as already pointed out, the data can be explained by differences in the assembly of mutant and normal haemoglobin. Here studies of haemoglobin synthesis on a number of cases would be of help.

α-thalassaemia. One way to explain the heterogeneity of the α-thalassaemia syndromes is by the presence of two α-chain loci. On this hypothesis α-thal-1 which completely suppresses α-chain synthesis due to the affected chromosome is due to the presence of two α-thalassaemia genes on it, while α-thal-2 which only partially suppresses the α-chain synthesis due to the affected chromosome has one α-thalassaemia gene and one normal α-gene on it. The various reported α-thalassaemia syndromes can then be categorized as follows.

Phenotype	Proposed genotype	
	one α-locus	two linked α-loci
α-thal-1 trait t = complete suppression	α^t/α^A	$\alpha^t\alpha^t/\alpha^A\alpha^A$
α thal-2 trait t = partial suppression	α^t/α^A	$\alpha^t\alpha^A/\alpha^A\alpha^A$
α-thal-1 homozygote	α^t/α^t	$\alpha^t\alpha^t/\alpha^t\alpha^t$
Hb-H disease α-thal-1/α-thal-2	α^t/α^t	$\alpha^t\alpha^t/\alpha^t\alpha^A$

Without further information both models appear equally valid.

The reported patients with Hb-Q-H disease (α^Q/α-thal-1) and Hb-I/α-thalassaemia (Hb-I 80%, Hb-A 20%, α^I/α-thal-2) [2] are more easily explained on the basis of a single α-chain locus.

Comparison with the situation in β-thalassaemia is also relevant. That the synthesis of β-chains is controlled by a single locus is well documented and accepted, while the heterogeneity of β-thalassaemia is well known, different mutations giving rise to genes which either completely suppress β^A-chain synthesis or allow some β^A-chain synthesis to continue. For example in Hb-S-β-thalassaemia three distinct situations are found – no Hb-A synthesis, 10–15% Hb-A, and 20–30% Hb-A [29]. In this case different mutations give rise to three degrees of suppression of the (single) β-gene involved. These findings suggest that there is no *a priori* reason to postulate a duplication of the locus controlling synthesis of α-chains to explain the heterogeneity of the various α-thalassaemia syndromes.

However, the findings that the presence of Hb-CS is phenotypically similar to the α-thal-2 gene and that the affected chromosome directs the synthesis of both α^{A}-chains and α^{CS}-chains argues in favour of the two α-loci hypothesis. The cause of the heterogeneity of α-thalassaemia is therefore still undecided.

Conclusion

Although there is clear evidence for the occurrence of duplication of the α-haemoglobin locus in some families, there is also equally convincing evidence in other families against this idea. To decide which is the most common or what is the situation in individual populations further critical genetic data, as well as further data on the synthesis of α-chain mutant haemoglobins are required.

*

I am grateful to Dr. Beaven for help in compiling Figure 3.

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Beta-thalassaemia in Cuba

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The β-thalassaemia trait was found in 7 out of 961 secondary school students of Havana. β-thalassaemia heterozygotes were also found among patients admitted to the Haematological Departments of two hospitals. Haematological and clinical data of these subjects, as well as of cases of thalassaemia major and sickle-cell thalassaemia treated in the hospitals are given. These findings are discussed and compared with data from other countries.

Introduction

The Cuban population originates mainly from two racial groups, Caucasoids and Negroes. The Caucasoid population is almost completely of Spanish origin, the Negroes are descendents of slaves imported from Africa. The African origin is well represented by the high frequency of biochemical traits such as HbS and the A variants of G6PD, which have extensively been studied in the last few years [6, 10, 17]. On the other hand, little is known about the prevalence of β-thalassaemia, mainly because of the difficulties of its diagnosis in heterozygotes.

The presence of β-thalassaemia in Cuba can be predicted from the frequencies found in Spain and Africa, but it is impossible to anticipate its incidence because the available data are incomplete and indicate a great variability of the frequency of the β-thalassaemia gene in these regions [5, 9, 11, 13, 20].

Moreover no reliable data are available concerning the regions of origin of the Spaniards and Africans who emigrated to Cuba in the past centuries.

Until now only one case of Cooley's anaemia has been reported in Cuba [4]. During the last three years we found some other cases in the William Soler Children's Hospitals of Habana, suggesting that this disease was more frequent than previously thought.

It was thus decided to investigate the problem by analyzing all the cases of anaemia of unknown aetiology treated in the haematological units of an adult's and a children's hospital. Having found that most of them were β-thalassaemia carriers, we studied a random population to obtain quantitative data on the incidence of the condition. Results of these investigations will be reported below.

Materials and Methods

Source of samples. Patients displaying anaemia of unknown origin, admitted to the haematology departments of E. Cabrera adult's hospital and the William Soler children's hospital in the last three years were studied together with their families. In addition, 961 secondary school students ranging in age from 11 to 15 years, coming from different districts of Habana City were studied.

Collection of samples. Blood was obtained in all cases by vein puncture, and collected in a tube containing EDTA as anticoagulant, for the determination of the red cell indexes. For other haematological analysis blood was collected in a heparinized tube. For iron determination, blood was collected in a third tube without anticoagulant.

All the tests were performed within 12 hours after sampling.

Routine haematological data. Red blood cell counts were performed in an automatic counter (TOA Microcell Counter Mod. CC-1002) in whole blood at 1 : 50,000 dilution. Haemoglobin concentration was determined by the cyanmethaemoglobin method [16] and packed cell volume by a micromethod, using capillary tubes sealed over flame and centrifuged at 8000 r.p.m. for 15 minutes. Osmotic resistance was determined by a standard method [7].

Haemoglobin electrophoresis. A rapid screening test with starch gel and Poulik buffer at pH 8.6 was used, as described previously [17]. Quantitation of HbA and HbS was made by electrophoresis [12].

Determination of HbA_2. Quantitative determination of HbA_2 was performed by diethylaminoethyl (DEAE)-cellulose column chromatography, as described by Bernini [1].

Determination of HbF. Two methods were used for quantitation of HbF. Samples containing low percentages of HbF were analyzed by the alkali denaturation method of Betke et al. [2], whereas the method of Jonxis and Huisman was used for samples of high HbF content [7].

Determination of serum iron. The amount of serum iron was determined by the sulphonated bathophenanthroline method [8].

Results

1. Heterozygous β-thalassaemia

For the diagnosis of the β-thalassaemia trait the following parameters were taken into consideration: (1) Mean corpuscular volume (MVC); (2) morphological appearance of peripheral blood; (3) osmotic fragility; (4) percentage of HbF; (5) percentage of HbA_2; (6) serum iron level.

The most characteristic finding is the high percentage of HbA_2, a feature which is considered typical of the disorder, although some cases of β-thalassaemia, the so-called silent carriers, show a normal HbA_2 level [14]. Because iron deficiency may result in a reduced amount of HbA_2, and may produce the above

listed features, the serum iron level was determined in every case. In Fig. 1 the distribution of HbA_2 in a normal population and in the β-thalassaemia trait is shown. The normal range was 1.25–3.25% with a mean of 2.21%, whereas the pathological range varied between 3.50% and 6.75%, with a mean value of 4.76%.

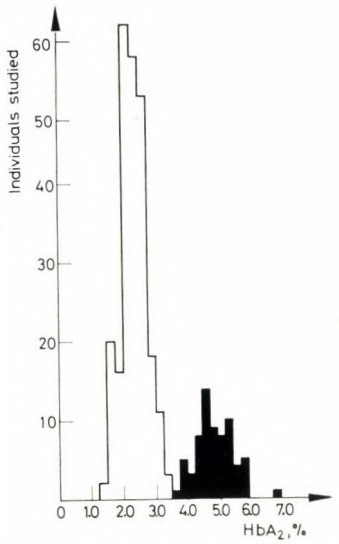

Fig. 1. Distribution of HbA_2 levels in normal and β-thalassaemic individuals

Table 1 summarizes the haematological data of β-thalassaemia heterozygotes found among the patients. Screening of the 961 students revealed cases of β-thalassaemia, as shown in Table 2. Their haemoglobin level varied between 8.6 and 14.7 g per 100 ml, with a mean value of 12.6 g per 100 ml; this was, as expected, higher than in the cases treated in the hospitals. None of these children had required medical care.

Table 1

Haematological data of patients heterozygous for β-thalassaemia and of their affected relatives

	Range	Mean value
Hb, g/100 ml	6.2–12.8	10
MCV, μ^3	63–85	70
MCH, pg	19–25	22
MCHC, %	26–34	30
Hb A_2, %	3.50–6.75	4.76
Hb F, %	0.50–5.0	2.40
Serum iron, μg/100 ml	52–155	106

Table 2

Haematological data of β-thalassaemia heterozygotes found among 961 children

Case	Age years	Hb g/100 ml	MCV μ^3	MCH pg	MCHC %	Hb F %	Hb A^0 %	Serum iron µg/100 ml	Osmotic resistance	Morpho-logy
1	12	8.6	69	23	28	0.7	3.7	95	increased	++
2	11	12.6	81	26	31	1.2	3.8	112	increased	+
3	12	12.7	63	21	32	1.5	5.5	—	increased	++
4	13	13.2	72	23	32	0.8	4.0	129	normal	+
5	12	13.4	82	25	29	0.4	3.8	84	increased	+
6	12	13.8	72	23	31	1.1	5.9	77	normal	+
7	12	14.7	75	25	34	0.9	3.8	86	increased	+

+ = Mild changes
++ = Moderate changes

2. Homozygous β-thalassaemia

The haematological data of the thalassaemia major patients and their relatives are shown in Table 3. Cases 1, 2 and 6 were typical of Cooley's anaemia. In Cases 3, 4 and 5 the disease was characterized only by the haematological findings; the clinical picture was mild, suggesting a diagnosis of intermediate β-thalassaemia.

3. Haemoglobin S-β-thalassaemia

Cases of sickle-cell β-thalassaemia had been expected in view of the high frequency of the β^s gene in the Cuban population [17]. The haematological data of these patients and their families are shown in Table 4.

Discussion

Up to now, no estimate of the frequency of β-thalassaemia in Cuba has been available. According to recent findings, the condition is present in the population but it cannot be regarded as a public health problem. Of 27 patients admitted in the last three years for mild or moderate anaemia the origin of which had not been revealed by routine laboratory tests, approximately 75% were high HbA_2 β-thalassaemia heterozygotes. Moreover, some cases of Cooley's anaemia have been found in the same period.

1. Heterozygous β-thalassaemia

The majority of the cases of minor type thalassaemia showed only anaemia, and few of them had also mild chronic jaundice. Asymptomatic cases were identified among the relatives of these patients. Family studies indicated that the

Table 3

Haematological and clinical data of β-thalassaemia homozygotes and their families

Family	Race	Age, years	Relationship	Clinical feature	Hb g per 100 ml	MCV μ^3	MCH pg	MCH per cent	Retic. per cent	HbF per cent	HbA_2 per cent	Serum iron μ per 100 ml	Osmotic resistance	Morphology*
1	White	34	Father	—	12.2	78	24	30	1.2	0.5	4.8	78	Increased	+
	White	30	Mother	—	9.5	73	22	29	2.8	2.0	3.9	57	Increased	++
	White	5	Index case #	Cooley's anaemia	5.7	69	21	29	6.5	74.3	1.0	147	Increased	+++
2	Mestizo	32	Father	—	12.6	80	23	29	1.4	1.7	4.4	126	Increased	+
	Mestizo	29	Mother	—	9.1	73	21	29	1.3	1.2	4.6	62	Increased	++
	Mestizo	3	Index case #	Cooley's anaemia	4.0	51	11	22	10.4	64.0	2.9	72	Increased	+++
3	Mestizo	32	Father	—	12.6	80	23	29	1.4	1.7	4.4	126	Increased	+
	White	26	Mother	—	10.0	73	22	30	2.0	2.1	4.0	85	Increased	+
	Mestizo	3	Index case	Intermediate thalassaemia	7.9	71	23	32	5.4	82.6	1.0	79	Increased	++
4	Negro	40	Father	—	12.8	72	21	30	1.0	0.7	5.6	155	Incresaed	++
	Negro	38	Mother	—	11.2	79	24	30	3.0	3.7	4.5	100	Increased	+
	Negro	10	Index case	Intermediate thalassaemia	6.5	60	16	27	7.0	66.0	3.0	127	Increased	+++
5	Negro	50	Father	—	12.2	77	24	31	0.9	1.1	5.6	95	Increased	++
	Negro	46	Mother	—	11.4	75	24	30	1.2	1.3	4.7	91	Increased	++
	Negro	25	Brother	Intermediate thalassaemia	8.5	72	22	30	4.0	72.0	3.5	115	Increased	+++
	Negro	22	Index case	Intermediate thalassaemia	8.0	73	23	31	6.0	64.5	2.3	103	Increased	+++
6	White	35	Father	—	11.1	77	25	30	2.0	0.5	2.5	100	Increased	+
	White	35	Mother	—	7.3	73	23	29	2.2	0.2	3.8	50	Increased	++
	White	3	Brother	Intermediate thalassaemia	6.6	68	22	28	2.2	43.0	2.7	192	Increased	+++
	White	4	Index case	Cooley's anaemia	6.3	69	23	29	10.0	68.0	2.9	184	Increased	+++

The father of families 2 and 3 is the same; these two related cases have been reported elsewhere (de la Torre et al., [3])
* + = mild changes, ++ = moderate changes, +++ = marked changes.

Table 4

Haematological and clinical findings in sickle-cell thalassaemia

Family	Race	Age, years	Relationship	Clinical feature	Hb g per 100 ml	MCV μ^3	MCH pg	MCH per cent	Retic. per cent	HbA per cent	HbF per cent	HbA_2 %	Serum iron μg per 100 ml	Osmotic resistance	Morphology
1	Negro	35	Father	—	10.7	70	24	27	2.4	—	0.8	4.6	87	Increased	++
	Negro	31	Mother	—	11.5	86	27	31	0.8	60	0.5	2.4	94	Normal	+
	Negro	10	Index case	Severe	6.6	69	21	30	30.6	0	1.2	4.3	190	Increased	+++
2	Negro	49	Father	—	12.8	90	30	32	1.0	62	0.4	2.5	—	Normal	Normal
	Negro	41	Mother	—	11.7	72	20	27	3.0	—	4.6	7.8	105	Increased	+
	Negro	19	Index case	Mild	10.6	67	23	29	5.6	?	16.0	—	—	Increased	++
3	Negro	38	Father	—	9.5	74	20	29	1.0	—	0.5	5.0	52	Increased	+
	Negro	35	Mother	—	14.0	89	27	32	2.0	60	—	2.0	95	Normal	Normal
	Negro	12	Index case	Severe	4.8	72	22	27	18.0	10	4.5	4.3	74	Increased	+++
4	Negro	30	Father	—	13.2	87	28	32	—	58	0.4	2.3	—	Normal	Normal
	Negro	28	Mother	—	11.3	71	24	29	3.0	—	1.5	4.8	—	Increased	+
	Negro	7	Brother	Mild	9.9	80	27	32	3.2	0	12.2	4.5	—	Increased	++
	Negro	5	Index case	Mild	9.5	78	25	30	4.0	0	9.5	—	—	Increased	++
5	Negro	50	Father	—	11.9	92	28	31	2.0	59	1.0	2.0	103	Normal	Normal
	Negro	40	Mother	—	10.3	77	25	29	2.2	—	1.3	3.8	110	Increased	+
	Negro	7	Brother	Very mild	8.5	71	25	29	8.0	18	6.2	3.9	100	Increased	++
	Negro	12	Index case	Very mild	9.9	69	24	33	5.2	20	5.3	4.0	100	Increased	++
6	Negro	47	Father	—	12.8	80	19	28	—	—	0.8	5.0	128	Increased	+
	Negro	43	Mother	—	11.6	87	28	32	1.0	57	—	2.6	115	Normal	Normal
	Negro	18	Index case	Severe	10.1	78	25	31	4.8	Present	—	—	123	Increased	++
7	Negro	40	Father	—	12.3	94	28	32	--	—	1.2	2.2	—	Normal	Normal
	Negro	35	Mother	—	10.8	79	26	33	1.4	—	1.0	5.0	98	Increased	++
	Negro	10	Brother	Severe	8.8	77	24	29	4.6	0	10.0	—	79	Increased	+++
	Negro	6	Index case	Severe	6.4	76	24	30	5.8	0	12.4	—	86	Increased	+++

haemoglobin levels were unrelated to the clinical picture even in the same family. These findings were in contrast to previous reports [19] and indicate that some non-genetic factor(s) may be of importance in producing the clinical picture. In some cases, the precipitating factor was found to be iron and/or folate deficiency, but many cases remained without explanation.

As previously reported, in all cases there was a reduction in MCV, the range of which was $63-82\ \mu^3$ with a mean value of 70.

The appearance of peripheral blood was as reported from other countries; the majority of our cases showed alterations typical of the thalassaemic syndrome but it was not uncommon to find almost normal blood smears.

On the other hand, our findings concerning osmotic fragility were not consistent with the observation that it is usually reduced and that this reduction may be observed even when other haematological findings are minimal [19]. Contrary to these reports, and in agreement with more recent data [15] we found some cases showing a normal osmotic resistance.

HbF levels ranged from normal values up to 5% in agreement with other reports in different parts of the world [19].

As already reported [18], we have observed that heterozygous β-thalassaemia in the Negro takes very mild form. The majority of our cases was identified in relatives of patients heterozygous for both the thalassaemia and sickle-cell genes. Some of them were of the β^0 type, as demonstrated by the absence of HbA in the sons (see Table 3): They always showed haemoglobin levels higher than the cases heterozygous for a β^+-gene, suggesting that the normal β-gene compensates better in the presence of a non-functioning β-thalassaemia gene than in the presence of a partially functioning allele. In fact, in the cases in which it was possible to demonstrate the presence of a β^0-gene, mean corpuscular haemoglobin ranged between 24 and 28 pg, whereas in the cases in which a β^+ was functioning, the MCH ranged from 16 to 20 pg.

2. Homozygous β-thalassaemia

Of the six cases of Cooley's anaemia, three showed typical haematological and clinical features and were recognized in early childhood. Case 4, on the other hand, showed a mild clinical picture with a normal physical development until the age of 4 years, and even more striking was Case 5, a Negro woman studied at the age of 22 years, when she was carrying a normal pregnancy. Both she and her brother are healthy, showing only moderate anaemia so that, although the haemoglobin pattern is typical of thalassaemia major, as indicated also by the high HbA_2 in both parents, from the clinical point of view they should be considered examples of intermediate thalassaemia. Case 6 displayed exactly the same characteristics.

Family 6 presented some special interest. The index case showed all the haematological and clinical features of thalassaemia major, but the father was a carrier of the silent type with a normal HbA level (see Table 3). Similar cases have

been reported [19], and it seems reasonable to regard this case as Cooley's anaemia. The striking and unexplained observation was that a brother, who showed the same haematological pattern, presented a mild clinical picture and has been almost free from symptoms until now.

3. Sickle-cell β-thalassaemia

Sickle-cell β-thalassaemia cases also showed a high degree of heterogeneity; some of them had been diagnosed as sickle-cell anaemia, others were almost asymptomatic. This variability has already been observed [19], and it was suggested that the severity of the condition was related to the absence of HbA, the less severe cases being characterized by the presence of 25–35% of normal haemoglobin. In our cases this observation has not been confirmed; many displayed a different pattern, and cases without HbA and with a mild clinical picture were not unusual.

The frequency of β-thalassaemia carriers was 0.73% in a sample of Habana City corresponding to a gene frequency of 0.0036%. It should, however, be noted that it is not possible to extend this figure to the whole country, because of the variable racial composition of the different provinces.

The problem of the origin of the β-thalassaemic gene(s) imported to Cuba is difficult to assess since the genetic admixture makes it impossible to obtain a true evaluation of the race of each subject. The data reported here only indicate that different thalassaemic genes are present in the population. The apparently Negro stock shows both β^0 and β^+ genes, the type(s) carried by the Caucasians has not been identified as to whether they produce β-chains, due to the lack of carriers of an interacting abnormal haemoglobin.

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Conformation of Human Globin

The Significance of Haem for the Structure of the Haemoglobin Molecule

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By comparing the properties of human globin with the atomic model of haemoglobin constructed by Perutz, conformation of the globin molecule is suggested and the importance of haem groups for the structure and stability of haemoglobin tetramer is shown.

In our previous work we were dealing with some physico-chemical properties of human globin solutions [2, 6, 7, 16] and isolated subunits [5, 15]. The findings allowed to conclude to the conformation of human globin and the possible role of haem-protein interactions in the structure of haemoglobin in solution [7, 17]. Additional information resulting from the comparison of human haemoglobin and globin sulphhydryl [8, 14, 18] and phenolic [18] group reactivity and from the CD spectra [18], as well as the recent detailed description of the contact regions between the building units of haemoglobin oligomer [1, 9, 11—13] have supported the previous conception.

Three-dimensional structure of the haemoglobin molecule

Analysis of horse haemoglobin crystals by X-ray crystallography at 2.8 Å resolution has led Perutz to design the atomic model of that protein [11, 13]. The haemoglobin molecule consists of two identical $\alpha\beta$ units which are related by dyad axis symmetry. The four polypeptide chains are practically identical in conformation. They consist of eight α-helical regions denoted A to H which are separated by short unordered segments AB to GH. Each of the chains carries one haem group which is a planar structure inserted in a kind of pocket formed by a hydrophobic core of the chain. The haem is attached to the protein molecule through the linkage between the iron ion and the N_5 of the histidyl residue at F 8 position and further through van der Waal's contacts of about 60 atoms of the polypeptide chain with the porphyrin ring. The haemoglobin tetramer is stabilized by many interchain interactions forming two large regions of non-polar contacts. The contacts* $\alpha 1\beta 1$ and $\alpha 2\beta 2$ are made up of 34 residues and about 110 atoms

* The individual chains of the tetrameric haemoglobin molecule are denoted α^1, α^2, β^1, β^2; the intersubunit contact regions, $\alpha 1\beta 1$, $\alpha 2\beta 2$, $\alpha 1\beta 2$, $\alpha 2\beta 1$; and the dimeric units formed on symmetrical splitting, $\alpha^1\beta^1$ and $\alpha^2\beta^2$.

lying within a distance of 4 Å. The contact regions $\alpha 1\beta 1$ and $\alpha 2\beta 2$ are smaller and therefore weaker; they consist of 19 amino acid residues and include about 80 atoms in each region.

Two conformational forms of haemoglobin exist in dependence on the electronic configuration of the prosthetic group iron ions [1, 9, 12]. These are commonly denoted as liganded and non-liganded or unconstrained and constrained conformations (conformer) or also the oxy and deoxy structures. The non-liganded form of haemoglobin is more rigid and firm due to the six electrostatic interactions of oppositely charged polar groups. In the liganded conformer these interactions are disrupted, β subunits are closer and amino acid residues on C-terminals are free to rotate. The iron ions are in the plane of the porphyrin rings while in the deoxy structure they are approximately 0.8 Å out of that plane. The interactions between protein moiety and haem as well as the $\alpha 1\beta 1$ contacts are practically identical in both conformers, while there are marked differences in the $\alpha 1\beta 2$ contacts. The conformers differ also in their affinity to various ligands.

It is generally assumed that the above conformation of the molecule is characteristic of all mammalian haemoglobins and that its main features persist even in aqueous solutions. We have based our assumption of the globin conformation on the atomic model of horse oxyhaemoglobin. This haemoglobin differs from the human in 43 of 287 residues per $\alpha\beta$ subunit [4]. Comparison of the electron density maps of horse deoxyhaemoglobin crystals with maps of human haemoglobin at 3.5 Å resolution shows, however [9], that these replacements are practically without influence on the tertiary and quarternary structure of the molecule.

Globin conformation

Human globin behaves at neutral pH (on removal of denatured molecules) [16] as a homogeneous protein the molecular weight of which is approximately 31,000 [7, 19]. It has been proved by means of zone electrophoresis that it contains equal amounts of α and β chains [19]. The shape of the circular dichroic spectra and of the optical rotatory dispersion curves is analogous to that of haemoglobin; this fact indicates that the α-helix character of the molecule persists even after splitting off the haem groups [18]. The 20 to 25% lower mean residue ellipticity as well as mean residue rotation values suggest, however, a lower α-helix content of globin as compared to haemoglobin. It follows therefore that the human globin molecule consists, in contrast to haemoglobin, of only one α and one β chain, the secondary structure of the unit being α-helical in about 50%.

Positions of the haemoglobin molecule residues taking part in the interactions with haem groups and of those involved in the interchain contacts are shown in Fig. 1. It follows from this scheme that the splitting of haem groups off the globin influences the conformation of the short α-helical regions C and F, of the middle part of helix E, of the N-terminal region of helix G, and of the C-terminal part of helix H, equally in both types of chain. It has been proved [7, 17] that parallel to the splitting off of haem groups, a dissociation of the $\alpha_2\beta_2$ tetramer

in two identical $\alpha\beta$ subunits occurs. This effect apparently results from the interruption of the $\alpha 1\beta 2$ type contacts which are weaker than the $\alpha 1\beta 1$ interchain interactions. It is assumed that on dissociation of the haemoglobin molecule under the action of high salt concentrations a splitting along the $\alpha 1\beta 2$ contacts occurs which results in the formation of $\alpha^1\beta^1$ and $\alpha^2\beta^2$ dimeric units [11, 13].

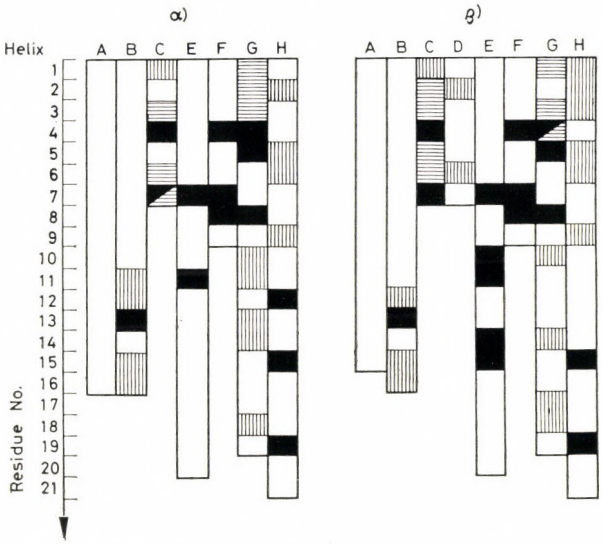

Fig. 1. Participation of the amino acid residues of α and β chains in the fixation of the liganded conformer of the tetrameric haemoglobin oligomer. Amino acid residues participating in haem-binding (black areas), residues forming interchain contacts $\alpha 1\beta 1$ (vertically shaded areas) and $\alpha 1\beta 2$ (horizontally shaded areas)

As follows from Fig. 1, many residues of helix C and of the N-terminal parts of helix G of both α and β chains take part in the $\alpha 1\beta 2$ contacts. Disruption of these interactions therefore influences the conformation of the same regions so as does the interruption of the haem-protein bonds. Each contact both between haem and the particular subunit and between the individual subunits is usually formed by several atoms of the amino acid residues involved. If we assume that formation of the globin molecule from haemoglobin $\alpha_2\beta_2$ oligomer changes the conformation of these residues to such an extent that they are no longer suitable for α-helix, then helices C and F should be completely disrupted as well as approximately one third of the helices E, G and H (see Fig. 1). On the other hand, helices A and D and nearly the whole of helix B should remain intact.

It follows that the secondary structure of globin must consist in approximately 54% of α-helices. This value is in good agreement with that obtained on the basis of chiroptical data. Employing the formulas by Chen and Yang [3] we calculated for five mammalian globins values from 49 to 53% for the α-helix content on the basis of the mean residue ellipticities at 222 nm and from 46 to 54%

from mean residue rotations at 233 nm. The mean value was 50% of α-helices for both types of measurements [18].

Residues involved in $\alpha 1\beta 1$ contacts mostly fall in the regions which we presume are maintained in the globin molecule (Fig. 1). In agreement with this theory, globin is a dimer of the $\alpha^1\beta^1$ type. To support the assumption of the maintenance of $\alpha 1\beta 1$ type contacts in the globin molecule, we compared the -SH group reactivity of human haemoglobin and globin. In haemoglobin, one of the three sulphhydryls (related to $\alpha\beta$ unit), namely Cys F 8 (93) β, is freely reactive, while the remaining two, Cys G 11 (104) α and Cys G 14 (112) β, are non-reactive due to their participation in $\alpha 1\beta 1$ contacts. Similarly, in the human globin molecule solely Cys F 8 β is freely accessible to sulphhydryl reagents while the reactivity of the remaining sulphhydryls is very low [8, 14]. Cysteinyls located in the $\alpha 1\beta 1$ contact region are then as weakly reactive as in haemoglobin. Therefore, these contacts seem to be maintained even in the globin $\alpha\beta$ molecule.

We have attempted to obtain additional information by comparing the reactivity of tyrosyl residues of both proteins. Their molecules contain 6 tyrosyls (related to $\alpha\beta$ unit) located at positions B 5 (24) α, C 7 (42) α, H 23 (140) α, C 1 (35) β, H 8 (130) β and H 23 (145) β. Three of them, C 7, H 23 β and H 23 β appear therefore in the regions where, in our opinion, conformational changes on globin formation occur. We found [18], however, in agreement with Yip and Bucci [20] that the number of freely reactive tyrosyls does not change on splitting off of haem groups and the consequent dissociation of the tetrameric molecule. It is difficult to explain the above discrepancy since even in the haemoglobin molecule the readily reactive and unreactive tyrosyls have not been identified unequivocally.

The binding energy of the non-polar interchain contact regions as estimated by Perutz [10] from the number and types of the contacts does not seem to be higher than 5 kcal · mole^{-1}. In agreement with this estimate is the value of 4.9 kcal · mole^{-1} found by us [7] for the standard molar free enthalpy of the dissociation of $\alpha 1\beta 1$ contacts of the globin molecule at low pH.

Horse, bovine, rabbit, canine and rat globins exhibited a behaviour similar to that of human globin on investigation of their oligomeric state in neutral and acid media, of their chiroptical properties and of the reactivity of sulphhydryl and phenolic groups [18]. It appears reasonable to assume that the above considerations concerning the conformation of human globin may be extended to the globins of other mammalian species.

The role of haem in the conformation of the haemoglobin oligomer

It follows from the above considerations that, as in the solid state also in solution, the haem groups play the key part in the conformation of the hydrophobic core of the tetrameric haemoglobin molecule subunits, in the $\alpha 1\beta 2$ contacts and therefore in the stability of $\alpha_2\beta_2$ oligomer. It appears on the other hand that the prosthetic groups do not influence the $\alpha 1\beta 1$ contact regions. This theory is supported by the findings of a low reactivity of both haemoglobin

and globin sulphhydryl groups located in $\alpha_1\beta_1$ contact region. The decrease observed in α-helix content after the dissociation of haem groups, agrees well with the value calculated on the assumption that on the interruption of the contacts between haem group and polypeptide chains and of interchain contacts $\alpha 1\beta 2$, the α-helical structure of these regions breaks down. As suggested by the similar physico-chemical properties of human, horse, bovine, canine, rabbit and rat globins, the previous conclusions presumably hold true for all mammalian haemoglobins.

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Functional Disorders of Some Haemoglobins Mutated in the Haem Pocket

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Ten different abnormal haemoglobins mutated in the haem pocket have been studied systematically and the results have been compared with data in the literature. A clear structural identification has been performed together with functional studies. A stereochemical classification is tentatively proposed, based on the three-dimensional position of the abnormality.

Introduction

The existence of more than 150 structural variants of haemoglobin has provided a unique material for the comprehension of the structure-function relationship of this molecule. The first informations were reported in 1968 by Perutz and Lehmann [1] after the construction of an atomic model of the haemoglobin molecule. The stereochemical alterations in structure, likely produced by known mutations, were related to clinical symptoms and abnormal properties. Later, a conformational change during the oxy-deoxy transition was demonstrated by Perutz [2]. A tentative explanation of the modified oxygen affinity of some abnormal haemoglobins has been offered by Morimoto et al. [3]. In a few cases the abnormal haemoglobin was purified and studied by X-ray diffraction; these cases supplied more accurate informations. Nevertheless, such a study has been performed only in a limited number of cases; among them some variants involving the C-terminal of the β chain and the $\alpha_1\beta_2$ contact are of special interest.

In a much larger number of cases, some of which were associated with severe functional disturbances, such a direct molecular study has not been possible, since the abnormal component could not be crystallized nor isolated without denaturation. Another approach had therefore to be used for the study of the abnormal molecule, based on a knowledge of the intra-erythrocytic regulatory mechanism and of its possible disorders.

In 1967, Benesch and Benesch [4] and Chanutin and Curnish [5] demonstrated in the red cell the major importance of 2,3-diphosphoglycerate (2,3-DPG) as an effector of the oxygen equilibrium. In the following years, the involvement of

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such regulatory mechanism has been demonstrated in various physiological or pathological conditions. It has been observed during adaptation of the haemoglobin function to extra-erythrocytic disturbances [6]. It may function also when the abnormal haemoglobin molecule itself is the primary disorder [7].

The intra-erythrocytic study of abnormal haemoglobins always supplies information about their behaviour in physiological conditions. Moreover, it is sometimes the only way to understand a modified and up to now not isolated protein. In the present work, attention has been focused on an important group of abnormal haemoglobins, all of them substituted in the haem pocket and more or less unstable.

A large number of abnormal haemoglobins substituted in the haem surrounding has been described since the first identification of haemoglobin Köln in 1966 by Carrell et al. [8]. The existence of pathological changes has been a constant feature of the known substitutions, this part of the haemoglobin molecule being electively sensitive to any structural modification. Theoretically, this is due to the molecule's conformation. Under normal conditions, the haem is deeply buried between the folded helices, the whole area being hydrophobic and tightly packed. More than sixty hydrophobic bonds exist between the haem molecule and the amino-acid side-chains of the globin, most of these amino-acids being invariant. Any substitution modifying the steric conformation will of course modify some of these bonds.

It is also known that the iron atom of the haem is the starting point of the reversible transition from oxy to deoxy conformation, several points being defined with precision: The relative position of the iron atom and of the tetrapyrrol plane, the change of inclination of the tetrapyrrol plane itself, the displacement of the distal histidine with the sliding of the different helices.

The functional disorders following mutations in this area have been summarized by Carrell and Lehmann [9]: Increased oxidation, loss of haem, instability. Nevertheless, the severity varies from one case to another, and so do the symptoms. The question arises of the relationship between the pathophysiological disturbances and the location as well as of the nature of the substitution. Our pertaining findings have been compared with data in the literature.

Methods of functional studies

We had the opportunity to study ten different abnormal haemoglobins mutated in the haem pocket, of which three have been described recently. Haemoglobin Toulouse β 66 (E10) Lys → Glu involves a substitution of a glutamic residue for the lysine normally bound to the propionic acid of the haem molecule [10]. The two other variants were characterized by deletions: Haemoglobin Tours [Thr β 87 (F3) deleted], and Haemoglobin St Antoine [Gly→Leu β 74 – 75 (E18 – 19) deleted] [11]. In the other cases, the structural abnormality had been identified, but no complete functional study has been published. For most of them we were

Table 1

Location of the abnormal haemoglobin described in this study

Abnormal haemoglobin	Substituted residue			Authors
Hb Genova	B10	β 28	Leu→ Pro	Sansone et al. [33]
Hb Hammersmith	CD1	β 42	Phe → Ser	Dacie et al. [34]
Hb M Saskatoon	E7	β 63	His → Tyr	Gerald and Efron [35]
Hb Toulouse	E10	β 66	Lys → Glu	Rosa et al. [10]
Hb St Antoine	E18–19	β 74–75	Gly→Leu del.	Wajcman et al. [11]
Hb Tours	F3	β 87	Thr del.	Wajcman et al. [11]
Hb Santa Ana	F4	β 88	Leu→ Pro	Opfell et al. [36]
Hb M Iwate	F7	α 87	His → Tyr	Shibata et al. [37]
Hb Köln	FG5	β 98	Val → Met	Carrell et al. [8]
Hb Casper	G8	β 106	Leu→ Pro	Jones et al. [31]

able to study independently discovered new cases. Table 1 summarizes the location of the studied abnormal haemoglobins.

The approach was as follows. No functional study was performed without a clear structural identification. Oxygen equilibrium curves were determined on intact cells, lysates, and often on phosphate-free pure components according to Benesch et al. [12]. The 2,3-DPG intra-erythrocytic concentration was assayed according to Krimsky as modified by Beutler [13]. Artificial modifications of the concentration of this substrate were induced in the red cells by incubations and the consequences upon the oxygen affinity were studied [14, 15]. Phosphate-free pure components were tested at various pHs and in the presence of different amounts of 2,3-DPG. Usually, the intra-erythrocytic pH was measured. Spectrophotometric studies were performed to establish haem defects, abnormal haem-binding and oxidation rate. As a second step, special investigations were made, such as haem titration [16], electron spin resonance [17], kinetic study of haemichrome formation [18, 19]. The subunit dissociation equilibrium was studied by differential gel filtration adapted from Gilbert [20].

Functional abnormalities, an attempt at stereochemical classification

The studies revealed that the only common feature of all these abnormalities is some degree of haemolytic anaemia related to the molecular instability, but even this varies widely from case to case ranging from a discrete anaemia to a dramatic haemolytic syndrome. The presence of Heinz bodies and splenomegaly are the non-specific consequences of the phenomenon, but none of the other functional disorders are constant symptoms. The proposed successive steps, increased oxidation rate, haem loss, globin precipitation into Heinz bodies, could therefore not be accepted as a general mechanism [18]. Another alternative was the existence in each case of a specific disorder pattern [21]. The increasing number of thoroughly

studied variants allowed a tentative systematic classification in a few groups, according to the three-dimensional position on the abnormality. As a basis for this classification, we looked in each case for the dominant feature.

1. The loss of haem, which has been described as a major sign, is found in the first group including the most common unstable haemoglobin, haemoglobin Köln. We found a similar pattern in haemoglobin Santa Ana and haemoglobin Tours. In these cases, the abnormal component was easily separated in pure form and proved to carry only two molecules of haem per tetramer. All the other abnormal properties seem to be related to this fact: An increased oxygen affinity with a diphasic curve in the red cells, a very high oxygen affinity with loss of cooperativity in the pure abnormal component [22]. These properties are the same as those described in artificial semi-haemoglobins [23] and also in haemoglobin St Etienne [24] and Gun-Hill [25] which are natural semi-haemoglobins. A shift of the subunit's dissociation equilibrium towards the dimers was found only in haemoglobin Köln and could be due to the involvement of the concerned residue in the $\alpha_1\beta_2$ contact. Such a dehaeminization was reported in some other cases, too. The data concerning this group are summarized in Table 2. It appears that all the substitutions are located in the same area, namely helix F and segment FG.

2. Another group, one of the best known, includes the M haemoglobins. Some of them have been studied at the molecular level. These abnormal haemoglobins are natural valency hybrids, having the abnormal chains irreversibly blocked in the ferric state. A complete review has been recently published by Pulsinelli [26]. Due in these cases to the direct involvement of the haem iron, the observed disturbances are related to the concerned α or β subunit rather than to the proximally or distally substituted histidine.

Our study on intact cells and on the lysate offered evidence of the role of 2,3-DPG in this hypoxic condition. Its concentration *in vivo* was very high, and its regulatory action in shifting the oxygen equilibrium curve to the right could be demonstrated [17].

Table 2

Abnormal haemoglobin characterized by a loss of haem

Abnormal haemoglobin	Substituted residue			Authors
Hb Tours	F3	β 87	Thr del.	Wajcman et al. [11]
Hb Santa Ana	F4	β 88	Leu → Pro	Opfell et al. [36]
Hb Sabine	F7	β 91	Leu → Pro	Schneider et al. [38]
Hb St Etienne*	F8	β 92	His → Gln	Beuzard et al. [24]
Hb Gun-Hill*	F7–FG2	γ 91–95	del.	Bradley et al. [25]
Hb Köln	FG5	β 98	Val → Met	Carrell et al. [8]
Hb Nottingham	FG5	β 98	Val → Gly	Gordon-Smith et al. (39)

* These two variants are natural semi-haemoglobins unable to bind the haem.

The X-ray study of some M haemoglobins has demonstrated that the tertiary configuration of the abnormal chains, particularly the F helix, is stabilized in the deoxy form.

3. A facilitation of the deoxy structure is found in another group of variant haemoglobins, the instability being always associated with cyanosis. A typical example is haemoglobin Hammersmith. We have studied two unrelated patients and demonstrated in both cases two main features. This haemoglobin has a low oxygen affinity with decreased cooperativity and normal regulation by 2,3-DPG. It is oxidized quickly, giving rise immediately to haemichromes. These haemichromes, although chemically reducible, are not reduced by the intra-erythrocytic system, as demonstrated by the use of a highly purified NADH-methaemoglobin reductase. Their oxidation is therefore likely to occur *in vivo* and explains their exceptionally high instability [19].

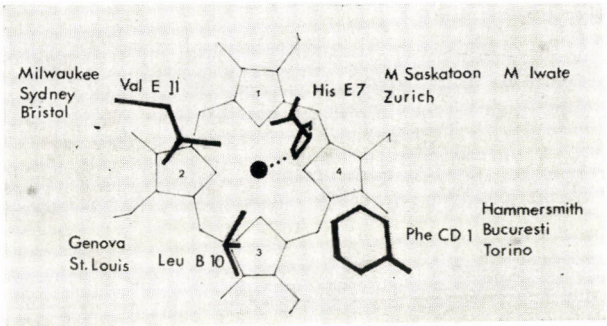

Fig. 1. Projection on the distal side of the haem plane of the four amino-acid side-chains whose substitution can be responsible for abnormal oxidation. Haemichromes have been described in the case of Hb Hammersmith, St Louis and Zürich [40]

Two other substitutions of the same CD 1 residue have been described. Although less severe, the observed syndrome is comparable. Haemichrome formation is, however, a more general process. It has been thoroughly studied in haemoglobin St Louis [27] and demonstrated in the haemoglobins Freiburg and Riverdale-Bronx [18] as well. It occurs in many other variants like haemoglobin Köln [28] but at a slower rate. The dissociation into subunits has been considered an explanatory mechanism in some cases but cannot be always relevant in the above-described variants. The position of the substituted amino-acid relative to the haem plane and the distal histidine have to be taken into account, allowing probably the formation of a direct bond (Fig. 1).

4. An accelerated oxidative process may be the dominant fact, but giving rise to a normal methaemoglobin. This was the case for haemoglobin St Antoine and haemoglobin Toulouse, where no haemichrome formation could be demonstrated even after two years' storage. In these cases the high oxidability was an isolated symptom without any other functional disorder. This could be explained

by a stereochemical alteration of the haem pocket modifying its hydrophobic character and allowing the entry of water. Both these substitutions take place at the end of the E helix. Some other variants have been described in the same zone: Haemoglobin Bristol and haemoglobin Sydney [29] are also characterized by an accelerated oxidation. In the case of haemoglobin Shepherd's Bush, a defect in the binding of 2,3-DPG has been proved, but no data concerning the oxidation properties have been published.

5. Finally, an increased oxygen affinity can be a characteristic feature of the abnormal haemoglobin. The substitution of leucine for phenylalanine at position G5 in haemoglobin Heathrow is the most demonstrative of them [30]. In this case the oxy tertiary conformation of the subunit should be highly facilitated by the disappearance of the phenylalanine ring which stabilizes the deoxy configuration. In haemoglobin Casper [31] a similar mechanism is likely to explain the high oxygen affinity, by introducing a proline residue in the immediate neighbourhood of the phenylalanine.

An increase of oxygen affinity has also been observed in haemoglobin Genova. The fact that the abnormal protein could not be isolated without denaturation allowed only an intra-erythrocytic study. The abnormal oxygen affinity could be concluded from the results of oxygen equilibrium studies, 2,3-DPG estimations and intra-erythrocytic pHs. The molecular mechanism remains unclear, but a careful investigation has ruled out the above-described mechanisms [32].

Conclusion

Our results as well as data in the literature allow to draw some conclusions. The unique instability mechanism formerly proposed is obviously oversimplified, even if partially true. It is also unlikely that each variant should represent a specific and unique process. Comparison is sometimes made difficult by the variable

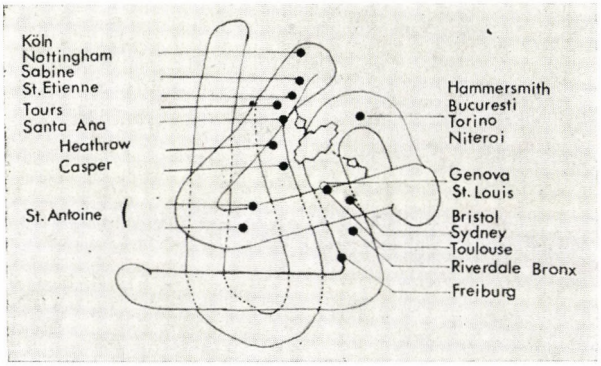

Fig. 2. Schematic view of one subunit of haemoglobin with the stereochemical position of some variants substituted in the surrounding of the haem

approaches used in the functional studies. Some similar patterns are, however, emerging from standardized investigations. When relating these patterns to the three-dimensional location of the abnormalities, a tentative systematic classification can be proposed, at least provisionally (Fig. 2). The proximal side of the haem plane, especially the F helix, is fundamental in the haem-globin binding. Conversely, a strong abnormal haem-binding giving rise to abnormal derivatives is more frequent when substitution occurs on the distal side. Structural abnormalities located on the E helix are less severe, giving usually rise to an increased oxidability only. When the main feature is an abnormal oxygen affinity, it is mainly due to some residues involved in the tertiary transition from oxy to deoxy form. Haemoglobin Hammersmith and haemoglobin Heathrow are particularly demonstrative examples, but other residues could be involved in a similar way. A substitution at a functionally important site may not give rise to important disorders, if the newly introduced residue is able to form bonds stabilizing a near normal conformation. The discovery of other mutants, the possibility of crystallizing some of them will be necessary to confirm such hypotheses.

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Use of the Sequenator for the Study of Abnormal Haemoglobins

Application to Haemoglobins Saint Louis [β28 (B10) Leu → Gln] and Lyon [β17—18 (A14—15) Lys → Val deleted]

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During the description of haemoglobin Saint Louis (unstable, and always oxidized haemoglobin) and of haemoglobin Lyon (with deletion of two amino acids), classical methods such as fingerprints and ion-exchange chromatography failed to give fully satisfactory results. Attempts to use automatic Edman degradation with a sequenator have solved the problems. The importance and interest of this technique in such cases is discussed.

Introduction

Numerous abnormal human haemoglobins have been described, the majority of which belongs to the class of punctual mutants. Of these, haemoglobin S is the first and the most studied example [1]. Until now, only seven cases of deletion have been described [1, 2]; these deletions lead to important modifications of the spatial structure, and as a consequence to a gross instability.

In this paper we describe the structure of a new ferri-unstable haemoglobin, haemoglobin Saint Louis, and of an eighth deleted haemoglobin, haemoglobin Lyon. The abnormal structures were elucidated by automatic Edman degradation. The description of haemoglobins Saint Louis and Lyon has clearly shown the advantage of this new method over the more classical techniques such as fingerprints and ion-exchange chromatography of peptides.

Material and Methods

Blood samples were collected in heparin and immediately refrigerated. Basic haematological studies were done with routine techniques [3]. After preparation of haemolysates, electrophoresis was performed either on starch gel or on cellulose acetate strips in Tris-EDTA-borate buffer pH 8.6 [4]; quantitative data were obtained by scanning the strips in a Cellomatic (CGA-Milano). Haemoglobin stability was studied by incubation in 0.1 M phosphate buffer pH 7.4 at 50°C for 2 hours, as described by Dacie et al. [5], by incubation in 17% isopropanol

in 0.1 M Tris-HCl buffer pH 7.4 at 37°C for half an hour according to Carrell and Kay [6], and by alkylation by paramercuribenzoate (PMB) according to Rosemeyer and Huehns [7].

The abnormal haemoglobins were separated by starch block electrophoresis. Globins were prepared by acid-acetone precipitation, the chains were isolated by column chromatography on CM-cellulose in 8 M urea followed by aminoethylation with ethylene-imine [8].

Fingerprints of tryptic hydrolysates were performed on cellulose Whatman CC 41 thin-layers [4, 9]. Peptides were isolated by ion-exchange resin column chromatography on Aminex A 5 (Bio Rad) according to Jones [10] using a linear gradient of pH and pyridine (pH 3.1, 0.2 M to pH 5.0, 2.0 M or pH 2.5, 0.04 M to pH 4.0, 0.85 M). All peptides were repurified by column chromatography on AG 1 X 2 [11]. Amino acid analysis was accomplished with a Jeol JLC 5 AH amino acid analyzer.

Manual Edman degradations were performed following the procedure described by Hermodson et al. [12] with slight modifications. Automatic Edman degradation was carried out in a Beckman 890 B Sequencer according to Edman and Begg [13] with the quadrol double cleavage program D. Reagents and solvents were purchased from Beckman (Sequencer grade). The PTHs were identified by gas-liquid chromatography on Chromosorb W-HP (100–120 mesh) with 10% SP 400 (Supelco) with Beckman GC 45 gas chromatography according to the procedure of Pisano and Bronzert [14], directly and as silyl derivatives. Identification of all the PTHs was performed by thin-layer chromatography on 6.3×6.3 cm silica gel sheets following a procedure described previously [15]. PTH Arg and PTH His were identified by specific staining of the aqueous phase.

Results and Discussion

I. Haemoglobin Saint Louis

1. Case report

The patient was first admitted to hospital Saint Louis of Paris in 1958, when he was 6 years old. He had a severe haemolytic anaemia requiring splenectomy. Thereafter, cyanosis was found in addition to the persistence of chronic haemolytic anaemia; numerous Heinz bodies appeared in the red cells and 20% of methaemoglobin was detected, though the reducing enzymatic system was normal. The severity of the disease at 6 years contrasted with the absence of complications during early infancy. Though no survey was performed during this period, it was assumed that the synthesis of foetal haemoglobin persisted for several years after birth and thereby reduced the symptoms, as it was found in subjects carrying some special type of unstable haemoglobin [3, 16]. Physical and psychical development of the patient was normal. He is now 22 years old, and has a normal activity.

We have recently performed a new study of the patient's haemoglobin, and succeeded in detecting an unstable haemoglobin which was assumed to be respon-

sible for the anaemia and the cyanosis. The patient's parents and siblings were found to have a normal haemoglobin, and since no indication of false paternity was found, the event of a new mutation seems to be probable.

2. Detection of an abnormal unstable haemoglobin

At pH 8.6 the electrophoretic pattern of the patient's fresh haemolysate (Fig. 1) was identical with that of a mixture of 70% normal and 30% ferri-haemoglobin. After addition of KCN a normal pattern was observed. After oxidation by ferricyanide, in addition to normal methaemoglobin a slower band representing 30% of the total haemoglobin was found. This electrophoretic behaviour is different from those of normal methaemoglobin, and of the haemoglobins M [17].

The spectrum of this band (Fig. 2) was similar to that described by Rachmilewitz and Harari [18] for haemichromes (low spin compounds in which the sixth coordinance of the haem iron is bound to an imidazole group). Our studies [19, 20] have indicated that these abnormal properties are due to haemichromes.

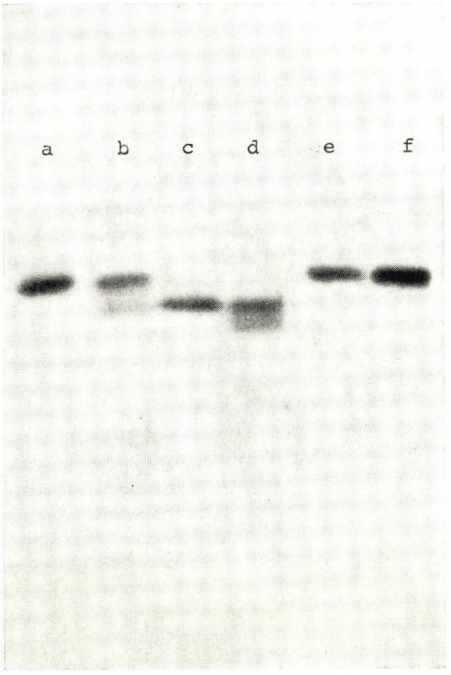

Fig. 1. Starch gel electrophoresis of haemoglobin Saint Louis and normal haemoglobin as reference after application of 7 volt/cm for 2.5 hours. Electrophoresis was performed in an EDTA, boric acid, Tris discontinuous buffer [4]. Samples are: a = normal adult haemolysate; b = patient's haemolysate; c = normal after addition of ferricyanide; d = patient's haemolysate after addition of ferricyanide; e = normal haemolysate after addition of potassium cyanide; f = patient's haemolysate after addition of potassium cyanide

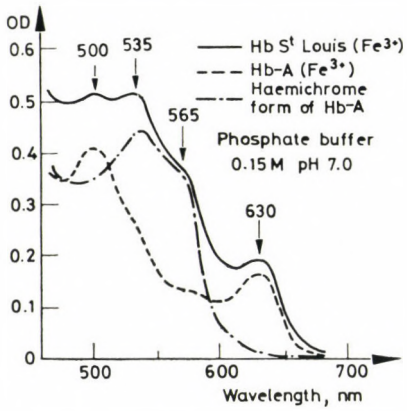

Fig. 2. Comparison of spectra of haemoglobin Saint Louis treated with potassium ferricyanide (———), normal methaemoglobin (- - - - - -) and normal haemichrome (— · — · —) suggests that the mutant haemoglobin is present as a hybrid of ferri- and haemichrome forms [20]

The abnormal haemoglobin was found to be thermo-unstable. Its reaction with PMB gave rise to the specific precipitation of the mutated β-chain.

3. Structure of the abnormal β-chain

The abnormal β-chain precipitated by PMB was found to have a normal charge. Fingerprints of the tryptic digest of aminoethylated $\beta^{\text{Saint Louis}}$ chains showed that all the peptides were in a normal position except for the βT3 peptide; this peptide had a normal electrophoretic mobility, but a slightly lower R_f value indicating a higher polarity (Fig. 3). The elution pattern of the tryptic peptides isolated by ion-exchange resin column chromatography was normal. All the pep-

Table 1

Amino acid composition of normal peptides found in haemoglobin Saint Louis*

Amino acid	Normal βT3	βT3 found in Hb Saint Louis
Arg	1	0.8
Asp	2	2.0
Glu	2	*3.1*
Gly	3	2.6
Ala	1	0.9
Val (1)	3	2.6
Leu	1	*0.1*

* after 72-hour hydrolysis

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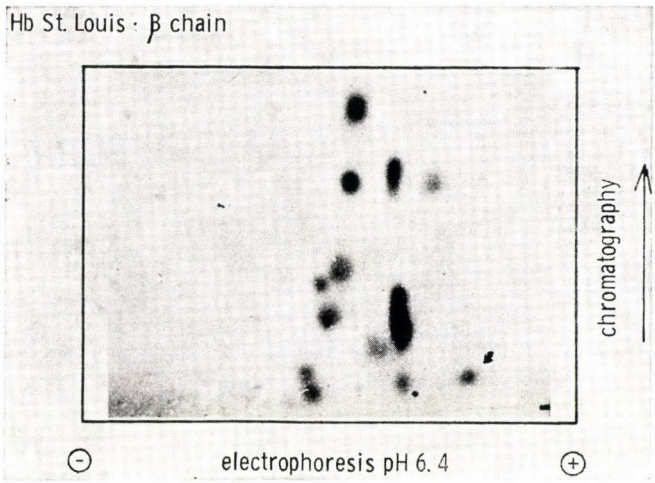

Fig. 3. Fingerprints of tryptic digests of S-AE-$\beta^{\text{Saint Louis}}$ chains made on 20×20 cm cellulose thin-layer plates [4, 9]. In the mutated β-chain all the peptides were at this normal site except for the βT3 peptide which has a weakly modified R_f value (indicated by an arrow)

tides had a normal amino acid composition, except for peptide βT3, in which a substitution Leu → Glx occurred in position 28, since in this peptide there is only one leucine residue in that position (Table 1).

4. Nature of the substitution Leu → Glx

Indirect evidence for the Leu → Glx instead of the Leu → Glu substitution was obtained from the normal charge behaviour of the whole $\beta^{\text{Saint Louis}}$ chain and of the peptide containing the amino acid substitution; in addition the genetic code would only allow the replacement of leucine by glutamine by a single base substitution.

Residue β28 occupies the 11th position in peptide βT3; it cannot be reached easily by manual Edman degradation, exopeptidases, or specific chemical and enzymatic cleavages. Direct evidence of the nature of Glx β28 was obtained by automatic Edman degradation of 36 residues of the intact $\beta^{\text{Saint Louis}}$ chain: a glutamine was found in the 28th position.

5. Mechanism of instability

The B10 (β28) residue has been shown to be in an internal position, with its side chain pointing towards the distal histidine E7 [21]. X-ray analysis of haemoglobin Saint Louis [Anderson, personal communication] indicates that the side chain of glutamine B10 is linked by a hydrogen bond to a molecule of water and this is bound to the histidine E7; this affects the polarity of the haem pocket leading to instability and to haemichrome formation.

II. Haemoglobin Lyon

1. Case report

P. Z., a male patient, 13 years of age, had always been in good health with a normal psycho-motor development. He was examined for a slight pallor. His physical condition was normal, he had no splenomegaly. Haematological examination revealed a slight anaemia (haemoglobin 11.0 g/100 ml), with decreased packed red cell volume (37%) and microcytosis (VGM = 77 μ^3). Heinz bodies were found only after incubation with acetyl-phenylhydrazine. A routine haemoglobin electrophoresis showed an abnormal band.

His father (of Spanish and North African extraction) and two brothers are carriers of the same abnormal haemoglobin and have similar haematological values. His mother and a third brother are normal.

2. Detection and isolation of the abnormal haemoglobin

Starch gel electrophoresis as well as cellulose acetate strip electrophoresis showed a fast-moving component representing 37% of the total haemoglobin migrating as haemoglobin J. Thermostability tests in phosphate as well as in isopropanol buffers revealed a slightly decreased heat-stability. Incubation with PMB gave rise to slight precipitation. Nevertheless, precipitation of the abnormal tetramer or the abnormal chain was not complete. The abnormal haemoglobin was then isolated from the major component by preparative starch block electrophoresis.

3. Structural studies

After preparation of globin, constitutive chains were separated and aminoethylated. Fingerprints of abnormal β-chains revealed the absence of peptides βT2 and βT3, but no additional peptide was found (Fig. 4). By specific stainings,

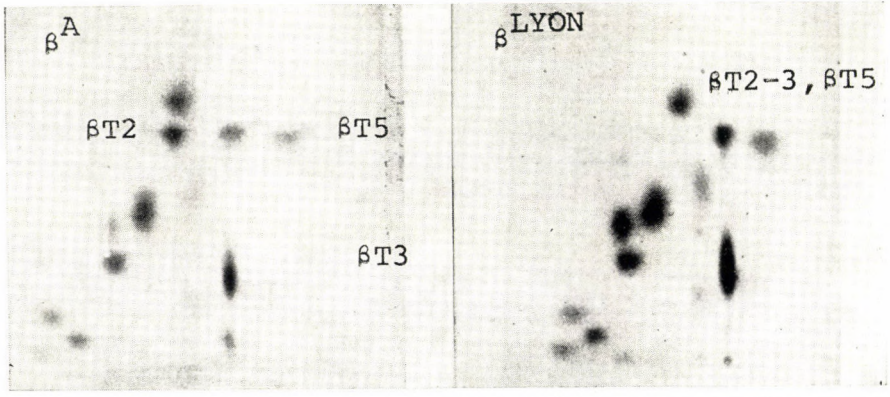

Fig. 4. Fingerprints of tryptic peptide from β^{Lyon} chain (on the right) and β^{A} chain (on the left). After staining with ninhydrine and specific stains, one can conclude to the absence of βT2 and βT3 and their fusion in one peptide βT2−3 confused with peptide βT5

methionine as well as tryptophane and arginine were found in a spot corresponding to the position of normal peptides βT5, indicating that the cleavage between βT2 and βT3 did not occur, and that the new βT2–3 overlaps with βT5.

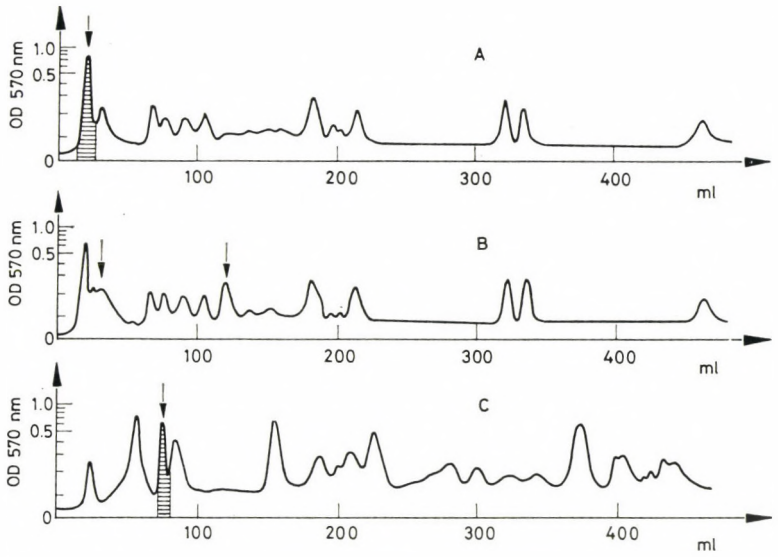

Fig. 5. Tryptic peptide elution patterns on Aminex A 5 resin according to Jones [10].
A: AE-β^{Lyon} chain } gradient pyridine, acetic acid pH 3.1 to 5.0, 0.2 to 2.0 M
B: AE-β^{A} chain } in pyridine
C: modified gradient of pyridine, acetic acid pH 2.5 to 4.0, 0.04 to 0.85 M in pyridine, for resolving acid peptides of the AE-β^{Lyon} chain

The elution pattern of tryptic peptides isolated by column chromatography on Aminex A 5 (Fig. 5A) provided the same features: no peak was found in the position of normal βT3 and βT2, and the height of the peak corresponding to βT5 was increased. Rechromatography on AG 1 X 2 failed to separate βT5 from βT2–3.

Finally, separation of the two peptides was achieved by another chromatography on the same resin A 5 with modified gradient, i.e. using buffers with decreased pH and molarity (Fig. 5C). This step was followed by repurification on AG 1 X 2; in this way, peptide βT2–3 could be obtained in pure state. Its amino acid composition showed the absence of one lysine and one valine residue from the sum of residues of peptides βT2 and βT3 (Table 2). All the other peptides were normal in composition.

Thus, a loss of two amino acids occurs in haemoglobin Lyon; one lysine which must be β17 (A14), and one valine. Since four valine residues were present in peptides βT2 and βT3, the sequence was determined. By manual Edman degra-

dation of peptide βT2–3, valine β11 was found to be present, and the sequence 17–19 was established Asn → Val → Asp (or Asn) instead of Lys → Val → Asn in the normal sequence. Valines 20 and 23 (the 12th and 15th positions of the peptide) could not be determined. Thus the substitution lysine–asparagine could be assumed in position β17 by manual Edman degradation of peptide βT2–3 since its amino acid composition indicated the loss of one lysine and one valine (Table 3).

Table 2

Amino acid composition of abnormal peptides found in haemoglobin Lyon**

	Abnormal βT2–3 found in Hb Lyon	Sum of residues of βT2 and βT3
Lys	*0.1*	1
Arg	1.0	1
Asp	2.3	2
Thr	0.9	1
Ser	1.1	1
Glu	1.7	2
Gly	4.0	4
Ala	2.7	3
Val (1)	*2.8*	4
Leu	2.0	2
Trp (2)	+**	1

* after 72-hour hydrolysis
** estimated by specific staining of a spot of peptide

In order to solve this contradiction, the intact aminoethylated β Lyon chain was submitted to automatic sequencing for 29 cycles. The results pointed to a deletion of lysine 17 and of valine 18, and to the presence of the other valine residues of βT2–3, since degradation reached the βT4 peptide (Table 3).

4. Mechanism of instability of haemoglobin Lyon

Examination of the haemoglobin tridimensional model [21] indicates that Lys A14 β forms a very strong salt bridge with Asp GH4 (121) β, which stabilizes the tertiary structure of the β-chain. The absence of that lysine in haemoglobin Lyon leads to the absence of that important salt bridge which gives rise to less stable β-chains.

Therefore the study of haemoglobin Lyon shows the importance of the AB corner. In the same locus the substitution of lysine β17 (A14) by glutamic acid was described previously in haemoglobin Nagasaki [22]. Although this was not studied in every detail, one may assume that haemoglobin Nagasaki has the same properties.

Table 3

Amino acid sequence of the N-terminal part of Lyon β chain as compared to the normal β chain

	1	2	3	4	5	6	7	8	9	10	11	12	13
Hb A	VAL –	HIS –	LEU –	THR –	PRO –	GLU –	GLU –	LYS –	SER –	ALA –	VAL –	THR –	ALA –
Hb Lyon	VAL –	HIS –	LEU –	THR –	PRO –	GLU –	GLU –	LYS –	SER –	ALA –	VAL –	THR –	ALA –
Manual Edman									⟶	⟶	⟶	⟶	⟶
Sequenator	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

	14	15	16	17	18	19	20	21	22	23	24	25
	LEU –	TRP –	GLY –	LYS –	VAL –	ASN –	VAL –	ASP –	GLU –	VAL –	GLY –	GLY –

	14	15	16			17	18	19	20	21	22	23
	LEU –	TRP –	GLY –			– ASN –	VAL –	ASP –	GLU –	VAL –	GLY –	GLY –
	⟶	⟶	⟶			⟶	⟶	⟶				

....

	26	27	28	29	30	31	32	33
	GLU –	ALA –	LEU –	GLY –	ARG –	LEU –	LEU –	VAL –

	24	25	26	27	28	29	30	31
	GLU –	ALA –	LEU –	GLY –	ARG –	LEU –	LEU –	VAL –

....

⟶ determined by manual degradation of peptide βT2–3

..... determined by Edman automatic degradation of Lyon intact aminoethylated β chain.

5. Genetic mechanism of haemoglobin Lyon

The most simple mechanism to be imagined is the deletion of one triplet (or more) as a consequence of an unequal crossing-over between two regions presenting sufficiently high homology [1, 2].

Such homology is evident in cases where the identical sequences are repeated in the protein as in haemoglobin Gun Hill [23]. On the other hand, the existence of these repeated sequences represents a supplementary difficulty for the establishment of the structure of deletion (e.g. in the case of haemoglobin Gun Hill [23] three different structures are compatible with the obtained results).

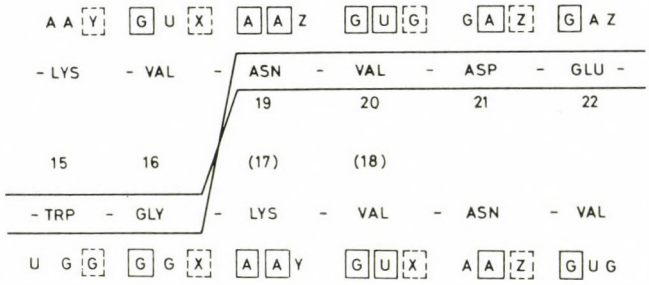

Fig. 6. Mechanism of deletion in haemoglobin Lyon. Deletions are the result of an unequal crossing-over during meiosis. This scheme indicates that a zone of marked homology exists in the messenger RNA of haemoglobin in the $\beta 17$ region. The bases of mRNA are shown taking into consideration the genetic code and punctual mutations described up to now in this part of the molecule. Where the bases are identical on the two genes, they are surrounded by a full line; when they may be identical or different, by a dotted line. X = U, C, G or A; Y = A or G; Z = U or C

In the particular case of haemoglobin Lyon the same fact has been demonstrated; the sequence Val → Asx is repeated and on putting the sequence of mRNA, more than 50% of the bases were found to be identical (Fig. 6).

III. Use of a sequenator in the description of abnormal haemoglobins

In the study of haemoglobin Saint Louis the methods used in the description of abnormal haemoglobins were not efficient: The fingerprints [9] indicated solely a weak difference at the limit of accuracy; the ion-exchange chromatographic method of Jones [10] failed to give satisfactory results, since the elution pattern of peptides was normal, and since the substitution was only found after determination of the composition of all the peptides; the exact nature of the substitution was only elucidated after automatic Edman degradation.

In the case of haemoglobin Lyon, the use of thin-layer fingerprints has easily shown the fusion of peptides βT2 and βT3. This proves the efficiency of that simple reproducible technique [9] in such a case.

Ion-exchange preparative chromatography according to Jones [10] did not give any interesting results. In fact, by this method the acid peptides were insufficiently resolved and were difficult to obtain in a pure state. The existence of a new mutated peptide has largely complicated the problem and it was impossible to isolate it. The same difficulty was met with by Schneider et al. [24] when studying haemoglobin P. The use of a modified elution gradient of the ion-exchange column permitted the isolation of the modified peptide but only with great effort and a feeble yield.

The abnormal peptide showed the absence of two amino acids. Determination of its amino acid sequence led to the inverse conclusion of there being only a simple substitution of lysine β17 by an asparagine. Such discordance implicates that one must be prudent in conclusions concerning the structural anomaly of an abnormal haemoglobin. The old errors in the establishment of the structure or in localizing the mutation of some haemoglobins have recently been corrected by the use of modern refined techniques as in the case of the haemoglobins Hiroshima [28], Oak Ridge [26], Seattle [27], Rainier, and Bethesda [25]. In the case of haemoglobin Lyon all difficulties were overcome by the use of the sequenator; it permits the determination of the amino acid sequence of the β-chain up to peptide βT4, the 29th residue, revealing the deletion of 17th lysine and 18th valine.

Until now this method has only been used for a few abnormal human haemoglobins. When the amino acid substitution is localized in the N-terminal segment of the chain (up to residue numbers 40 to 50), results are obtained without any difficulty. This was the case with the haemoglobins I [29], D Iran [30], and also with the haemoglobins Saint Louis and Lyon. When the mutation is localized farther, specific cleavages leading to large peptides must be found, such as Br CN cleavage in the case of haemoglobin Seattle [27]. Studies are in progress in this laboratory in sequencing the haemoglobin α-core using a unique specific chemical cleavage of Asp $\rightarrow$ Pro 94–95 peptide-bound [Cohen-Solal, unpublished data].

The usefulness of this new strategy in the description of abnormal haemoglobins has clearly been demonstrated in the case of haemoglobin Lyon. This rapid but difficult method will, however, fully be appreciated when considering the cost and time spent on the numerous chromatographies necessary for the isolation of peptide βT2–3, and even without arriving at definitive conclusions.

*

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Physiological Activity of Fresh and Preserved Erythrocytes

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The rate of reduction of ferrihaemoglobin within the erythrocytes and the oxygen affinity of red cells have been measured as a function of storage time of the blood; moreover the effect on oxygen binding properties of suspending fresh erythrocytes in different isotonic media has been reported.

1. The efficiency of the ferrihaemoglobin reducing systems decreases with time of storage as measured in the presence of glucose or lactate as substrate.

2. The well-known increase in oxygen affinity of stored blood is observed when it is determined in Tris-HCl but not in phosphate buffer; this is explained as being due to the replacement of the originally haemoglobin bound 2,3-diphosphoglycerate by phosphate ions entering the erythrocytes.

3. The oxygen affinity of fresh erythrocytes does not change significantly in the presence of different passively penetrating ions, whereas the addition of a non-diffusible polyanion, such as citrate, to the surrounding medium affects the pH; this phenomenon is only in part due to the classical Bohr effect.

In carrying out its respiratory function, blood reveals itself as a perfectly adapted physicochemical mechanism. Central in every aspect of the respiratory function of blood is the erythrocyte, which may be considered a working unit; basically, the red cell is to be thought of as a bag containing haemoglobin, within which the respiratory function of the protein is essentially the same as if all the erythrocyte components were present in solution.

There are, however, phenomena of considerable complexity which result from confining the haemoglobin in the erythrocyte. They involve a reciprocal transfer of materials back and forth between cells and plasma as the blood passes through the respiratory cycle: not simply the oxygen and carbon dioxide, directly involved in respiration, but water and inorganic ions as well. Moreover, in respect to the factors influencing the maintenance of gas transport of the red cell the active metabolism is of primary importance; it is responsible, among others, for modulating the function of haemoglobin and keeping the haemoglobin in reduced form. These phenomena must be regarded as part of the respiratory process, indeed as inherent in the design of the respiratory mechanism itself.

In this paper we shall report on observations on red cells carried out in our Institute. They mainly involve modifications of the rate of reduction of methaemo-

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globin within the erythrocytes and of red cell oxygen equilibrium occurring during blood preservation. The effect of suspending the erythrocytes in different isotonic media on their oxygen-binding property has also been studied. Some of the results to be reported are in press elsewhere [1].

Decrease in rate of methaemoglobin reduction on storage of human blood

The methaemoglobin reduction rate may be considered to indicate mean cell age, i.e. the enzymatic youth of the erythrocyte [2]. Therefore, the reduction rate of red cells ageing *in vitro* has been investigated.

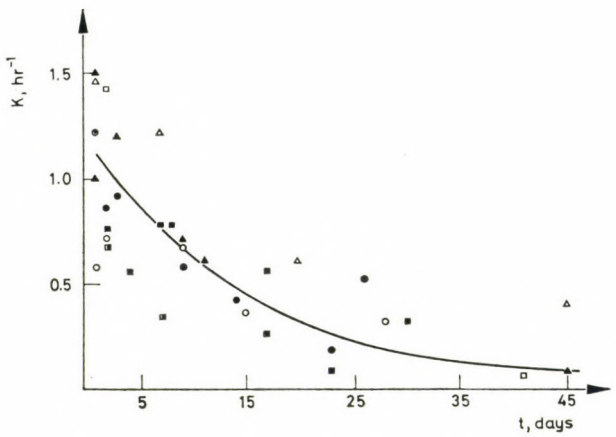

Fig. 1. Rate constant of ferrihaemoglobin reduction as a function of storage time in presence of 20 μM methylene blue with glucose as substrate. Symbols refer to different samples of blood

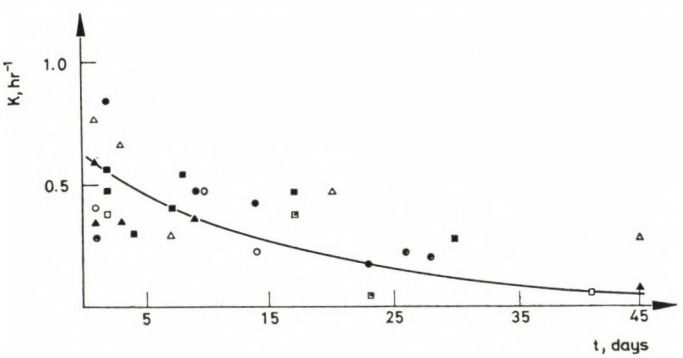

Fig. 2. Rate constant of ferrihaemoglobin reduction as a function of storage time in presence of 20 μM methylene blue with lactate as substrate. Symbols refer to different samples of blood

The methaemoglobin reduction rate of nitrite-treated red cells, determined with a method described previously [3], decreases with the duration of blood storage; this decay is evident in the presence of glucose or lactate as substrate and occurs with these two substances at the same rate at any time.

On adding methylene blue (20 μM) to the reaction mixture, the effect of the two substrates is seen to be different, the absolute rate measured with glucose

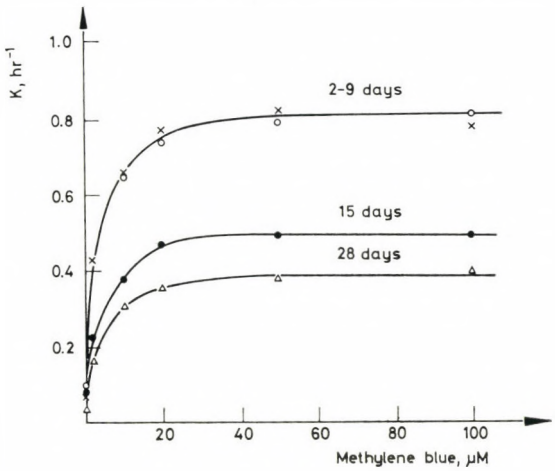

Fig. 3. Rate constant of ferrihaemoglobin reduction as a function of methylene blue concentration in presence of glucose as substrate

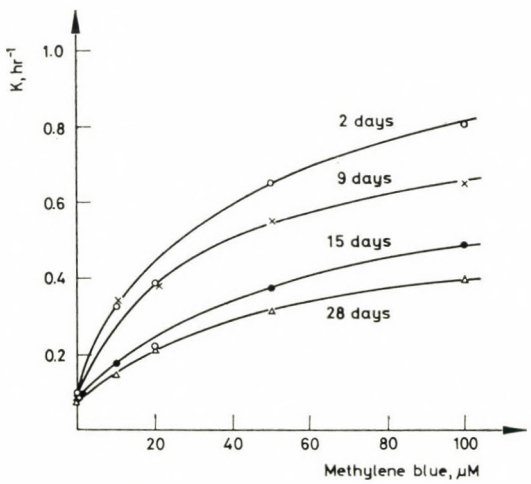

Fig. 4. Rate constant of ferrihaemoglobin reduction as a function of methylene blue concentration in presence of glucose as substrate

being at any time greater than that with lactate (Figs 1 and 2). Under these conditions the trend as well as the half-time (about 15 days) of the decay process are, however, the same for both substrates (see Figs 1 and 2).

In order to investigate the different behaviour of the dye observed in the presence of glucose and lactate the rate of reduction was measured as a function of increasing methylene blue concentration. The results, reported in Figs 3 and 4, indicate that the pseudo-first-order rate constant rises in both cases with the dye concentration up to a maximum, which depends only on the duration of blood storage. The maximum velocity of reduction is achieved with glucose at a methylene blue concentration of 20 μM which is exactly that used in the experiments reported in Figs 1 and 2, and five times lower than the 100 μM required with lactate. This evidence may be related to the postulated presence of two methaemoglobin-reducing systems in the erythrocyte; the first one is NADH-dependent and the second one NADPH-dependent.

Increase of oxygen affinity on storage of human erythrocytes

Satisfactory red cell preservation implies that the erythrocyte membranes are not irreversibly damaged and the preserved red cells are able to transport oxygen [4]. The major functional change during the shelf-life of erythrocytes is their increased oxygen affinity; the importance of this parameter to tissue oxygen supply has been recognized since long [5]. As shown in Fig. 5, the position of the oxygen dissociation curve of preserved red cells (determined spectrophotometri-

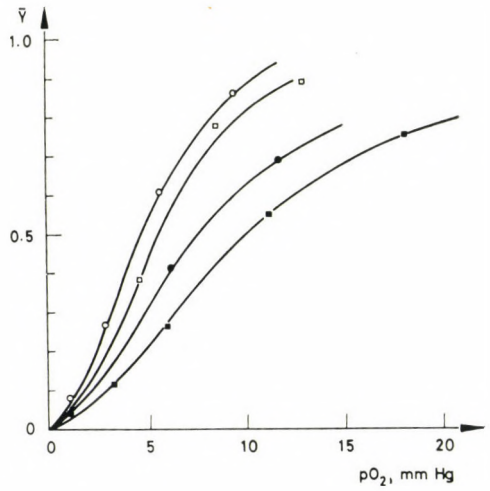

Fig. 5. Oxygen-binding curves of erythrocytes at different storage times. Conditions: 0.05 M Tris-HCl buffer pH 7.3, plus 0.1 M NaCl, 20°C. Duration of storage: ■ = 1 day; ● = 3 days; □ = 8 days; ○ = 85 days

cally [6] until the 85th day of blood shelf-life) is shifted to the left upon increasing storage time; after 10 days of ageing there is, however, little, if any, further increase in oxygen affinity.

Comparison of decrease in methaemoglobin reduction rate and increase in oxygen affinity with storage of human blood

The effect of methaemoglobin on the physiology of respiration is of great importance, in that its presence not only reduces the oxygen-binding capacity of blood, but also alters the remaining ferrihaemoglobin's oxygen dissociation curve, which becomes less sigmoid and is shifted to the left [7]. In other words, the presence of methaemoglobin affects the oxygen transport of the blood. It is generally accepted that methaemoglobin is continuously formed in the erythrocytes, even under physiological conditions, and is reduced to the ferroderivative by enzyme

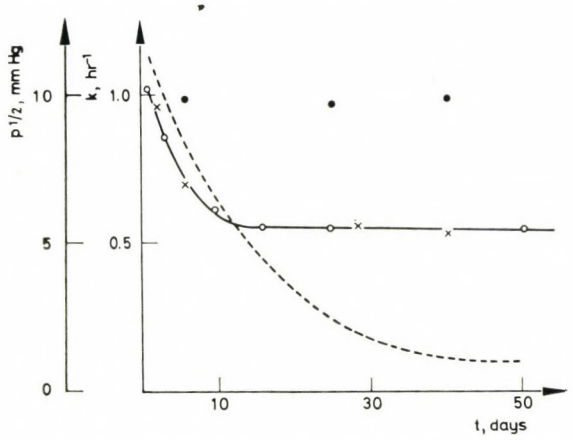

Fig. 6. Change during storage in oxygen affinity (continuous line) and in behaviour of ferrihaemoglobin-reducing systems in the presence of 20 μM methylene blue with glucose as substrate (dashed line). Conditions: 20°C; ○, $\bar{\times}$ = in 0.05 M Tris-HCl buffer pH 7.3, plus 0.1 M NaCl; ● = in isotonic phosphate buffer pH 7.3

systems active in carbohydrate metabolism. A large amount of methaemoglobin may be present *in vivo* due to (a) a congenital deficiency in reducing enzyme systems; (b) a congenital abnormality of the haemoglobin molecule; (c) the introduction of foreign substances.

Figure 6 shows that the changes in the reduction velocity of methaemoglobin and in oxygen affinity with time of storage are parallel during the first week of shelf-life of the blood. However after eight days the oxygen affinity appears to have reached its maximum value, while the reduction rate continues to decrease.

It is well-known that 2,3-diphosphoglycerate (DPG) decreases the affinity of haemoglobin to oxygen [8] and its level in the erythrocytes drops sharply with storage, falling in 10 days to 25% of the original concentration and continuing to decrease *in vitro* with time [9]. Thus, the initial exponential increase in the affinity of blood to oxygen may be explained by the decrease in the DPG level on storage. The constancy of p1/2, observed after a week of shelf-life, may be due to the interaction of haemoglobin with chloride ions of the buffer used (0.15 M Tris-HCl, pH 7.3). In fact, as expected on the basis of the general features of the interaction between anions and haemoglobin [10], the increase in oxygen affinity with storage is not observed in phosphate buffer (Fig. 6), due to the replacement of the originally haemoglobin-bound DPG by phosphate ions, which have a higher affinity to haemoglobin than have chloride ions. The initial parallelism of the changes in the two parameters studied (oxygen affinity and methaemoglobin reductase activity) is only a phenomenological observation. A possible correlation between the metabolic pathway important in the regulation of oxygen affinity and that involved in the reducing systems must await further experiments.

Oxygen affinity of fresh human erythrocytes suspended in different isotonic media of penetrating ions

The oxygen affinity of haemoglobin both in solution and in the erythrocytes depends on its interaction with the components of the surrounding (protons, carbon dioxide, anions, etc.); effects of this kind (heterotropic effects) [11] represent the basis of the regularoty mechanism of gas transport at the molecular level.

Table 1

Oxygen affinity values (as log p1/2) of human eyrthrocytes suspended in various isotonic solutions of penetrating ions at 20°C

Type of salt	Log p1/2	
	pH_{ex} 7.3	pH_{ex} 6.1
Sodium chloride	0.9	1.3
Potassium chloride	0.97	
Lithium chloride	0.9	
Tris-HCl	0.9	1.24
bis-Tris-HCl	0.86	
Magnesium chloride	1.05	
Calcium chloride	0.98	
Sodium acetate	1.0	1.3
Calcium lactate	1.05	
Sodium phosphate	1.03	1.2

Fresh human blood, collected under sterile conditions in bottles with ACD, was stored at 4°C for 10 hours; during this time no significant change occurred in the DPG level. In the physiological concentration range the effect of anions appears to be due primarily to polyphosphates (DPG and adenosine phosphate) normally present in human erythrocytes; in fact, as shown in Table 1, the oxygen affinity of erythrocytes does not change significantly in the presence of different penetrating ions.

Oxygen affinity of fresh human erythrocytes in presence of non-penetrating anions in the external medium

The distribution of passively penetrating ions between erythrocytes and the surrounding medium may be explained in terms of the Gibbs–Donnan theory of membrane equilibrium and therefore depends on the total concentrations and net charges of non-penetrating solutes in the intra- and extracellular compartment [12]. Thus, normally DPG is present in human erythrocytes at a concentration at which its effect on the oxygen affinity of haemoglobin in solution has been found to be almost maximal; when in intact erythrocytes an elevation of the DPG content occurs (see, for instance [13]), this leads to a further decrease of the oxygen affinity *via* the Bohr effect, because the non-penetrating anion affects the Donnan distribution ratio of penetrating ions between cells and plasma and thereby alters the intracellular pH [14]. Therefore, the addition of a non-diffusible polyanion, such as citrate or EDTA, to the external medium must lead to changes of ion distribution, and then of intracellular pH, by neutralizing the effect of the non-diffusible anions of the erythrocyte. As shown in Table 2, the distribution of protons between the intra- and extracellular compartment is affected in the predicted way by the presence of the non-diffusible polyanions in the surrounding medium with a resultant change in the oxygen affinity.

Table 2

Oxygen affinity value (as log p1/2) of human erythrocytes suspended in isotonic solutions of non-penetrating ions at 20°C

Type of salt	pH_{in} (corresponding to) pH_{ex} 7.3	Log p1/2
Sodium EDTA	7.9	0.5
Sodium citrate	8.2	0.3

To check if the effect of these polyanions on the oxygen affinity of haemoglobin in the erythrocyte is due to the classical Bohr effect, that is, to a decreased acidity in the interior of the red cell, the citrate effect has been studied over three

pH units. Figure 7 shows the values for log p 1/2 in the presence of citrate, as a function of the external and internal pH of the red cells. Even after correction for the pH gradient across the membrane, there still remains a significant difference between the curves in the presence and the absence of citrate. Experiments for the rationalization of this phenomenon are in progress.

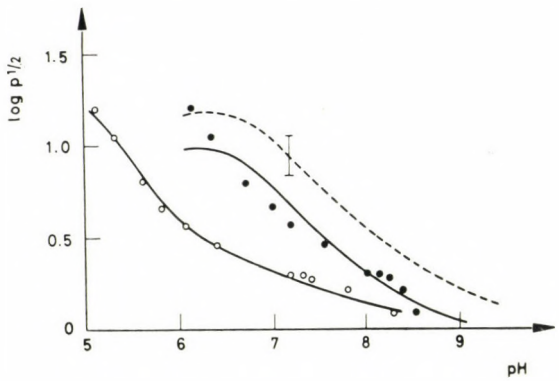

Fig. 7. Effect of pH on the affinity to oxygen (as log p 1/2) of fresh erythrocytes in presence of 0.075 M sodium citrate. Empty circles refer to extracellular pH and full circles to the same values in oxygen affinity but as function of intracellular pH. The dashed line represents the Bohr effect of fresh erythrocytes in presence of isotonic solutions of different salts

Conclusions

As a structure of much larger order of magnitude with respect to haemoglobin, larger in terms of mass by a factor of nearly 10^9, the red cell represents a working unit of the blood. There is no significant change in the overall capacity of haemoglobin to carry either oxygen or carbon dioxide, when this protein is confined to the erythrocytes. However, certain interconnected phenomena involving the metabolism and the exchange of materials between cells and plasma must be regarded as part of the respiratory process.

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Formation and Cleavage of Mixed Disulfide of Hemoglobin-Glutathione in Intact Erythrocytes*

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A marked decrease in the level of GSH was observed in human erythrocytes incubated with glucose in the presence of acetyl phenylhydrazine. All the GSH lost could not be accounted for as GSSG. After washing and reincubation of the erythrocytes with phosphate-saline containing glucose, almost 80% of the GSH could be recovered. The rate of appearance of GSH was much faster when inosine and adenine were also incorporated in the incubation medium. The results of these studies, taken together with our previous studies of GSH-Hb mixed disulfide, suggest that in the presence of acetyl phenylhydrazine, a portion of GSH oxidized becomes bound to haemoglobin or other proteins in the form of mixed disulfide. GSH from the mixed disulfide is released in the presence of NADPH and glutathione reductase.

Allen and Jandl [1], Huisman and Dozy [2], and Birchmeier et al. [3] have demonstrated that hemoglobin can form complexes with GSH. These investigators have suggested that such complexes are probably mixed disulfides of GSH with the β-93 cysteine of hemoglobin.

In previous studies we prepared mixed disulfide of hemoglobin and GSH by incubating hemolysate with GSH and acetyl phenylhydrazine at 37°C for 2 hours [4]. Using ^{35}S GSH we were able to demonstrate that under the conditions used, a large quantity of GSH becomes bound to hemoglobin [4]. Glutathione reductase, from red cells or from yeast, cleaves this mixed disulfide to GSH and hemoglobin-SH in the presence of NADPH:

$$\text{Hb-SSG} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{reductase}]{\text{glutathione}} \text{Hb-SH} + \text{GSH} + \text{NADP}$$

In 1956, one of us [5] first used acetyl phenylhydrazine to oxidize GSH in the GSH stability test. Within 2 hours of incubation of glucose-6-P dehydrogenase deficient whole blood or glucose depleted red cells with acetyl phenylhydrazine, almost all the GSH had disappeared. Much of the GSH lost could not be accounted for as GSSG [6]. However, regeneration of GSH in the presence of glucose was not studied at that time.

* This work was supported in part by NIH Grant HL 07449.

In this report, using improved methods, we could confirm that a large proportion of GSH lost after incubation of red cells with acetyl phenylhydrazine without glucose, cannot be accounted for as GSSG. Much of the GSH lost during incubation with acetyl phenylhydrazine can be recovered on incubation with glucose of the GSH-depleted cells. Our findings suggest that in intact red cells, as in hemolysates, GSH is probably boundto hemoglobin and is released by glutathione reductase in the presence of glucose which generates NADPH.

Materials and Methods

All the fine chemicals were purchased from Sigma Chemical Company, U. S. A. ^{35}S GSH was purchased from Schwarz/Mann, New York, U. S. A.

GSH and GSSG were determined as described previously [7, 8]. Blood from normal donors was collected by venipuncture and centrifuged at 1000 *g* at 4°C for 30 minutes. Plasma and buffy coat were aspirated and the red cells were washed two times with isotonic phosphate saline (sodium/potassium phosphate buffer 0.1 M, pH 7.0, 1 part + NaCl, 0.145 M, 9 parts). A 25% suspension of washed red cells in phosphate saline pH 7.0, was transferred into a 25 ml conical flask containing 50 mg acetyl phenylhydrazine per 12 ml suspension. The flasks were incubated in a Dubnoff shaker at 37°C and agitated at about 100 oscillations per minute for 2 hours. Samples were drawn for GSH, GSSG, and haemoglobin determinations [7, 8] at intervals. After 2 hours the suspension was centrifuged and the top layer aspired. The erythrocytes were then washed two times as before, with about 8 volumes of phosphate-saline. In the first set of experiments a 25% suspension of erythrocytes in phosphate-saline solution pH 7.0, containing 5.5 mM glucose was incubated for 2 hours. In the second set of experiments, a 25% suspension of erythrocytes in phosphate-saline solution, pH 7.0, containing 55 mM glucose, 10 mM inosine, and 1 mM adenine was re-incubated for 2 hours. Additional samples were drawn at intervals and analyzed for GSH, GSSG [7, 8] and hemoglobin.

Results

The results of the first experiment are presented in Fig. 1. In the presence of acetyl phenylhydrazine a marked decrease in the GSH level and increase in the GSSG level was observed. However, in about two hours, the total GSH (GSH + 2 × GSSG) decreased by about 45% as compared to the initial GSH level. After incubation with glucose, GSH in the red cells increased and the GSSG decreased (Fig. 1). However, the increase in GSH level exceeded that which could be accounted for on the basis of GSSG disappearance. Total GSH (GSH + 2 × GSSG) increased to about 80% of the initial value.

Since it is known that incubation with acetyl phenylhydrazine leads to depletion of red cell ATP [9], and hence to loss of capacity to phosphorylate glucose,

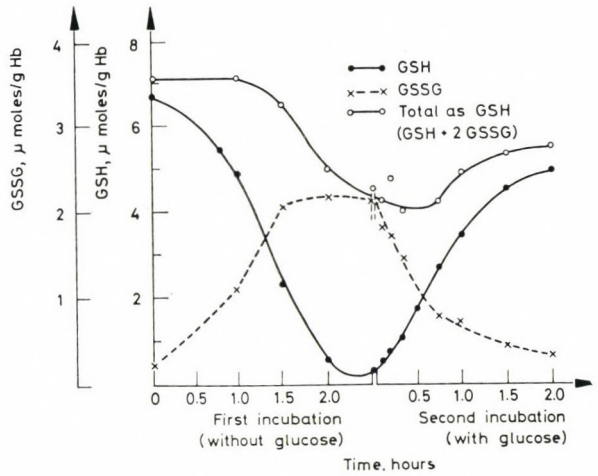

Fig. 1. Oxidation of GSH in the presence of acetyl phenylhydrazine in glucose depleted red cells and regeneration of GSH in the presence of 5.5 mM glucose. The experimental conditions are described in the text

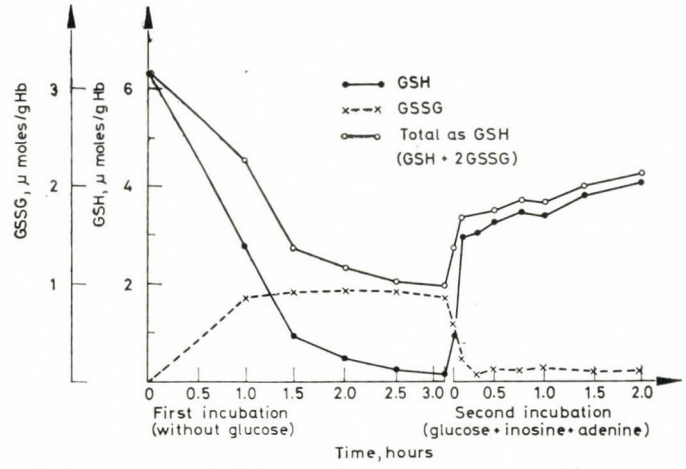

Fig. 2. Oxidation of GSH in the presence of acetyl phenylhydrazine in glucose depleted red cells and regeneration of GSH in the presence of 5.5 mM glucose + 10 mM inosine and 1.0 mM adenine. The experimental conditions are described in the text

another experiment was conducted using glucose, inosine, and adenine as substrates. Much more rapid regeneration of GSH was observed under these circumstances (Fig. 2). Almost half of the total glutathione (GSH + 2 × GSSG) which had been lost on incubation with acetyl phenylhydrazine was recovered in this experiment.

Discussion

It is known that GSH forms mixed disulfides with hemoglobin [1 – 4]. Thus, the loss of total GSH (GSH + 2 × GSSG) during a two-hour incubation of red cells with acetyl phenylhydrazine was probably due to the formation of mixed disulfide of GSH and hemoglobin. Washing the red cells after the first incubation removed most of the remaining acetyl phenylhydrazine. In the presence of glucose, NADPH is regenerated:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + \text{ADP}$$

$$\text{Glucose-6-P} + \text{NADP} \xrightarrow{\text{G-6-PD}} \text{6-phosphogluconic acid} + \text{NADPH} + \text{H}^+$$

$$\text{6-phosphogluconic acid} + \text{NADP} \xrightarrow{\text{6PGD}} \text{ribulose-5-P} + \text{NADPH} + \text{H}^+$$

G6PD, glucose-6-P dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase.

The NADPH formed reduces GSSG to GSH and haemoglobin-SSG to hemoglobin-SH and GSH in the presence of glutathione reductase. The reduction of GSSG occurs quickly, while the cleavage of hemoglobin-SSG by glutathione reductase in the presence of NADPH is known to be rather slow [4].

The rate of reduction of NADPH in red cells is limited by the phosphorylation of glucose by hexokinase. Even under optimal conditions, this reaction is rather slow, and it is greatly retarded when red cell ATP has been depleted, as in our first experiment. Inosine, however, is very rapidly metabolized even by red cells lacking in ATP. It forms organic phosphate when it undergoes cleavage by nucleoside phosphorylase into hypoxanthine and ribose-1-phosphate [10]. The ribose-1-phosphate is converted, through a complex series of reactions, to fructose-6-phosphate and glyceraldehyde-3-phosphate. Since fructose-6-phosphate is in equilibrium with glucose-6-phosphate, it is immediately available as substrate for the glucose-6-phosphate dehydrogenase reaction. Indeed, when inosine was supplied as substrate, a much more rapid initial increase of the GSH and decrease of the GSSG level was observed than was the case when glucose alone was the substrate. Nonetheless, the subsequent regeneration of GSH occurred more slowly. It is clear that the GSH formed toward the end of incubation with substrate must have been derived from a source other than GSSG, since GSSG was virtually absent from the red cells. The most likely source of this GSH is the mixed disulfide of hemoglobin and possibly of other red cell proteins.

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Protective Effect of Formate on GSH Concentration and Heinz Body Formation: a Preliminary Model Study

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The effects of H_2O_2 and of formate on the concentration of GSH and on the formation of Heinz bodies were studied in 1. glucose-free human erythrocytes; 2. sheep blood; and 3. blood of patients with a G6PD defect. In glucose-free normal human red cells, formate protected the erythrocytes both from the H_2O_2-induced decrease of GSH and of Heinz body formation. There was a definite effect on osmotic resistance. Similar results were obtained with sheep blood which is characterized by extremely low G6PD and hexokinase activities. In preliminary studies on patients with G6PD defect there was an indication of less Heinz body formation. The mechanism of the beneficial effect of formate is unclarified.

Heinz bodies occur in a variety of haemolytic anaemias. They are presumably due to a direct attack of some extraneous toxic agent or its product on the haemoglobin in the red cells of peripheral blood. Heinz bodies are thought to consist largely of haemoglobin denaturated by an oxidative affection of its SH-groups. According to a theory [1], the irreversible denaturation of haemoglobin must involve the masked SH-groups which are presumably essential for structural integrity. With the denaturation of the haemoglobin there occurs a loss of haem with some kind of involvement of the cell membrane which is damaged in the process [2, 3]. There is also evidence of the cell membrane being the target of the oxidative attack [4, 5]. Thereby lipid peroxides are formed which in turn oxidize the haemoglobin. The occurrence of radical chain processes cannot be excluded, they may even constitute the predominant mechanism.

The appearance of Heinz bodies is particularly pronounced under the effect of phenylhydrazine or its derivatives. Heinz bodies also occur frequently in G6PD deficiency during a haemolytic crisis. Such a crisis may be provoked by a variety of agents including antimalarials, sulphonamides and analgetics, compounds which have in common the ability to form H_2O_2 [6, 7]. The precise mechanism of Heinz body formation is uncertain. There is a connection between their appearance and the behaviour of the red cells GSH [8, 9]. In many instances a fall of GSH coincidences with the occurrence of Heinz bodies, while the conditions that fully or partly prevent both occurrences are also similar. It is thought that GSH may be necessary for the reduction of disulphide and perhaps also the sulphoxy forms of the protein SH-groups, by a chain of reactions including disulphide

exchange, GSSG reductase, and G6PD. Such a mechanism would not be instrumental if oxidation of the SH-groups or their loss would occur.

There is some doubt as to the nature of the oxidative agent. H_2O_2 does not appear to be involved as its action on SH-groups leads to a reversible formation of disulphide, furthermore it is removed effectively by both catalase and GSH peroxidase. Radicals are likely to be of greater importance. Here a variety of species and various secondary reactions may be considered, superoxide [10] as well as organic radicals, among others.

Another unclarified area is the connection between the formation of methaemoglobin and of Heinz bodies [9]. The correlation is apparently only partial. While many oxidants are capable of oxidizing both the divalent iron and the SH-groups of haemoglobin, there are some significant differences. They include the higher reduction potential of the iron as compared with that of the SH-group, different steric requirements for the oxidation and the existence of two special systems for the reduction. The reductase system for methaemoglobin is NADH-dependent, while that for the reduction of GSSG is NADPH-dependent.

The significance of Heinz bodies appears to lie in the decreased life span of the erythrocyte containing them. This is due to two processes [2, 11]. One is the preferential destruction of Heinz body containing red cells in the spleen; the other, also in the spleen, is the removal of Heinz bodies, leading to the formation of microcytes of decreased viability. It follows from all the above that any measure which could decrease or prevent Heinz body formation would be of great clinical significance.

It has been reported earlier that the addition of formate reduces the formation of $^{14}CO_2$ from 1-^{14}C-labelled glucose [12]. A competition of H_2O_2 reduction via GSH peroxidase, GSSG reductase and G6PD on the one hand and catalase on the other was postulated [12]. Therefore it appeared of interest to study the effect of formate on the level of GSH and on the formation of Heinz bodies. Three types of system were utilized: 1. Glucose-free suspensions of normal human erythrocytes, which should imitate in their behaviour a complete G6PD defect; 2. sheep blood, the red cells of which are characterized by extremely low G6PD and hexokinase activity; and 3. the blood of patients with a G6PD defect.

Methods

The erythrocyte suspensions were prepared by washing 3 times with 0.9% NaCl solution. 2 ml of whole blood or erythrocyte suspension were incubated in a Warburg apparatus at 37°C under shaking (100 strokes/min) for 120 min. In the experiments on the influence of H_2O_2, 0.5 ml of 10% H_2O_2 were placed into the paraffined side arm [13, 14].

The measurement of GSH was performed according to Beutler et al. [15]. Osmotic resistance was measured according to a modification [16] of the method of Sacher and Miller [17]. Heinz bodies were visualized according to Dacie [18]

with methyl violet. Equal volumes of blood or of a mixture of one part erythrocyte suspension with one part of blood plasma were mixed with an equal volume of 0.5% methyl violet (Feinchemie K. H. Kallies KG) in 0.9% NaCl in a small test tube. After 20 min, slides were prepared and 1000 cells were counted on two slides. Only erythrocytes containing bluish-red granula were registered.

Results

1. Effect of formate on the GSH level of erythrocytes

In Table 1 are shown 3 experiments on the effect of formate on the GSH concentration of glucose-free human erythrocytes. The results demonstrate a decrease of GSH with H_2O_2 alone, while the addition of formate significantly reduced the decline of GSH. There were no great differences between the effects of 2 mM and 8 mM formate. Sheep erythrocytes (Table 2) gave essentially the same results.

Table 1

Protective effect of formate on GSH concentration of glucose-free normal human erythrocytes. GSH concentration in mM per L erythrocytes

Exp. No.	Control	+ H_2O_2	+ H_2O_2 + 2 mM formate	+ H_2O_2 + 7 mM formate
1	3.02	1.38	—	1.98
2	3.38	1.26	1.77	1.73
3	2.14	0.96	1.22	1.34

Table 2

Protective effect of formate on GSH of sheep blood. GSH concentration in mM per L erythrocytes. Experiments were performed on two animals

Exp. No.	Control	+ H_2O_2	+ H_2O_2 + 2 mM formate
1	1.61	1.06	1.24
2	2.29	1.54	1.94

2. Effect of formate on Heinz body formation

Tables 3–5 show the effects of H_2O_2 and the protection exerted by formate in the three systems studied. In Table 3 are shown the results for sheep blood. Here H_2O_2 produced a high percentage of Heinz bodies, which was nearly complete-

Table 3

Effect of formate on Heinz body formation in sheep blood.
Values given indicate the percentage of red cells containing Heinz bodies

Exp. No.	Control	+ H_2O_2	+ H_2O_2 + 2 mM formate	+ H_2O_2 + 8 mM formate
1	4	98	4	5
2	11	73	19	17

Table 4

Effect of formate on Heinz body formation in glucose-free human erythrocytes.
Values are expressed as in Table 3

Exp. No.	Control	+ H_2O_2	+ H_2O_2 + 2 mM formate	+ H_2O_2 + 8 mM formate
1	9	44	21	8
2	12	26	13	15

Table 5

Effect of formate on Heinz body formation in the blood of patients with a G6PD defect,
Values are given as in Table 3

Patient.	Control	+ H_2O_2	+ H_2O_2 + 2 mM formate	+ 2 mM formate	μMol/ml cells/h
H. B.	9	14	11	8	40
H. B.*	18	50	23	18	64
G. T.+	5	12	5	5	56
A.G.	11	19	12	13	0
G. G.++	4	6	2	3	234

* before transfusion
\+ 0.4% initially
++ heterozygote mother of A. G.

ly prevented by formate. The results were nearly as clear-cut in the two experiments with glucose-free normal human erythrocytes (Table 4). Least impressive was the effect on whole blood of patients with G6PD defect, both with respect to Heinz body production by H_2O_2 and to the effects of formate. They were, however, consistent with the protective effect of formate. Notable was the behaviour of the erythrocytes of patient H. B. They exhibited 18% Heinz bodies on incubation without H_2O_2, and 50% under the influence of H_2O_2. In the presence of formate, the percentage was decreased to 23%.

3. Effect on osmotic resistance

H_2O_2 exerted a deleterious and formate a protective effect on osmotic resistance (Fig. 1). The addition of cysteine reduced the osmotic resistance.

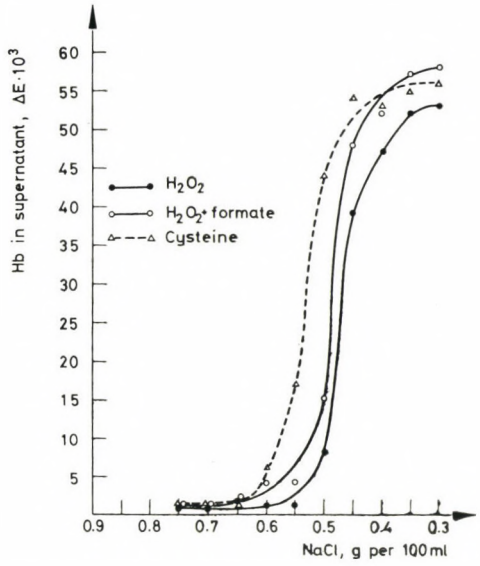

Fig. 1. Osmotic resistance of human erythrocytes

Discussion

The results presented are of a preliminary nature and require extension in several respects. The use of standardized models such as glucose-free erythrocytes and sheep blood has obvious advantages as compared with the variability of clinical cases. The model experiments have yielded clear-cut evidence of the beneficial effect of formate both on the maintenance of GSH and on the formation of Heinz bodies. Results for the blood of patients with G6PD defect were less consistent. While there was a diminution of Heinz body formation, there was no correlation between the degree of G6PD deficiency and the extent of Heinz body formation, nor were there consistent changes of GSH. Only in the case of patient A. G., who had a particularly severe G6PD defect, did the GSH level decline to 1.35 mM during incubation. The number of cases studied is too small for drawing definite conclusions. Nevertheless, the results seem sufficiently encouraging to extend the studies with a view to the application of formate in patients. Such considerations are supported by the innocuous nature of formate [19].

The mechanism of the beneficial action of formate is unclear. The original suggestion of a competition for H_2O_2 between the peroxidative function of catalase

with formate as co-substrate with the GSH dependent reactions appears to be invalid on theoretical grounds. It may be calculated from the appropriate rate equations [20] that formate has a weak effect on the rate of H_2O_2 removal by catalase. Its effect consists in a reduction of the overall rate of H_2O_2 consumption or in an increased steady state level of H_2O_2.

This conclusion has been arrived at in the following manner. The symbols used are those of Chance [20]: e, indicating the concentration of catalase; p_m, the concentration of the catalase-H_2O_2 complex at steady state; k_1, the rate constant for the formation of the catalase-H_2O_2 complex; k_4', the rate constant for the catalatic breakdown of the catalase-H_2O_2 complex; k_4, the rate constant for the peroxidative breakdown of the catalase-H_2O_2 complex with formate as substrate; x_m, the H_2O_2 concentration at steady state; a, the concentration of formate; $\frac{dx_n}{dt}$, the rate of H_2O_2 generation; $n = k_4'/k_1$, variously assumed to be 3 or 1.5 [20, 21]. According to Chance, at steady state

$$\frac{dx_n}{dt} = 2k_4' x_m p_m + k_4 a p_m \tag{1}$$

while

$$p_m = \frac{2}{1 + n + \frac{k_4 a}{k_1 x_m}} \tag{2}$$

Inserting the expression for p_m in equation (1) and keeping in mind that $k_4' = nk_1$, one obtains

$$\frac{dx_n}{dt} = \frac{(2nk_1 x_m p_m + k_4 a)\, e}{1 + n + \frac{k_4 a}{k_1 x_m}} \tag{3}$$

Rearrangement yields

$$\frac{dx_n}{e\,dt} = \frac{(2nk_1 x_m + k_4 a)k_1 x_m}{k_1 x_m + nk_1 x_m + k_4 a} = \frac{2nk_1 x_m + k_4 a}{(n+1)k_1 x_m + k_4 a} k_1 x_m \tag{4}$$

It may be seen that the concentration of the peroxidative substrate a, which is formate, influences the rate of H_2O_2 generation required for the maintenance of a steady state level of H_2O_2 in a small degree. Assuming a value of 3 [21] for n one obtains for $a = 0$

$$\frac{1}{e}\frac{dx_n}{dt} = \frac{2 \cdot 3x_m}{4x_m} k_1 x_m = 1.5 k_1 x_m \tag{5}$$

while for $a = \infty$

$$\frac{1}{e}\frac{dx_n}{dt} = k_1 x_m \tag{6}$$

Therefore, the value of x_m is influenced by formate only to a minor extent. Its effect is to increase the steady state concentration of x_m, i.e. of H_2O_2. Consideration of the activity of the GSH peroxidase reduces the influence of formate.

Therefore, the beneficial effect observed must find another explanation. One possibility is a competition of formate with NADPH for the removal of GSSG, another one could be a direct effect on the conformation of catalase, haemoglobin, or one of the GSH-dependent enzymes. Lastly, an effect on the formation or removal of radicals may be considered.

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Genetic Abnormalities in Glutathione Metabolism as a Cause of Drug-Induced Haemolytic Anaemia

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In some individuals, drugs may induce haemolytic anaemia. The sensitivity to drug-induced haemolysis is caused mostly by a disorder in the glutathione metabolism of the erythrocyte. These disorders are inherited and linked to the deficiency of six enzymes: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase, glutathione synthetase, gamma-glutamylcysteine synthetase and glutathione peroxidase. The drug-induced haemolytic anaemias are impressive models of the so-called pharmacogenetic diseases.

Glucose metabolism of the mature erythrocyte is restricted to two pathways. The major one is the Embden – Meyerhoff pathway, in which glucose is metabolized to lactic acid and energy is provided in the form of ATP. Although this pathway is the primary source of energy, lesions affecting it are characteristically not involved in drug sensitivity. The second pathway is the hexose-monophosphate (HMP) shunt, through which about 5 to 10% of the glucose utilized passes before reentering the main stream of glycolysis. This shunt is the only source for the generation of reduced NADP. The enzymes which are involved in the reduction of NADP catalyze the first two steps of the shunt.

Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate, and 6-phosphogluconate dehydrogenase (6PGD) catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate and CO_2. NADPH is the preferred cofactor for the reduction of oxidized glutathione (GSSG), which is catalyzed by the enzyme GSSG-reductase. Normally, nearly all glutathione is in the reduced form (GSH). The intracellular concentration of GSH is surprisingly high. GSH appears to play a role in preserving the sulphhydryl groups of proteins, for example hexokinase, triosephosphate dehydrogenase and the enzymes of glutathione metabolism themselves, the SH-groups of haemoglobin and of membrane proteins. It also seems to be important for detoxifying small quantities of hydrogen peroxide. The enzyme which reduces hydrogen peroxide to water by oxidation of GSH is GSH-peroxi-

* Dedicated to Professor Susan R. Hollán, Editor-in-Chief of Haematologia and Director of the National Institute of Haematology and Blood Transfusion, Budapest, Hungary

dase. This enzyme reacts with very small quantities of H_2O_2, whereas the catalase system, which is also present in the erythrocyte plays a minor role [14]. The turnover of glutathione is comparatively high, its half-life is about 3 days. No enzyme could be found which degrades glutathione in the cell. Recent findings reveal an active transport of GSSG out of the cell [37]. The tripeptide is synthesized by two enzymes, gamma-glutamylcysteine synthetase and glutathione synthetase. Both enzymes seem to be involved in drug-induced haemolytic anaemias. We have thus required five enzymes for maintaining GSH in its reduced state and at normal level, G6PD, 6PGD, GSSG-reductase GSH-synthetase, gamma-glutamylcysteine synthetase and, finally, GSH-peroxidase, which oxidizes GSH for reduction of H_2O_2. When any of these six enzymes is not functioning properly, the erythrocyte becomes excessively susceptible to drug-induced haemolysis.

Shortly after the introduction of antimalarial compounds into medical practice, some cases of haemolytic anaemia were observed to be associated with the administration of these drugs. In 1926, Cordes was the first to note manifestations of acute haemolysis in a subject given pamaquine naphthoate [16]. This was followed by many other reports on this type of haemolytic anaemia [4]. Family studies have revealed the genetic basis of the susceptibility to drug-induced haemolysis. Transfusion experiments with ^{51}Cr-labelled erythrocytes from sensitive persons into non-sensitive recipients and *vice versa* showed that the drug sensitivity was due to an intrinsic defect of the erythrocyte. Detailed enzymological and biochemical studies during the last 20 years have demonstrated that this defect was primarily caused by an abnormality in the glutathione metabolism of the erythrocyte. Very rarely, drug-induced haemolysis occurs also in diseases due to haemoglobinopathies.

The mechanism of drug-induced haemolysis is not completely clear. Most of these drugs or their degradation products are able to catalyze the oxidative denaturation of haemoglobin with molecular oxygen. It has been shown that the interaction of drugs with oxidized haemoglobin generates H_2O_2 [15]. The formation of free radicals of GSH through the action of peroxidase or by the direct action of drugs may lead to oxidation of GSH to the disulphide GSSG or complexing of the glutathione with haemoglobin to form a mixed disulphide. The mixed disulphide of haemoglobin and GSH is probably unstable, and the haemoglobin is irreversibly denaturated, and will precipitate as Heinz bodies. Cells containing Heinz bodies are rapidly eliminated from the circulation.

In the case of GSSG-reductase deficiency, direct enzyme inhibition may play a role in drug-induced haemolysis [39]. Recently, Kosower et al. have revealed that the formation of GSSG from GSH by means of methyl-phenyl-diazenecarboxylate (azoester) is a three-step non-enzymatic reaction independent of oxygen [21]. Thus, multiple mechanisms may exist for drug-induced oxidation of GSH within the cell. The same authors provided evidence that the action of azoester leads to the generation of free reactive radicals near the membrane and subsequently to damage of the membrane and haemolysis, the "GSH loss catastrophe" [22].

G6PD-deficiency

This deficiency is the most common prototype of the enzymatic deficiencies of the human erythrocyte. After the first report [12] on the basis of altered electrophoretic mobilities, kinetic- or physicochemical properties of the enzyme, more than 100 different variants of G6PD have been described and the biochemical characterization of the deficient enzymes has been standardized [41]. The difference between the normal enzyme (B) and the most common variant (A^+) has been attributed to a single amino acid substitution, asparagine for aspartic acid [42]. Many of the variants described are not associated with clinical effects and exhibit a normal activity. One variant shows an elevated activity [17]. The three most common variants with decreased activity, G6PD A^-, Mediterranean (B^-), and Canton, are present in clinically unaffected subjects. They are subject to drug-induced haemolysis and, in the case of Mediterranean and Canton, to favism. The spectrum of the drugs inducing haemolysis is not well defined. Some drugs act preferentially in the Mediterranean type (B^-). Favism is unknown in the Negro type, G6PD A^-. Some drugs act only in conjunction with viral or bacterial infections, acidosis or other conditions. A survey of the corresponding drugs has been given by Beutler [4]. It is obvious from the transmission of the defect that the G6PD gene is located on the X-chromosome. The locus appears to be closely linked to those for colour blindness and haemophilia A and B. According to Lyon's hypothesis, in human females a genetic mosaicism with two erythrocyte populations, normal and enzyme-deficient, could be demonstrated [2].

6PGD-deficiency

In contrast to G6PD-deficiency, only a few cases have been reported. Most patients exhibited a partial 6PGD deficiency [11, 24, 32, 35] and only one patient displayed a nearly complete absence of the enzyme [33]. However, in this severely affected subject, clinical investigations and drug challenge have not been carried out. One case with a partial deficiency was associated with a severe non-spherocytic haemolytic anaemia, an increased instability of GSH and Heinz body formation [24], but in another case these signs of susceptibility were absent. Only a slight destruction of erythrocytes occurred in another subject with partial deficiency, healed with primaquine [18]. Thus the cause-and-effect relationship between the deficiency and haemolysis seems to be questionable. Some electrophoretic variants have been described [33]. 6PGD appears to be controlled by an autosomal gene.

GSSG-reductase deficiency

The spectrum of clinical disease associated with this deficiency varies widely. There is not only drug-induced haemolysis [13], non-spherocytic haemolytic anaemia [26], but also pancytopenia combined with neurological disorders [40].

The drugs which produce acute haemolysis differ from those inducing haemolysis in G6PD deficiency [39]. By means of high voltage electrophoresis on cellulose acetate, GSSG-reductase could be separated into two bands [6]. GSSG-reductase has long been known to be a flavin enzyme, with a FAD prosthetic group [36]. In 1968, the activation of GSSG-reductase from riboflavin-deficient rats and humans could be demonstrated by the addition of FAD *in vitro* [19]. In many cases with reduced GSSG-reductase activity it is increased to normal level by administration of riboflavin *in vivo* or the addition of FAD to the haemolysate *in vitro* [3]. Reexamination of a number of individuals with low GR activity revealed that diminished enzymatic reduction due to an abnormal enzyme molecule was limited to a small number of subjects. In most cases with various clinical disorders and low GR activity, riboflavin administration resulted in a rapid increase of enzyme activity without correcting the disease, e.g. the haemolysis or pancytopenia. An insufficient response to riboflavin *in vivo* and FAD addition *in vitro* was found in a patient with pancytopenia, while a decreased affinity of GR for FAD was noted in two other individuals. An abnormal electrophoretic pattern of GR (absence of one band) was observed in one of the patients before and after the addition of riboflavin or FAD [27].

GSH-synthetase

In 1961, a patient with non-spherocytic haemolytic anaemia and a markedly decreased erythrocyte GSH was described [31]. Subsequent studies showed a lack of GSH synthesis and an increased red cell destruction after primaquine administration [34]. Few such cases have been reported [7, 8, 25]. It could be shown that GSH-synthetase was the rate-limiting enzyme of GSH synthesis and that in most cases a deficiency of this enzyme was responsible for the defect. The mode of inheritance is autosomally recessive. The heterozygotes exhibit only half the normal synthetase activity but a normal GSH content and no signs of disease [28]. Recently, a deficiency of gamma-glutamylcysteine synthetase with chronic haemolytic anaemia and neurological disorders has been described [20].

GSH-peroxidase

Few cases of GSH-peroxidase deficiency have been described. The first case was a newborn with transient haemolytic anaemia and a partial, presumably heterozygous, deficiency [29]. The subsequent reports revealed an autosomal recessive inheritance of the deficiency, with about half normal activity in heterozygotes. The clinical picture is sometimes characterized by chronic haemolytic anaemia, methaemoglobinaemia, the presence of Heinz bodies but normal GSH stability and increased sensitivity to drugs even in heterozygotes [9, 10, 30].

In the detection of abnormalities in HMP shunt, none of the routine haematologic tests is of a diagnostic significance. Only the increased number of Heinz

bodies may give a hint. There are, however, some improved methods to give information about the function of the shunt: generation of an increased number of Heinz bodies after incubation with acetyl-phenylhydrazine (APH) [1] and measurement of GSH content with DTNB [5]. GSSG is difficult to determine because

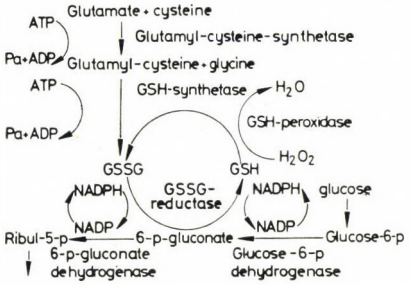

Fig. 1

of its rapid conversion to GSH and its low level in red cells. In G6PD-deficient cells, however, an increased level could be demonstrated [38]. In the azoester test developed by Kosower et al. the GSH is completely oxidized and, after addition of glucose, the regeneration of GSH is followed [23]. For determination of the special enzymes, many screening tests have been described [5]; we prefer the pyridine nucleotide-linked test systems [5]. GSH-synthetase and gamma-glutamyl-cysteine synthetase can be measured by means of isotopes [28].

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Erythrocyte Parameters During Induced Ca^{2+}-dependent Rapid K^+-efflux: Optimum Conditions for Kinetic Analysis

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Increased Ca^{2+}-dependent K^+-permeability of human erythrocytes is accompanied by changes in shape such as echinocytosis or stomatocytosis, a reduction in volume, adhesiveness, sedimentation and intracellular ion activity and an increase in intracellular haemoglobin concentration and osmotic resistance. These changes depend on the applied metabolic inhibitors and drugs, ionic composition of the medium, and incubation times. Standard conditions and procedures have been suggested suitable for kinetic analysis of the selectively enhanced K^+-transport.

Introduction

Cation permeability of the erythrocyte membrane is changed significantly by ATP depletion of the cells or by their treatment with propranolol or a few related compounds in the presence of Ca^{2+} [1—3, 6]. Permeability to K^+ increases selectively and, as a result — in the case of physiological ion distribution — a rapid K^+-efflux ensues unaccompanied by equimolar Na^+ uptake. Electroneutrality is maintained by the simultaneous efflux of Cl^- and OH^- ions and/or uptake of H^+-ions. It is not clear whether K^+ ions move by simple diffusion or their transport is carrier-mediated. A kinetic approach of the problem is complicated by the fact that, parallel with the changes in permeability, several characteristics of the red blood cell are also altered. In the present paper, these changes are analyzed with the aim of finding ideal conditions for kinetic analysis.

Methods

Washed erythrocytes from human defibrinated blood were used. Various metabolic inhibitors (IA + inosine, NaF, NaF + arsenate) were applied for ATP-depletion. In order to achieve complete phosphate-ester depletion, cells were treated as follows. For 2,3-DPG depletion they were incubated with 15 mM $NaHSO_3$ ± 2.5 mM IA at 37 °C for 5 hours; for subsequent ATP depletion, either with 2.5 mM IA + 10 mM inosine or with 8 mM NaF + 2.5 mM Na_2HAsO_4 at 37 °C for a minimum of 2 hours. All this was done in the presence of 1 mM EGTA to prevent rapid K^+-loss.

Cation fluxes were determined by flame photometry or by the use of ^{42}K. Morphology of the erythrocytes was assayed under the phase-contrast microscope. Haematocrit was determined by the conventional microtube technique after centrifuging the samples at 5000 g for 20 minutes or according to the isotope dilution principle by using ^{125}I-albumine.

Erythrocyte adhesiveness was determined according to a further modification of the Fahraeus method applied by Bicher [4]. The procedure was carried out as follows. Washed cells were incubated with metabolic inhibitors or the drugs to be tested at 37 °C for 2–3 hours. Alternatively, cells were incubated in serum and then washed three times. Finally, RBC were centrifuged at 8000 r.p.m. for 10 minutes and the supernatants and upper layers were discarded. To one volume of erythrocyte sediment two volumes of 4% dextran T-250 dissolved in physiological saline pH 7.4 and supplemented with 0.02% Na oleate were added. If metabolic inhibitors were used, the cells immediately became sphaerocytes in this medium. In the presence of propranolol and related drugs, 0.2% Na oleate was needed for perfect sphering. Three parallels were run of each suspension in perpendicular Westergreen tubes at 20–25 °C. In control erythrocytes, macroscopic aggregates developed within 15 minutes and this was followed by rapid sedimentation. Subsequently sedimentation slowed down and stopped. The final demarcation line of the cells lay below the 100 mm mark. If adhesiveness increased, rapid sedimentation started earlier; if it decreased, the onset of rapid sedimentation was postponed and the cells did not reach the 100 mm mark at the equilibrium. In extreme cases, no rapid sedimentation occurred.

Results

Shape changes, volume changes

Cell shape was affected by both ATP-depletion and propranolol treatment. In the former case there was a transformation in the direction of echinocytosis, in the latter case towards stomatocytosis (Fig. 1). RBC shape was transformed even in the absence of extracellular Ca^{2+}, without any significant change in cell volume. If Ca^{2+} was present in the medium, echinocytosis was slightly promoted and stomatocytosis hindered, and due to the changes in permeability (see later) a considerable shrinking took place.

Sedimentation

The presence or absence of volume changes by shape transformation is usually checked by haematocrit determination. We intended to control, however, whether the results obtained were not affected by the transformed cell shape. The results of the microhaematocrit tube test were controlled by the isotope dilution technique using ^{125}I-albumin. It turned out that shape changes developed in the

presence of EGTA did not significantly affect the reliability of the microhaematocrit tube technique. Conversely, in systems containing Ca^{2+}, parallel with the shape and volume changes the amount of fluid trapped in the microhaematocrit

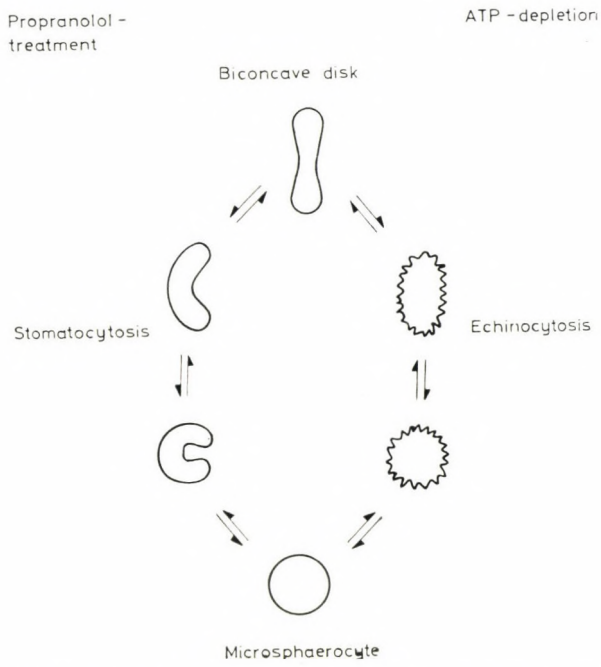

Fig. 1. Changes of erythrocyte shape during treatments inducing rapid K^+-efflux

Table 1

Haematocrit values in various systems after 3-hour incubation at 37 °C

Medium	Haematocrit		
	^{125}I-albumin dilution	Microhaematocrit tube technique	"Trapped fluid" per cent
Physiological saline			
2.5 mM IA + 10 mM inosine + 2.5 mM EGTA	27.8	28.5	2.5
2.5 mM IA + 10 mM inosine + 10 mM $CaCl_2$	14.5	20.2	28.2
Serum			
2.5 mM EGTA	36.9	38	2.9
2.5 mM IA + 10 mM inosine	24	32	25
8 mM NaF + 2.5 mM Na_2HAsO_3	29.8	33.2	12.5
16 mM NaF	27.5	34	19.1

tube was significantly altered (Table 1). Similar results were obtained with inulin, indicating that the changes were not due to an altered adsorption of albumin to the cells. In view of the varying amount of trapped fluid, the results read on the haematocrit tubes in these systems were not comparable. In propranolol (0.5 – 1 mM) + Ca^{2+} (0.5 mM) treated cells the trapped fluid increased significantly.

Adhesiveness

In contrast to the centrifugation method, shape changes in themselves caused a shift in sedimentation if Bicher's method was applied. In 1% dextran T-250 medium, loss of the biconcave shape and the consequent decrease in

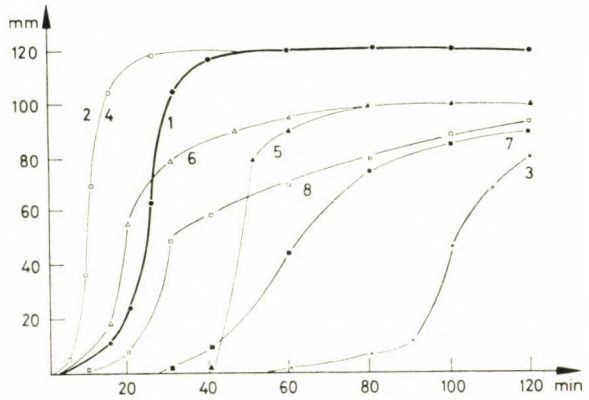

Fig. 2. Sedimentation of erythrocytes in 4% dextrane and 0.02% Na oleate. 1 = Control 2 = 2.5 mM EGTA, 3 = 8 mM NaF + 2.5 mM Na_2HAsO_4, 4 = 8 mM NaF + 2.5 mM Na_2HAsO + 2.5 mM EGTA, 5 = 16 mM NaF, 6 = 16 mM NaF + 2.5 mM EGTA, 7 = 1 mM IA + 10 mM inosine, 8 = 1 mM IA + 10 mM inosine + 2.5 mM EGTA. Incubation medium: serum; time: 3 hours; temperature: 37°C

rouleaux formation was reflected in a reduction of the sedimentation rate. In addition, a direct alteration of the RBC adhesiveness was found if the shape differences were abolished by agents inducing sphaerocytosis (lecithin, Na oleate, Na cholate). By using 4% dextran T-250 medium, sedimentation of these sphaerocytes could conveniently be enhanced and hence their adhesiveness could sensitively be tested. Under these circumstances, RBC adhesiveness increased on elimination of Ca^{2+} by EGTA-treatment, while it decreased significantly under the combined effect of Ca^{2+} and agents inducing ATP-depletion. IA and higher concentrations of NaF affected adhesiveness even if the Ca^{2+} effect had been eliminated (Fig. 2). The significant stomatocytosis caused by 0.5 mM propranolol and related compounds (1.5 mM pronethalol, 2.5 mM tetracaine) could only be compensated by a 10-fold elevation of Na oleate in the medium used for sedimentation studies.

Under these circumstances adhesiveness also decreased significantly. Due to membrane damage indicated by the incipient haemolysis these results could, however, be accepted only with caution.

Cation permeability, osmotic resistance

The rate of K^+-efflux elicited as described above depends on the extracellular Ca^{2+}-concentration up to a certain limit. Above a definite concentration it cannot be enhanced by further addition of Ca^{2+}. This critical Ca^{2+}-concentration is 10 mM when fresh erythrocytes are being ATP-depleted and 0.5 mM when fresh cells are treated with 0.5–1 mM propranolol, or when phosphate ester depleted cells are exposed to Ca^{2+}, or to Ca^{2+} and propranolol. After a certain time, even at optimum Ca^{2+}-concentrations the K^+-efflux slowed down and ceased in every case (Figs 3a and b).

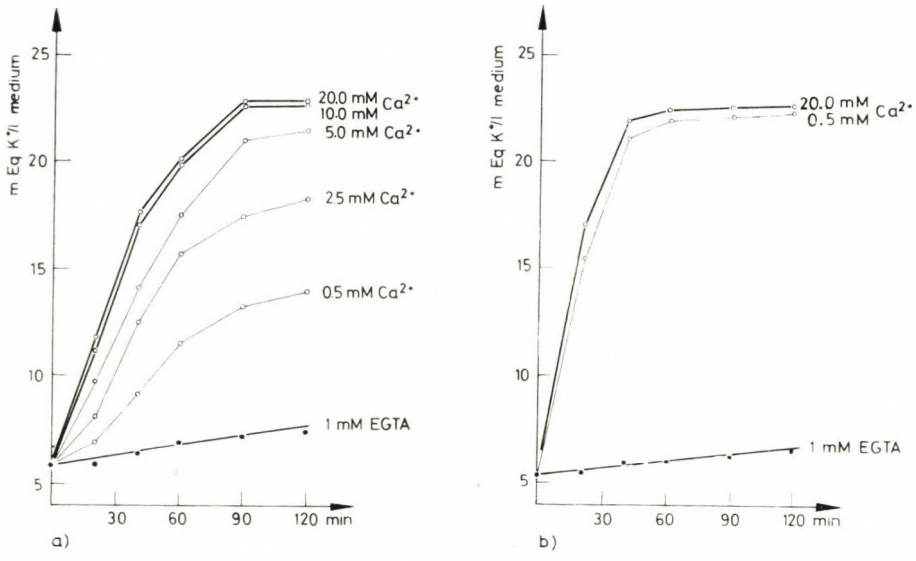

Fig. 3. Effect of Ca^{2+} on the rate of K^+ efflux. a) Fresh erythrocytes + 0.25 mM IA + 10 mM inosine. b) Phosphate ester depleted erythrocytes

The final state of the Ca^{2+}-dependent K^+-efflux does not represent an equilibration of K^+-ions. K^+-concentration in the cells is still significantly higher than in the medium. If cells are transferred into a low-K^+ medium completed with Ca^{2+}, no further K^+-efflux takes place. These cells are not haemolyzed by ice-cold distilled water, indicating a significant increase in osmotic resistance. If, however, the cells are further incubated in 0.16 M KCl completed with Ca^{2+}, a K^+-influx sets in indicating that the cell membrane remained permeable to Ca^{2+}.

Meanwhile the cells regain their original volume (Fig. 4). Their osmotic resistance also returns to normal.

These K^+-transport processes can be followed more exactly by the use of ^{42}K. If during rapid K^+-efflux the medium is completed with ^{42}K, the latter accumulates in the cells. If these cells are transferred into a low-K^+ medium after the standstill of rapid K^+-transport, isotope K^+ does not equilibrate. In high K^+ medium completed with Ca^{2+}, however, the isotope equilibrates very rapidly, while water uptake accompanying the rapid K^+-influx regenerates the normal

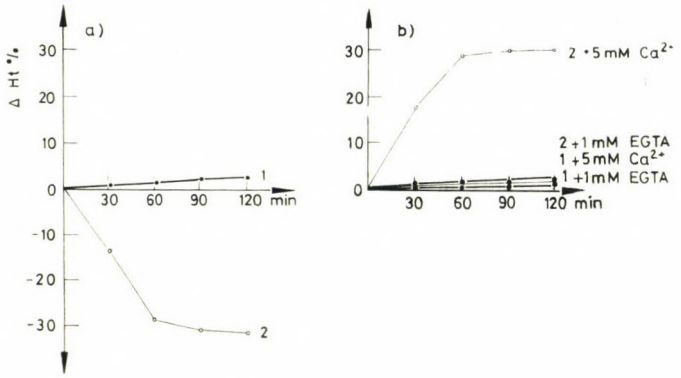

Fig. 4. Changes of haematocrit determined by ^{125}I-albumin during IA + inosine treatment. a) Incubation in 0.16 M NaCl. 1=1 mM EGTA, 2=5 mM $CaCl_2$. b) Incubation medium changed to 0.16 M KCl

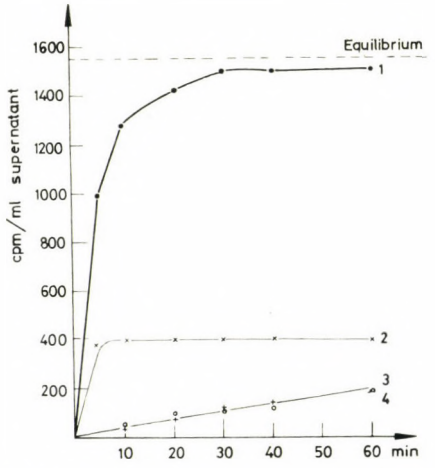

Fig. 5. ^{42}K-efflux into different media from cells preincubated with ^{42}KCl during the phase of rapid K^+-efflux. 1=0.16 M KCl + 2 mM $CaCl_2$, 2=0.16 M NaCl + 2 mM $CaCl_2$, 3=0.16 M KCl + 1 mM EGTA, 4=0.16 M NaCl + 1 mM EGTA

cell volume (Fig. 5). The final stage of rapid K^+-efflux is determined by the extent of shrinkage of the cells, as K^+-efflux stops at 60–65% intracellular haemoglobin concentration.

Discussion

Opinions diverge concerning the mechanism of the Ca^{2+}-dependent rapid K^+-efflux. According to Kregenow and Hoffman [5] and Manninen [6] it is a carrier-mediated transport. The main argument for this is the ouabain-sensitivity of the process described by the former authors. The validity of this interpretation has been refused by Lew [7], who demonstrated that K^+-efflux was ouabain-sensitive only in cells containing residual ATP. Hence, the explanation of the ouabain-sensitivity of the K^+-efflux is the Ca^{2+}-chelating effect of ATP spared by the ouabain-inhibition of transport-ATPase. According to Glynn and Warner [8], Manninen's results may be interpreted also without the assumption of a carrier-mediated transport. A reason for all these contradictions in the literature is that some research teams regard the red blood cells as too simple corpuscles and they do not attempt to achieve standard biochemical and physicochemical conditions suitable for the kinetic analysis of K^+-transport.

An important prerequisite to achieve this goal is a complete phosphate ester depletion of the cells. To this end we induced the breakdown of 2,3-DPG – a potential ATP source in erythrocytes – by $NaHSO_3$ and subsequently depleted the cells from ATP. The phosphate ester content of cells obtained in this way was very slight and their inorganic phosphate level low and no change in phosphate metabolism could be observed during the experiments. Ca^{2+} permeability of these cells surpassed by 10–15 times that of the fresh cells during the process of depletion [9]. The phosphate ester content of fresh, propranolol-treated cells is high, their phosphate metabolism is not in equilibrium, their Ca^{2+}-permeability is transitorily increased. Thus, the normal interactions between ATP and membrane seem to be altered. In cells completely depleted from phosphate-ester propranolol slightly reduces the high Ca^{2+}-permeability. This reduction, however, does not limit the rate of K^+-transport. At the same time it promotes the Ca^{2+}-membrane interactions advantageous for K^+-transport [9]. Thus, the preparations most suitable for kinetic analysis are the phosphate ester depleted cells, eventually completed with propranolol.

Even under such conditions the linear phase of K^+-permeability is restricted in time. To explain this, the volume reduction accompanying the rapid K^+-efflux and the consequent increase of intracellular Hb-concentration have to be considered. The change of Hb-concentration in *in vitro* systems and erythrocytes does not influence the ion activity, it does not cause a "bound water" effect within a wide range [10]. According to our results, above a 60–65% intracellular Hb-concentration, ion activity in the cell is dramatically reduced and diffusion practically stops. Riordan and Passow [11] demonstrated that after the rapid K^+-efflux the osmotic resistance of the cells increases. These cells do not haemolyze in ice-cold distilled water; in this medium they behave like the paracrystalline rat

erythrocytes [12]. Consequently, by kinetic calculations intracellular ion activities can be regarded to be proportional to the ion concentration only in a definite interval of K^+-transport.

By an analysis of the K^+-transport process, the K^+-content of the cell has to be computed. Doubts have, however, emerged concerning the reliability of values derived from calculations based on the results of the microhaematocrit test, it having been shown that the sedimentation rates decrease in cases where an echinocytic shape transformation has taken place [13]. These sedimentation differences are, however, reflected after centrifuging in a microhaematocrit tube only if permeability and volume changes also occur, as for example in the presence of Ca^{2+}. For simple shape changes, sedimentation techniques without centrifugation were found to be much more sensitive. One reason for this is obviously the reduced tendency for rouleaux-formation of the cells that have lost their perfect biconcave shape. By transforming the red blood cells into sphaerocytes with Na oleate, it could be demonstrated that there was even a direct change, namely a decrease in adhesiveness of cells treated with metabolic inhibitors and Ca^{2+}, as compared to the metabolically intact cells treated only by oleate. Thus, Ca^{2+} in erythrocytes, in contrast to tissue cells, does not enhance adhesiveness but causes an opposite effect. This behaviour of the red cells should be considered a useful adaptation. An adhesive property that can be enhanced with Ca^{2+} would lead in the Ca^{2+}-containing plasma to sludge-formation with unfavourable rheological consequences. Prevention of Ca^{2+}-accumulation completely abolished the changes of adhesiveness in the case of some metabolic inhibitor combination. However, the technique developed was sensitive enough to demonstrate that in addition to Ca^{2+}-penetration, adhesiveness was influenced by other effects (high concentrations of NaF, SH-reagents, etc.). Light transmission through an erythrocyte suspension is affected by the cell shape and volume which are unstable in our cases. This causes difficulties in the determination of adhesiveness by light transmission changes. On the other hand the adhesiveness of erythrocytes artificially transformed into sphaerocytes is so slight that minute differences could only be observed by the highly sensitive instruments based on light reflexion (syllectometry). With all this in mind the determination of adhesiveness based on the sedimentation rate under standard conditions should be regarded as a very convenient procedure.

Since in the course of Ca^{2+}-penetration due to the altered shape and adhesiveness, there was a shift in the amount of the fluid trapped in the microhaematocrit tubes, it has been concluded that for computing cell K^+, only cell volume data obtained by the isotope dilution technique can be used.

In view of the above results, in our kinetic analyses metabolically stable, depleted erythrocyte suspensions and optimum Ca^{2+}-concentrations were used, only the linear phase of K^+-efflux was taken into consideration and cell K^+ was computed based on isotope cell volume determinations.

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Life Span of Homogeneous Red Cell Population Formed after Thermal Injury

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1. The elimination curve of red blood cells (RBC) labelled with ^{15}N-glycine was examined. A 120—123-day mean life span was found for the RBC of healthy individuals, in good agreement with data in the literature. Most of the RBC died between the 104th and 140th day; elimination started approximately on the 70th day and was complete at 150 days.

2. Mean life span of RBC formed on the 8th day after thermal injury is 102—103 days. In this respect the individual cells differed significantly. Most of them were destroyed between the 75th and 152nd day, elimination started on the 40th day.

3. Mean life span of RBC formed on the 30th and 31st day after thermal injury amounted to 111 days. Their elimination curve was similar to the curve observed in the earlier stage of the illness.

4. The life span of RBC is only slightly shortened subsequent to thermal injury. This factor, as compared to the severe disorder of haemoglobin synthesis, plays a minor role in the pathogenesis of thermal anaemia.

Early hyperhaemolysis and a prolonged bone marrow dysfunction play a significant role in the pathogenesis of thermal anaemia. The biochemical features of bone marrow dysfunction have been discussed previously [1—7]. Mean life span of RBC is reduced in both the early and the late phase of thermal injury [10]. The aim of these investigations having been to estimate the life span of a mixed RBC population, all RBC were labelled with ^{51}Cr without distinction. It could not therefore be decided whether the shortening of the life span was due to direct injury of the circulating RBC, or the RBC formed after the injury were involved, or both factors had a role.

To decide the question, it was necessary to label solely the RBC unaffected by heat. Therefore, ^{15}N-glycine was chosen as a compound incorporated only by young immature cells (erythroblasts, reticulocytes). Previously, an average life span of 126 days was established in the only patient [12] with ^{15}N-glycine after thermal injury.

Material and Method

Examinations were performed in 2 healthy subjects and 2 burnt patients. They were given by mouth 2 g of ^{15}N-glycine dissolved in 100 ml water every 4 hours for 2 days. The total dose of ^{15}N-glycine amounted to 16 g.

The increase of excess ^{15}N-atom percentage in haem and the total ^{15}N content of haem was determined on 12 occasions in a period of 120–160 days.

Haem was isolated according to Shemin and Rittenberg [14, 15] and recrystallized according to Fischer [11]. Our one-step method [9] was applied for releasing nitrogen from haem. The released excess ^{15}N atom percentage was determined with a Varian MAT M-86 type high precision mass spectrometer. The total amount of ^{15}N incorporated in the haem molecule was calculated from the excess atom percentage and the circulating total amount of haem. From the results a curve was plotted.

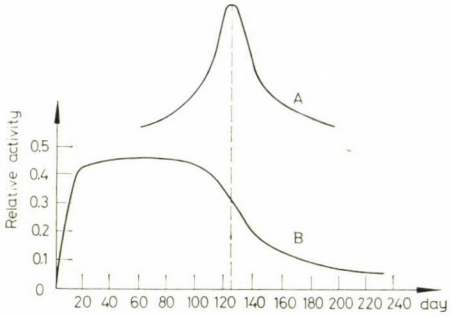

Fig. 1. Curve of incorporation and elimination (B). The rate of destruction (A) is indicated by the slope of the descending part and the differential quotient of the curve (B). (The peak of curve A shows the time of maximum destruction)

A large amount of the ingested ^{15}N-glycine is soon utilized for haemoglobin synthesis so that a comparatively homogeneous population of erythroid cells of approximately identical age becomes labelled. A further advantage of the method over labellling with ^{59}Fe is that ^{15}N-glycine is practically not reutilized [8, 13–15]. At present this is the most reliable procedure of RBC life span estimation.

The total amount of excess ^{15}N atom labelling a homogeneous population of RBC changes characteristically with time [N/N(t)]. The curve shows (a) an initial sharp increase; (b) a horizontal plateau and (c) a sharp decrease reaching or approximating the base line. Mean RBC life span is determined during the sharp decrease, where the rate of destruction is highest. Mathematically, this is the point of the peak value for the dN/dt differential quotient (Fig. 1). In view of the fact that not all the labelled RBC are formed at the same moment, as indicated by the initial part of the curve which does not rise vertically, Shemin and Rittenberg [14, 15], have approximated this part of the curve by an exponential function. Instead of determining the maximum of the differential quotient dN/dt, the necessary correction was completed by determining the maximum (i.e. time) of the expression

$$\frac{1}{\lambda} \cdot \frac{d^2N}{dt^2} + \frac{dN}{dt},$$

where λ is a constant denoting the exponent of the function describing the initial rise. This correction being complicated and inaccurate, correction for the non-simultaneous formation of RBC is performed by subtracting the time of the half maximum point of the rising slope of the curve from the time corresponding to the maximum of the first differential quotient. Calculation thus consists of the differentiation of the empirical curve.

Instead of applying this complicated procedure, we have determined the differentiated curve by an analogous electronic method. The empirical curve is read by a moving photocell detector, differentiation is performed electronically, and the differentiated curve is plotted together with the original one by an X – Y recorder.

The curves obtained in this way offer a more reliable information than do conventional calculations.

Results and Discussion

In the RBC (in the haem) of healthy subjects, after the ingestion of ^{15}N-glycine the excess ^{15}N atom percentage increases rapidly (Table 1, Fig. 2). The rate of incorporation is highest between the 3rd and 7th days. The maximum of incorporation is reached between the 12th and 14th days. Then the level remains practically constant until the 104 – 105th day, subsequently until the 114th day a slow and then a more rapid decrease takes place. On the 146th day a relatively low value is found (0.060 excess ^{15}N atom percentage). The rate of decrease is highest between the 121st and 126th days with the mean at the 123rd day. Thus, according to the above data, the mean life span of RBC is 123 – 5 = 118 days.

Table 1

Incorporation of ^{15}N into haem, and its elimination from blood circulation in a healthy subject

Date	Excess ^{15}N atom percentage in haem	Total amount of circulating haemoglobin g/100 ml	Total amount of circulating haem mg	Total amount of ^{15}N in haem mg
V. 15.	0.004	610	22.143	0.08
V. 24.	0.145	717	26.027	3.43
V. 28.	0.163	728	26.426	3.91
V. 31.	0.162	728	26.426	3.89
VI. 4.	0.166	650	23.595	3.56
VI. 29.	0.178	648	23.522	3.81
VII. 23.	0.167	616	22.361	3.39
VIII. 27.	0.159	692	25.120	3.63
IX. 6.	0.155	619	22.470	3.17
IX. 13.	0.136	628	22.796	2.82
IX. 20.	0.109	602	21.853	2.16
X. 9.	0.060	624	22.651	1.24

Even more accurate results are obtained if the mean life span of RBC is calculated from the total amount of ^{15}N in haem (Table 1, Fig. 3). The incorporation rate is highest between the 3rd and 7th days, with the peak on the 14th day. The plateau lasts until the 105th day. The rate of decrease is highest between 121 and 129 days. On the basis of these data, the mean life span of RBC of healthy subjects is 125 – 5 = 120 days.

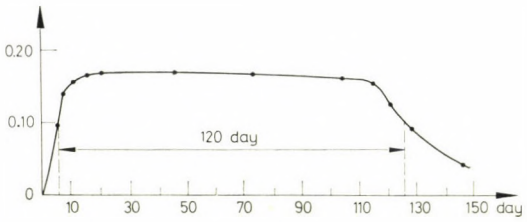

Fig. 2. Excess ^{15}N atom percentage in haem of a healthy subject after ingestion of ^{15}N-glycine

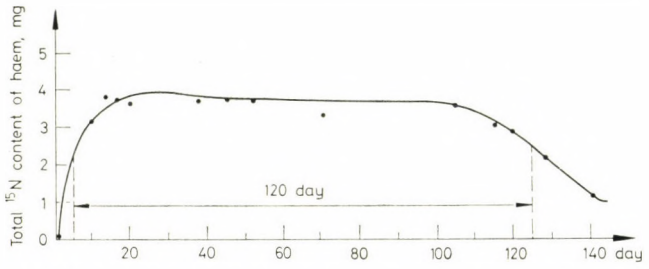

Fig. 3. Total ^{15}N in haem of a healthy subject, after ingestion of ^{15}N-glycine

The first patient, K. A., a 20-year-old male, had suffered 25% second degree and 15% third degree burns on 15th April, 1969. The history was non-contributory. He was transferred to our Hospital on the 7th day after injury. On admission: RBC, 4.9 million; haemoglobin, 15.8 g/100 ml; haematocrit, 47%; MCH, 32 $\mu\mu$g; MCHC, 34%. On the 30th day after injury: RBC count, 4.46 million; haemoglobin, 12.5 g/100 ml; haematocrit, 42%; MCH, 28 $\mu\mu$g; MCHC, 29.8%; plasma volume, 2.875 ml; RBC volume, 1.667 ml; total blood volume, 4.542 ml. The patient was kept on a diet of 110 g protein, 485 g carbohydrate and 95 g fat.

Table 2

Incorporation of ^{15}N into haem and its elimination from the blood circulation in a burnt patient (K. A. 20 years, male)

Date	Excess ^{15}N atom percentage in haem	Total amount of circulating haemoglobin g/100 ml	Total amount of circulating haem mg	Total amount of ^{15}N in haem mg
V. 15.	0.0045	568	20.618	0.08
V. 24.	0.318	583	21.163	6.12
V. 28.	0.320	458	16.625	4.84
V. 31.	0.295	458	16.625	4.46
VI. 4.	0.282	463	16.806	4.31
VI. 29.	0.241	624	22.651	4.96
VII. 23.	0.191	762	27.661	4.88
VIII. 27.	0.135	768	27.878	3.42
IX. 6.	0.120	757	27.479	3.00
IX. 13.	0.094	761	27.624	2.36
IX. 20.	0.079	754	27.370	1.97
X. 9.	0.042	798	28.974	1.11

On days 30 and 31, 16 g of ^{15}N-glycine was administered. It caused no complaint whatever.

On the 12th day after this treatment, i.e. 42 days after injury, excess ^{15}N atom percentage was maximum (Table 2, Fig. 4). It decreased fast between the 14th and 21st days, then slowed until the 114th day, to increase again to the 121st day. Finally, from the 121st until the 146th day, the rate of decrease was slow. According to the curve displaying the changes, RBC destruction was significant

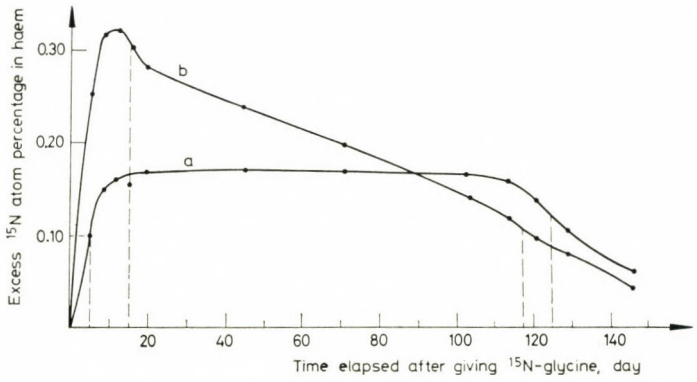

Fig. 4. Excess ^{15}N atom percentage in haem in a burnt patient after ingestion of ^{15}N-glycine (b); (a) shows the same curve for a healthy subject

as early as 14 days after ^{15}N-glycine intake. However, calculations based on excess atom percentage yield reliable data only if the rate of RBC formation – and thus the total volume of RBC – is constant during the period of investigation. If the total volume of RBC increased during this period the decreasing trend of the curve can be due either to an increased destruction of RBC or to isotope dilution caused by the appearance in the circulation of non-labelled RBC.

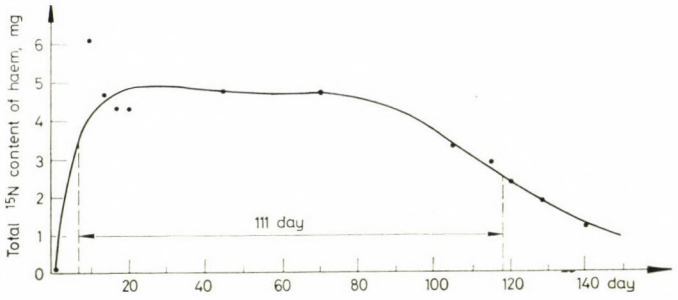

Fig. 5. Total ^{15}N in haem after ^{15}N-glycine ingestion, in a burnt patient

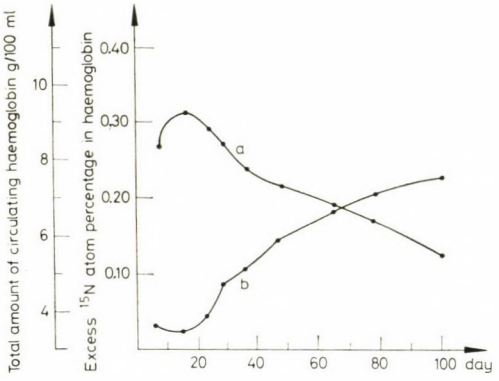

Fig. 6. Correlation between excess ^{15}N atom percentage (a) and haemoglobin content (b) in a burnt patient

In this patient, the circulating haemoglobin mass increased from 458 g to 798 g in 131 days. Therefore, to obtain correct results, mean RBC life span had to be calculated from the change in total ^{15}N content of haem (Table 2, Fig. 5).

The curve for total ^{15}N content of haem – similarly to that for the healthy subjects – also displays a plateau but the values are beginning to decrease from the 80th day and the rate of destruction attains its maximum between the 115th

Table 3

Incorporation of ^{15}N into haem and its elimination from the blood circulation in a burnt patient (Sz. I., 22 years, male)

Date	Excess ^{15}N atom percentage in haem	Total amount of circulating haemoglobin g/100 ml	Total amount of circulating haem mg	Total amount of ^{15}N in haem mg
X. 6.	0.270	369	13.395	3.28
X. 13.	0.318	347	12.596	3.64
X. 22.	0.292	377	13.685	3.63
X. 27.	0.277	476	17.279	4.35
XI. 3.	0.233	515	18.694	3.95
XI. 15.	0.220	580	21.054	4.21
XII. 3.	0.188	663	24.067	4.11
XII. 15.	0.168	714	25.918	3.96
I. 6.	0.125	738	26.789	3.04

and 121st days, with the mean at 118 days. Thus, the mean life span of RBC formed in the 5th week after thermal injury amounts to 111 days (118 − 7 = 111). This means that extensive RBC destruction starts about 20 days earlier than in healthy subjects. The descending slope of the curve is prolonged, i.e. after a thermal injury the life span of the individual RBC differs to a greater extent than under normal circumstances.

The second patient, Sz. I., a 22-year-old male, suffered 10% second degree and 15% third degree burns. ^{15}N-glycine was given on the 7th and 8th days following the burn. Maximum excess ^{15}N atom percentage in haem was reached 14 days later (Table 3, Fig. 6). Subsequently, ^{15}N concentration decreased rapidly until the 35th day, then slower until the 100th day.

The curve was similar to that for the first patient with the only difference that in the second case the fast rate of decrease persisted for 3 weeks instead of one week, as in the first patient. The phenomenon was explained by the difference in circulating total haemoglobin mass (Fig. 6). Figure 6 demonstrates that the curve for excess ^{15}N atom percentage and that for the total haemoglobin mass are almost mirror images; thus, the decrease in concentration is related to its increase in haemoglobin content (isotope dilution).

The curve displaying the total amount of ^{15}N in haem (Table 3, Fig. 7), after reaching a maximum between the 20th and 30th days, remained constant until the 75th day, then descended to be steepest between the 98th and 120th days with the mean on the 109th day. As calculated from this curve and of the value for half-time incorporation at 7 days, the mean RBC life span was 102 days.

Significant RBC destruction occurred from the 75th day on. The life span of individual cells showed significant differences in this case, too.

Derived curves obtained by the electronic analogue method provide more information than data obtained by conventional calculations. The derived curve for healthy subjects (Fig. 8, curve b) shows little differences in individual RBC life span.

A slow rise can be noted from about the 70th day, on the 104th day the curve becomes steep to reach the maximum on the 128th day. Finally, between the 140th and 145th days it flattens again. Thus, the curve for the differential quotient is approximately symmetrical to the vertical drawn through the peak. Elimination

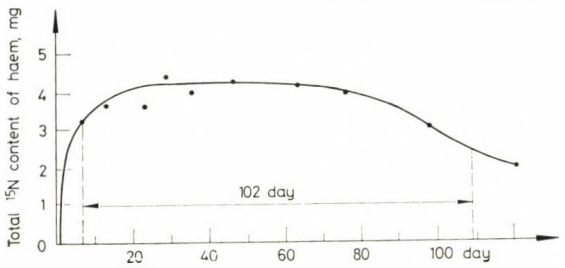

Fig. 7. Total ^{15}N in haem after ^{15}N-glycine ingestion, in a burnt patient

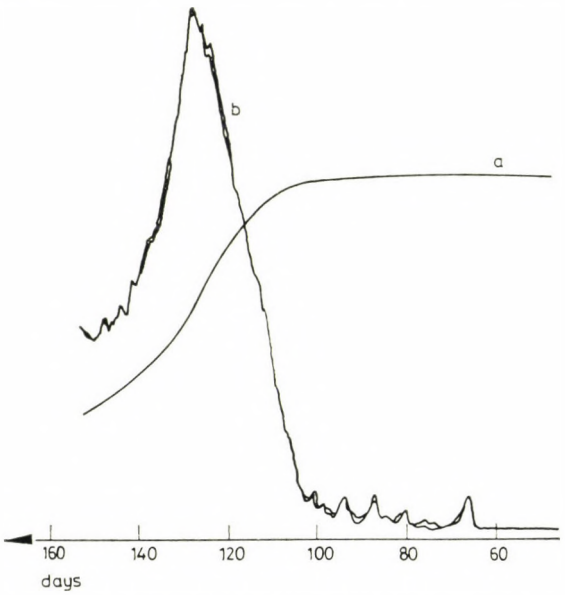

Fig. 8. Elimination curve of ^{15}N (a) and its derived curve (b) in a healthy subject

of RBC starts on the 70th day, and most of the RBC are destroyed between the 104th and 140th days according to the Gauss distribution, and their mean life span amounts to 123 days.

The derived curve of the second patient differed from the curve for healthy subjects (Fig. 9, curve b). The slow rise started on the 40th day. The slope became

steep on the 75th day to reach the maximum on the 110th day. From this time onwards the curve descended at a fast rate, although slower than it rose in the first period, then it became flat. Thus, the derived curve for this burnt patient was broad and asymmetrical. In other words, the life span of individual cells differed to a greater extent than under normal conditions. The rate of destruction was also higher between the 75th and 110th days than between the 110th and 152nd days. Most of the RBC disappeared from the circulation between the 75th and 152nd days.

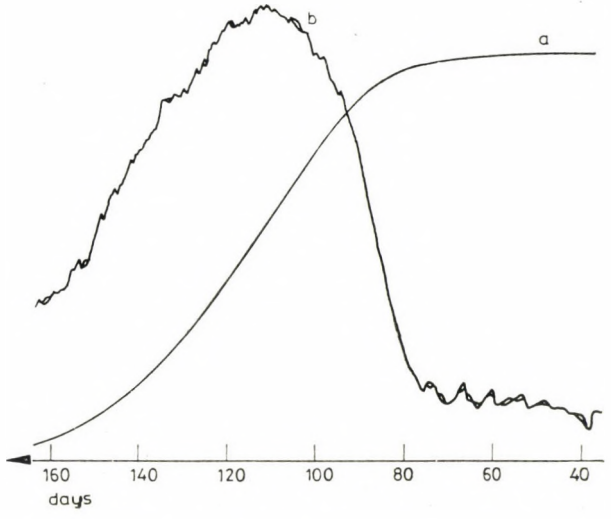

Fig. 9. Elimination (a) and derived (b) curve for ^{15}N in a burnt patient

Thus, mean RBC life span as calculated from the maximum of the derived curve and the incorporation half-time, amounted to 103 (110 – 7) days. As the curve is asymmetrical, the real life span must be somewhat longer.

Shemin and Rittenberg's definition of the mean life span is not necessarily identical to the value obtained by mathematical calculations, as in pathological cases the derived curve is not always symmetrical. This fact calls for a method for estimating the real life span by means of electronic analogous and computer techniques.

Thus, mean life span of the RBC of burnt patients was found to be shortened only slightly. This means that the increased rate of RBC destruction cannot play a decisive role in the pathogenesis of thermal anaemia and is of secondary importance in comparison with the severe disturbance of haemoglobin synthesis [1 – 7]. In view of the latter, however, even a moderate shortening of the mean RBC life span must be considered a factor maintaining anaemia and even aggravating it in the late period following a thermal injury.

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Перманентный интраваскальный гемолиз (ПИГ) при гипопластических состояниях кроветворения

(клинико-морфологические параллели)

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Возникновение перманентного интраваскального гемолиза (ПИГ) у больных гипо-апластической анемией описано в различные сроки после удаления селезенки, произведенного в связи с тромбоцитопеническими кровотечениями.

Приводим кратко наши наблюдения.

Больная К. в возрасте 37 лет подверглась спленэктомии по витальным показаниям в связи с профузными маточными кровотечениями, возникшими на фоне миелогипоплазии с тромбоцитопенией (в костномозговом трепанате 70% жира). Через 11 лет у больной в связи с гемотрансфузиями впервые появились гемолитические кризы с гемоглобинурией. В настоящее время (1972 г.), спустя 15 лет от начала заболевания, у больной наблюдается развернутая картина ПИГ с типичной клиникой болезни Штрюбинга—Маркиафава: выраженная гемологическая анемия и желтуха, а также гиперретикулоцитоз и нормобластоз на фоне парциально-гиперпластического, эритробластического костного мозга, гипергемоглобинемия, постоянная гемосидеринурия («железный диабет»), гемоглобинурия в период кризов, гипосидеремия. В последнее время возник гиперкоагуляционный синдром (флеботромбозы); определяются резко положительные кислотная и сахарозная пробы.

Эволюция болезни со сменой клинических масок (Верльгоф—Маркиафава) может наблюдаться и спонтанно, без спленэктомии.

У больного А. с 7-летнего возраста возникают тромбоцитопенические кровотечения. Через 13 лет в связи с очередной гемотрансфузией развилась тяжелая форма ПИГ с частыми гемоглобинурийными кризами. В костном мозге в период тромбоцитопении наблюдался выраженный мегакариоцитоз, в период ПИГ — выраженный эритробластоз. Спустя 3 года после обнаружения первых признаков ПИГ наступил летальный исход при явлениях тяжелейшего гемолитического и абдоминального сосудисто-тромботического криза.

Подобная трансформация болезни, очевидно, может происходить в плане единого процесса — дисплазии кроветворения (дисгемопоэза), протекающего как бы двухфазно. Первая фаза — миелогипопластическая, протекающая нередко под маской болезни Верльгофа, ее гипопластического варианта. Вторая фаза — парциальная эритробластическая гиперплазия костного мозга, имитирующая эритромиелоз с развернутой клинической картиной болезни Штрюбинга—Маркиафава, т. е. ПИГ, возникающего в связи с поступлением в циркуляцию подверженных внутрисосудистому гемолизу

«комплементсензитивных» эритроцитов, продуцируемых в костном мозге аномальной популяцией «ПИГ-эритробластов».

Применение простых диагностических тестов — определения содержания гемоглобина плазмы, кислотной и сахарозной проб — позволяет диагностировать «болезнь мембраны эритроцитов» в ее ранней, гипопластической, фазе развития, еще задолго до выраженных клинических проявлений мочевого ПИГ-синдрома — гемосидеринурии и гемоглобинурии.

Специфичность сахарозной пробы подтверждается данными Л. И. Идельсона и соавт., получивших положительный результат этой пробы у 41 больного болезнью Штрюбинга—Маркиафава, которым ставили эту пробу, наряду с отрицательным результатом у 46 больных гемолитической анемией других видов. При этом сахарозная проба оказывалась положительной и в ранней стадии болезни, при отсутствии мочевого синдрома, на фоне гипопластического костного мозга, когда единственным лабораторным критерием интраваскального гемолиза являлся лишь несколько повышенный уровень ретикулоцитов ($>1{,}2\%$) и гемоглобина плазмы (>4 *мг%*).

В настоящее время еще не представляется возможным утверждать облигатную двухфазность болезни Штрюбинга—Маркиафава. Тем не менее выявление с помощью специфических лабораторных тестов ранних признаков ПИГ при гипопластических состояниях кроветворения позволяет рассматривать как попытку к регенеративному гемопоэзу соматическую мутацию, приводящую к появлению «ПИГ-клеток» в гипо-апластическом костном мозге.

В соответствии с современной клоновой теорией патогенез ПИГ на основе миелогипоплазии может рассматриваться как процесс постепенной, длящейся годами «оккупации» костного мозга патологической популяцией «ПИГ-эритробластов», продуцирующих «комплементсензитивные» эритроциты, подверженные интраваскальному гемолизу. В начальной фазе клинические проявления ПИГ еще не выражены, и определяемый при помощи специальных тестов ПИГ-синдром носит чисто «лабораторный» характер. Констатация ПИГ-синдрома на фоне миелогипоплазии в этом периоде носит не столько диагностический, сколько прогностический характер, предвещая перспективное развитие болезни Штрюбинга—Маркиафава, разумеется, при условии, что больной переживет фазу миелодепрессии. В противном случае, если больной погибнет в анемической коме от профузных тромбоцитопенических кровотечений, при патологоанатомическом исследовании обнаруживается типичная картина миелогипоплазии или аплазии без признаков интраваскального гемолиза (гемосидерин в почках, как правило, отсутствует).

Б о л ь н о й Н., 21 года, страдал подострой гипопластической анемией, осложненной профузными тромбоцитопеническими кровотечениями, приведшими к летальному исходу через 10 месяцев от появления первых симптомов болезни. В результате клинико-лабораторных исследований при жизни больного обнаруживали латентный без пароксизмов гемоглобинурии ПИГ-синдром: при слегка повышенном ретикулоцитозе (1,5—3%) и уме-

Дифференциально-диагностические признаки болезни Штробинга—Маркиафава и ПИГ-синдрома при гипоплазии костного мозга (по Г. А. Алексееву и Г. Б. Берлинеру)

Дифференциально-диагностические критерии		Болезнь Штрюбинга-Маркиафава	ПИГ-синдром при гипоплазии костного мозга
Клинические	Гемолитический (гемоглобинурийный) синдром	++	—+
	Тромбоцитопенический геморрагический синдром	—	+
	Гипосидеремический синдром («железный диабет»)	+	—
	Гиперкоагуляционный синдром (сосудистые тромбозы)	+	—
	Тромбогеморрагический синдром	+	—
Морфологические	Панцитопения	+—	++
	Сидероцитоз в крови	+	+
	Сидеробластоз в костном мозге	—+	++
	Эритробластическая пролиферация костного мозга	++	—+
	Жировое перерождение костного мозга	—	+
	Гемосидероз кожи, печени, селезенки, костного мозга, лимфатических узлов (по данным биопсий, включая удаленные селезенки, и некропсий)	—+ Как правило, посттрансфузионная болезнь	++
	Изолированный гемосидероз почек	++	—+
Биохимические	Гипербилирубинемия	+	—
	Гемоглобинемия	++	—+
	Гиперсидеремия	—	+
	Свободный трансферрин	+	—
	Десфераловый тест	—	+
	Гемосидеринурия	++	—+
Радиометрические	Утилизация Fe^{59} костным мозгом	+	—
	Демонстрирование Fe^{59} в печени	—	+
	Секвестрация меченных Cr^{51} эритроцитов в селезенке и печени	—+	+—
Терапевтические	Усиление гемолиза в связи с гемотрансфузиями и под влиянием гемостимулирующих средств	++	—+
	Эффективность спленэктомии	—	—+

ренной гиперсидеремии (207 *мкг*% по Генри), свойственной миелогипоплазии, отмечались гемосидеринурия, гипергемоглобинемия (15 *мг*%), положительные кислотная и сахарозная пробы (данные Л. И. Идельсона и В. И. Бенисовича). Несмотря на многократные гемотрансфузии, в том числе иногруппной крови (у больного кровь IV группы, резусотрицательная), ни разу не наблюдалось острого интравазального гемолиза (не было пароксизмальной ночной гемоглобинурии — ПНГ). Клинический диагноз: болезнь Маркиафава—Микели, гипопластическая фаза. На секции найдено тотальное жировое перерождение костного мозга. При микроскопическом исследовании (прозектор Б. С. Сидоров, консультант Н. М. Неменова) в плоских костях на фоне резкого преобладания жира отмечаются отдельные участки эритропоэза, состоящие из эритронормобластов. Незначительный гемосидероз почек. Патологоанатомический эпикриз: подострая апластическая анемия, осложненная тромбоцитопеническими кровотечениями. Вторичный ПИГ-синдром.

В данном случае больной погиб в первой, гипопластической, фазе болезни, когда еще не было развернутой картины ПИГ-синдрома, последний определялся только лабораторными исследованиями.

Дифференциально-диагностические критерии ПИГ-синдрома при гипоплазии костного мозга и болезни Штрюбинга—Маркиафава в ее развернутой фазе представлены в таблице.

ПИГ-синдром при гипоплазии костного мозга характеризуется в основном весьма умеренными проявлениями внутрисосудистого гемолиза, присоединяющимися к обычной картине миелогипоплазии.

Преобладающими клинико-морфологичекими синдромами являются: выраженная панцитопения, тромбоцитопенические кровотечения, жировое перерождение костного мозга, гемосидероз органов, костномозговой сидеробластоз (Г. Б. Берлинер и соавт.) наряду с гиперсидеремией и повышенным отложением железа в тканях, что доказывается положительным десфераловым тестом (Л. А. Аполлонова и Л. И. Идельсон; Н. С. Турбина и соавт.), депонирование радиоактивного железа в печени при нарушенной его утилизации костным мозгом (Г. Б. Берлинер и соавт.), секвестрация эритроцитов в печении и селезенке. Собственно симптомы внутрисосудистого гемолиза не столь выражены и нередко носят латентный характер. Пароксизмальная ночная гемоглобинурия, как таковая, в ряде случаев вообще не возникала. Напротив, в развернутой фазе болезни Штрюбинга—Маркиафава ведущими клинико-морфологическими синдромами являются гемолитический (гемоглобинурийный) и гиперкоагуляционный, обусловленные массивным внутрисосудистым распадом крови на фоне выраженной эритробластической гиперплазии костного мозга при обычном или редуцированном содержании жира, с наличием зон опустошения. Характерны изолированный гемосидероз почек с картиной «железного диабета», массивная гемосидеринурия, частые кризы (ПИГ), гипосидеремия, иногда «pica chlorotica». Костномозговой сидеробластоз не выражен, десфераловый тест отрицателен, утилизация радиоактивного железа костным мозгом не нарушена, депонирование радиоактивного железа в печени и повышенная секвестрация эритроциров в печени и селезенке, как правило, отсутствуют. Указан-

ные различия по существу отражают не столько различные нозологические состояния, сколько разные фазы одного и того же патологического процесса.

ПИГ-синдром, позволяющий диагностировать «болезнь мембраны эритроцитов», обнаруживается не только при гипопластических состояниях кроветворения, он может определяться также при гиперпластических и миелопролиферативных процессах, в частности при остеомиелосклерозе. По статистическим данным (Hansen и Killman), у каждого пятого больного остеомиелосклерозом можно выявить признаки перманентного внутрисосудистого гемолиза со снижением ацетилхолинэстеразной активности эритроцитов (АХЭ) при положительном тесте Хэма. Мы наблюдали в клинике больного остеомиелосклерозом с эритробластической метаплазией увеличенной селезенки (форма Воган) и ПИГ-синдромом в виде постоянной гемосидеринурии.

ПИГ-синдром описан также при миелокарционозе (раковых метастазах в костный мозг); в этом случае вторичный характер внутрисосудистого гемолиза не вызывает сомнений. Поэтому наряду со специфическими пробами, документирующими «болезнь мембраны эритроцитов», важнейшее значение в нозологическом диагнозе мы придаем морфологической картине костного мозга, полученного путем трепанобиопсии подвздошной кости. Морфологическим критерием болезни Штрюбинга—Маркиафава в развернутой стадии интраваза́льного гемолиза является парциальная эритробластическая гиперплазия костного мозга при отсутствии жира и наличии зон опустошения, образованных расширенными венозными синусоидами, заполненными эритроцитами, как целыми («костномозговой эритроцитоз»), так и находящимися в различных стадиях распада (эритрорексис, эритролиз).

Разумеется, морфологический критерий не является абсолютным. Однократная и «одноместная» трепанобиопсия не всегда отражает масштабность гемопоэза в целом. Обнаружение жирового костного мозга в илиакальном трепанате при наличии лабораторных признаков интраваза́льного гемолиза не дает права отрицать присутствие в сохранившихся очагах кроветворения популяции аномальных эритробластов, продуцирующих «комплементсензитивные» ПИГ-эритроциты.

Необходимо также учесть, что при резком сокращении плацдарма гемопоэза в связи с квазитотальной аплазией костного мозга интраваза́льный гемолиз может протекать без признаков гемолитической желтухи и мочевого синдрома (поскольку уровень свободного гемоглобина плазмы не превышает гемоглобинсвязывающей способности гаптоглобина). Этим же обстоятельством — редукцией общей массы эритробластов на первоначальном этапе болезни при отсутствии гемосидеринурии — объясняется гиперсидеремия в начальной, гипопластической, стадии болезни.

Хотя гемолиз при рассматриваемом заболевании происходит внутрисосудисто, участие элементов ретикуло-гистиоцитарной системы (РГС) в патологическом процессе имеет большое значение. Важнейшим показателем

заинтересованности РГС является отмечаемая у ряда больных спленомегалия. Увеличение селезенки при ПИГ достигает иногда внушительных размеров.

В литературе имеются сообщения об удаленных селезенках весом 2 *кг* (Донат), 1,8 *кг* (Topp и Hart), 900 *г* (Andersson). Мы (1969) наблюдали селезенку весом 1 *кг*.

Основным субстратом спленомегалии, как показывают гистологические исследования, является выраженная гиперплазия ретикуло-гистиоцитарных элементов пульпы, что наряду с атрофией лимфоидных фолликулов изменяет обычный рисунок строения селезенки. Причина столь выраженной гиперплазии ретикуло-гистиоцитарных элементов селезенки при ПИГ не вполне ясна. Полагают (Dacie), что спленомегалия возникает в порядке рабочей гипертрофии органа, «выхватывающего» из кровяного потока обрывки («тени») эритроцитов и гаптогемоглобиновые комплексы лизированных эритроциров. В пользу этой концепции свидетельствуют данные радиологических исследований, в результате которых у ряда больных обнаружено повышенное накопление Cr^{51} в селезенке (Motulsky и соавт.; Lewis и Dacie; Beal и соавт.).

Не отрицая известной «подсобной» роли селезенки в процессах внутрисосудистого гемолиза, мы на основе своих наблюдений допускаем возможность увеличения селезенки также в связи с ее миелоидной метаплазией. Приводим наше наблюдение.

Б о л ь н о й С. в возрасте 21 года впервые отметил появление «черной» мочи, особенно в ночное и утреннее время, развитие тяжелой анемии и желтухи. Ежегодно на протяжении 15 лет госпитализировался в клинику в связи с тяжелыми обострениями болезни. При последней госпитализации (ноябрь 1968 г. — февраль 1969 г.) больной слегка желтушен (билирубин 1,4 *мг*%, реакция непрямая), резко анемичен (Hb 2 *г*%, эр. 610 000, ретикулоц. 3,9%, нормобл. 7 на 100 лейкоцитов), при лейкопении [л. 2200, из них нейтрофилов 52%, э. 2%, б. 3%, лимф. 36%, мон. 6%, гемоцитобл. (!) 1%] и тромбоцитопении (15 250). Отмечены положительные кислотная и сахарозная пробы, выраженное увеличение печени и селезенки, постоянная гемосидеринурия. В трепанатах подвздошных костей (трепанобиопсия 1965 и 1969 гг.) среди миелокариоцитов опеределяется преобладание клеток красного ряда; наблюдаются скопления ретикулярных клеток, мегакариоциты. На последнем этапе болезни у больного развились абдоминальные тромбозы, профузные носовые и десневые кровотечения.

Данные патогистологического исследования (прозектор Н. Н. Вакуленко, консультант Н. М. Неменова): костный мозг плоских костей и диафизов длинных костей темно-красного цвета, сочный. Количество жира резко редуцировано. При микроскопическом исследовании отмечена выраженная гиперплазия красного костного мозга с преобладанием эритробластических элементов. Среди клеток гранулопоэза преобладают незрелые формы — миело- и промиелоциты. Мегакариоциты в обычном количестве.

Селезенка значительно увеличена (вес 1 *кг*). При гистологическом исследовании отмечены резкое полнокровие, значительная редукция фолликулов с зонами начинающегося опустошения в зародышевых центрах; лимфоидная ткань сохранилась лишь вокруг трабекул. Периваскулярная плазмоклеточная реакция. Зоны периваскулярного склероза (по типу портальной гипертензии). Гиперплазия ретикулярных элементов пульпы («огрубение стромы»), эритрофагоцитоз. Выраженная миелоидная метаплазия, эритронормобласты, гранулоциты (миелоциты), единичные мегакариоциты.

Печень значительно увеличена (вес 3 *кг* 750 *г*). На фоне дискомплексации печеночных клеток отмечается выраженный эритрофагоцитоз в купферовских клетках. Внутри капилляров и в глиссоновой капсуле встречаются миелоидные клетки, преимущественно эритронормобласты. При исследовании почек отмечен выраженный гемосидероз.

Особенности течения болезни (значительная гепатоспленомегалия) и данные патогистологического исследования (гиперплазия ретикулярных элементов печени и селезенки, очаги экстрамедуллярного гемопоэза в этих органах), выходящие за рамки обычной картины интраваскального гемолиза, позволяют рассматривать данный случай как терминальную фазу болезни Штрюбинга—Маркиафава с возможным исходом в миелопролиферативный синдром.

Возможность исхода гипопластического состояния кроветворения через стадию красноклеточной гиперплазии, осложненной ПИГ-синдромом, в системный гиперпластический процесс доказывается наблюдениями ряда авторов, описавших исход ПИГ в острый миелобластный лейкоз (Holden и соавт.; Kaufman и соавт.), а также в эритромиелоз с «мегалобластоидным» сдвигом эритропоэза (Thsevrenis и соавт.). Подобные случаи позволили Dameshek рассматривать ПИГ в качестве «кандидата» в группу миелопролиферативных заболеваний. В аспекте эволюции патологического процесса развитие гипоцеллюлярного («малопроцентного» по Dameshek) миелопролиферативного синдрома представляет собой завершающую, III фазу болезни Штрюфинга—Маркиафава.

Учение о «фазности» болезни Штрюбинга—Маркиафава имеет значение не только для диагностики и прогнозирования, но что весьма существенно, и для определения тактики лечения и профилактики опасных для жизни осложнений.

В данной статье можно только подчеркнуть основные принципы терапии, вытекающие из учета стадийности патологического процесса. В I, гипопластической, стадии показан весь арсенал средств, включая спленэктомию, применяемый в лечении «обычных» (без ПИГ-синдрома) гипопластических состояний кроветворения, осложненных тромбоцитопеническими кровотечениями. Во II стадии (развернутый внутрисосудистый распад крови с гемоглобинурией и тромботическими осложнениями) врачебная тактика сводится к поддержанию на оптимальном уровне показателей красной крови и профилактике сосудисто-тромоботических осложнений. В III стадии могут возникнуть показания к применению цитостатических, противолейкозных препаратов.

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Permanent Intravasal Haemolysis (PIH-Syndrome) in Hypoplastic Conditions of Haemopoiesis

The appearance against the background of depression of bone marrow haemopoiesis of a syndrome of permanent intravasal haemolysis (haemoglobinaemia, haemosiderinuria with positive acid and saccharose test) is regarded as a second phase of a "biphasic" disease of the Strübing—Marchiafava type. Development of PIH-syndrome up to paroxysms of nocturnal haemoglobinuria was caused by the appearance of an anomalous population of PIH-erythrocytes producing complement-sensitive erythrocytes subject to intravascular haemolysis. The morphological expression of this process is a dynamic transformation of the hypoplastic bone marrow into a partially hyperplastic one with a prevalence of cells of the erythroid series imitating the picture of erythromyelosis. The characteristic peculiarity of the PIH-syndrome is the presence in the bone marrow of "depletion zones" with fat reduction. Development against the background of a prolonged permanent intravasal haemolysis with considerable splenomegaly on account of a hyperplasia of reticular elements and myeloid metaplasia of the spleen is regarded as the third, terminal phase of Strübing—Marchiafava's disease with a potential transformation into the systemic myeloproliferative syndrome.

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Anaemias Associated with Schizocytosis

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Formation of schizocytes is due to mechanical injury. The most frequent aetiological factors are artificial heart valve, formation of microthrombi in capillaries, and the pitting function of the spleen. It is an interesting feature that red blood cell fragmentation only occurs in certain thrombohaemorrhagic syndromes such as thrombopenic thrombotic purpura and the haemolytic uraemia syndrome. Diseases associated with schizocytosis are discussed and the importance of detecting these cells in the smears is stressed. This besides being a valuable method of differentiation is indispensable for clarifying the pathogenesis of enigmatic anaemias.

The origin and clinical significance of schizocytes or fragmentocytes have only been discovered recently. The fragmentation of red blood cells is due to mechanical injury. An artificial cardiac valve, total or partial stenosis of the capillaries due to microthrombi and the pitting function of the spleen are the most frequent aetiological factors. In certain haemoglobinopathies and in microangiopathic haemolytic anaemia the occurrence of numerous schistocytes in peripheral blood is a regular phenomenon. Their detection, besides being a valuable aid in differentiation, is indispensable for clarifying the pathogenesis of enigmatic anaemias.

After the application of artificial heart valves often an unexpected complication arises: fragmentation of RBC and haemolysis. This was naturally thought to be caused by the prosthesis. Modifications were therefore attempted and the silicone balls were replaced by metal ones with Teflone. Follath et al. [1] and others have then showed that the latter cause haemolysis even more often. Similar symptoms were observed after the application of plastic blood vessels.

As immune reactions are exceptional, a mechanical injury of RBC was assumed; this was supported as follows. The life time of RBC is more frequently and more considerably shortened in cases of stenosis than those of insufficiency [2] and a strong blood stream, physical exertion and tachycardia all increase the fragmentation of RBC. When haemolysis occurs, schizocytes are always detectable in the peripheral blood smear, as a further evidence of mechanical injury. Their differentiation from other deformed RBC such as poikilocytes, echinocytes and acanthocytes is sometimes a problem. Severe haemolysis is rare and, if latent, often the only sign indicating the shortening of the RBC life span.

Determination of lactate dehydrogenase (LDH) has proved the most useful test for revealing RBC injury, and a definite correlation was established between the increase of LDH activity and the shortening of RBC life span. Naturally, other causes of increased LDH activity have to be excluded.

March haemoglobinuria occurs after long marching and is always associated with a lordotic lumbal spine. During increased muscular activity the spine is strained and renal venous outflow is inhibited, so the direct cause was assumed to be a renal congestion. Martin and Noviczki [3] assumed an acquired RBC injury on the basis of the presence of schizocytes in march haemoglobinuria. The actual site of the RBC injury was then revealed by Davidson [4] and Buckle [5] who found that the RBC are damaged in the sole and if the patient wears elastic in-soles or shoe-soles, no haemolysis occurs.

Microangiopathic anaemia is characteristic of some forms of thrombohaemorrhagic disease. The term has been proposed by Selye et al. [6] for the syndrome consisting of small blood vessel thrombi associated with haemorrhagic diathesis. Hitzig [7] identified this syndrome with the Sanarelli–Shwarzman phenomenon the histological and clinical equivalent of which is the well-known consumption coagulopathy. The only difference between the two conditions is that in the latter the underlying factor is unknown. Maak and Rogner [8] listed the thrombopenic thrombotic syndrome, the haemolytic uraemia syndrome, Waterhouse–Friederichsen's syndrome, fulminant purpura and the defibrination syndrome occurring in carcinoses and as an obstetric complication with the thrombohaemorrhagic disease group. Subsequently, these symptoms were, however, observed in association with malignant hypertension, ulcerative colitis, incompatible transfusion and graft rejection. Independently of the aetiology, both profuse and localized haemorrhages throughout the body and the tissue lesions associated with microthrombi are induced by an identical mechanism.

An interesting feature is that schizocytes occur only in association with thrombopenic thrombotic purpura and in the haemolytic uraemia syndrome. In other thrombohaemorrhagic diseases, schizocytes are infrequent. The cause of this is not clear and one may speculate whether the RBC are injured only with an extensive formation of microthrombi or when many renal tufts are occluded by thrombi and there is even a possibility that in the cases with slight haemorrhagic diathesis and fibrin precipitation in the capillaries few examinations have been carried out to detect schizocytes, and the few deformed RBC found in the smears were identified as poikilocytes.

Thrombopenic thrombotic purpura. Localization of the microcirculation disorder and the character and site of the haemorrhagic diathesis are characteristic. The microthrombi induce haemorrhagic necrosis in the kidney, liver, lungs, central nervous system, etc. Echinoses and suffusions are more frequent than purpuras in the skin and mucous membranes. Gastric, intestinal, renal and cerebral haemorrhage may occur. The main symptoms are increasing haemolytic anaemia, schizocytes in peripheral blood, haemorrhagic diathesis, azotaemia, paralyses and seizures. The haemolytic anaemia is severe, often associated with haemo-

globinaemia. The most characteristic feature of the RBC is their fragmentation. Rubenberg et al. [9] showed that the formation of schizocytes was due to mechanical injury. A high reticulocyte count and the presence of nucleated erythrocytes in the peripheral blood indicate that the RBC of shortened life span are destroyed in high numbers. Erythropoietic activity is considerably increased but still unable to substitute the loss. There is no close correlation between the schizocyte count and the degree of anaemia, since not only RBC fragmentation but a functional disturbance of the clotting factors also have a role in the pathomechanism of the anaemia.

The thrombocyte defect is both quantitative and qualitative. The thrombocytopenia is of peripheral origin: In the bone marrow the megakaryocyte count is slightly increased, the defect of thrombocyte formation is sporadic. In the centre of the intravascular aggregates the structure of the thrombocytes remains intact for a long time. At the edge of the thrombus the thrombocytes are disintegrated, alpha granules are released and thus the formation of fibrin is increased. The activity of clotting factors V, VIII and XIII (fibrin stabilizer) is often decreased. Fibrin degradation products accumulate. The increase of compensatory fibrinolysis indicates a considerable decrease of the fibrinogen level.

The aetiology of the disease is unknown. It has been ascribed to infection and this seems to be supported by the marked leukocytosis with toxic granulations. Other authors assumed an autoimmune process but antibodies to RBC, platelets of the vessels could not be detected. Some authors presume the possibility of a disseminated autoimmune mechanism; in systemic lupus erythematosus the perivascular reaction is, however, more extensive and in cases of periarteritis nodosa, the initial proliferation is more pronounced.

Examination of RBC, platelets and clotting factors allow only a conditional diagnosis. For the definitive diagnosis, a histologic examination is necessary. The most characteristic changes are microthrombi, endothelial hyperplasia and lesions in the end-arteries and capillaries. Alcorn and Fadell [10] and Brown and Taylor [11] corroborated the diagnosis by lymph node biopsy. Sternal biopsy also identifies the disease, but skin biopsy does not confirm the diagnosis.

The *haemolytic uraemia syndrome* observed by Gasser et al. [12] in a child has recently been reported also in adults. Not all authors regard this condition as an independent disease: Habib et al. [13] and Vocel et al. [14] consider it to represent a form of thrombotic purpura frequently associated with renal injury. The classical triad is an acute renal disturbance, haemolytic anaemia, and haemorrhagic diathesis. Renal insufficiency is the central symptom; the oliguria may increase to anuria associated with unrelievable vomiting and eclamptic seizures. Haemoglobinuria is also common but the renal injury is a result of capillary thrombosis. Microthrombi may occur in other organs also. Detection of schizocytes is important for distinguishing the condition from renal insufficiency of other origin. Thrombocytopenia, the decrease of fibrinogen and of other clotting factors is a sign of consumption coagulopathy.

Gasser et al. [12] found cortical necrosis of both kidneys in a child who had

died of haemolytic uraemia syndrome. Later it has been shown that the necroses resulted from thrombotic vascular obstructions. The glomerular changes vary according to the intensity and extension of the local procedure. In most cases, the microthrombi of the capillaries cause tiny infarctions. Lieberman et al. [15] found intracapillary thrombus formation and glomerular necroses by renal biopsy. In advanced cases the proliferative changes were so extensive that the glomerular structure was unrecognizable.

As the aetiological factor, bacterial or viral infection is assumed as in children enteritis or tonsillitis has been followed by the syndrome.

From among the *haemoglobinopathies* in thalassaemia and in anaemias associated with inclusion bodies schizocytes may be found. In beta-thalassaemia major no or few beta-chains are formed, while alpha-chain formation is increased 40–50-fold. The latter are precipitated in reticulocytes and erythrocytes [16] and appear as inclusion bodies. Koyama et al. [17] showed that the spleen eliminates these inclusion bodies from the RBC (pitting function) and thus bizarre schizocytes are formed. In beta-thalassaemia minor, there are much less superfluous alpha-chains, and so inclusion bodies are exceptional [18].

The cause of schizocyte formation is similar in both haemoglobinopathies. As it has been shown by Hollán [19], the RBC are able to discharge their inclusion bodies.

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Laboratory Parameters in the Diagnosis of Iron Deficiency*

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Measurements of hemoglobin, red cell protoporphyrin, and ferritin concentrations have been carried out in 95 adult females selected on the basis of a transferrin saturation of <16%. A good correlation was observed between the measurements, substantiating the usefulness of all parameters. However, with each measurement there was a broad distribution extending from normal to abnormal values. Far greater reliability was observed in those individuals who were so severely iron deficient as to manifest anemia. It is concluded that multiple measurements are essential to identify the presence of iron deficiency with precision in the non-anemic individual.

Introduction

Iron deficiency has been detected in various ways, depending somewhat on the purpose of the study. It is customary for the clinician to start with anemia and to differentiate its cause. While no difficulty is encountered when anemia is severe, problems arise with mild anemia. A significant overlap exists in the distribution of hemoglobin concentration between normal individuals and those who are mildly iron deficient, and any one point of separation will result in appreciable error. For example, in a study in which WHO criteria of 11 g/100 ml were employed to define anemia in pregnant women, it was shown that 33% classified as anemic were normal and that 33% of those classified as normal were anemic [1].

In recent years emphasis has been placed on measurement of the *iron supply for erythropoiesis*. Transferrin saturation with iron rather than plasma iron *per se* has been shown to be the most significant biologic parameter of iron supply; a percent saturation <16 is inadequate to maintain a basal level of erythropoiesis [2]. Likewise a red cell protoporphyrin concentration >about 100 μg/100 ml red cells reflects iron-deficient erythropoiesis [3]. These two measurements have been shown to detect about twice the number of iron-deficient subjects as would be found by first identifying anemia and then demonstrating a deficiency in iron

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supply. One difficulty with these two methods, however, is that they do not differentiate between a decrease in total body iron and an internal iron block as occurs with infection.

Measurements of serum ferritin have recently been described which promise to add a valuable parameter to the diagnosis of iron deficiency [4, 5]. Serum ferritin levels decrease when iron stores are decreased, even though plasma iron and red cell protoporphyrin may remain unaffected [6]. Furthermore, while plasma ferritin is decreased with iron deficiency, it increases with inflammation [7]. In the present study we have examined serum ferritin along with other parameters of iron status in 95 otherwise normal women who were selected from a nutritional survey on the basis of a low transferrin saturation.

Materials and Methods

The patients studied comprise 95 women between 18 and 45 years of age who were selected from the Washington State Nutritional Survey (1968–1970) on the basis of a transferrin saturation of $<16\%$. A total of 5692 individuals participated in that survey. The mean family income of the survey population was similar to that reported for the State of Washington in 1970, although the original sampling had been designed to select a disproportionate number of low income families. Blood samples were drawn between 2 p.m. and 8.30 p.m. from subjects who had not been fasting.

Methods employed in this study include an immunoradiometric assay for serum ferritin [5], an abbreviated protoporphyrin method [8], plasma iron according to the method of Young and Hicks [9], and total iron-binding capacity determined by the same method after removal of saturating iron excess with $MgCO_3$ [10]. Hemoglobin determination was carried out by a model S Coulter counter.

Results

The mean laboratory values for 95 women with iron-deficient erythropoiesis compared to average values in a group of 195 non-iron-deficient adult females are shown in Table 1, and in Fig. 1 the cumulative plots of individual values are displayed. Values for transferrin saturation were distributed between 4.1 and 15.8%. Serum iron values showed a relatively uniform distribution over the range of 5 to 65 μg/100 ml with 10% of the individuals having concentrations between 65 and 92 μg/100 ml. Total iron-binding capacity showed a distribution between 335 and 520 μg/100 ml for 80% of the subjects with 8% at lower levels and 12% above. All of these measurements were affected by the initial criteria of patient selection (transferrin saturation $<16\%$). Red cell protoporphyrin concentration showed a fairly uniform distribution over a range from 55 to 140 with 19% of the subjects lying between 145 and 265. Ferritin values for 59% of the individuals

Table 1

Laboratory measurements of iron deficiency

Parameter	Normal values*		Women with iron-deficient erythropoiesis	
	Mean	± 1 SD	Mean	±1 SD
Hemoglobin (g/100 ml)	13.8	1.0	12.5	1.3
Serum iron (μg/100 ml)	116	34	47.7	14.5
Total iron-binding capacity (μg/100 ml)	374	67	444	91
Transferrin saturation (%)	31.2	8.6	11.0	3.3
Serum ferritin (ng/100 ml)**	34.0	17.6—66.3	11.8	4.2—32.9
Red cell protoporphyrin (μg/100 ml RBC)	74.4	10.6	118	53

* Values obtained in 152 females between 20 and 50 years of age selected from the Northwest Nutritional Survey on the basis of 1) absence of anemia (Hb > 12 g/100 ml), 2) normal transferrin saturation (saturation > 20%), 3) normal red cell protoporphyrin (below 95 μg/100 ml).

** Calculated on logarithmic scale.

Table 2

Correlation coefficients in 95 women with iron-deficient erythropoiesis

Parameter	Hemoglobin	Serum iron	Total iron-binding capacity	Transferrin saturation	Red cell protoporphyrin	Serum ferritin*
Hemoglobin	—					
Serum iron (μg/100 ml)	.433	—				
Total iron-binding capacity (μg/100 ml)	−.180	.234	—			
Transferrin saturation (%)	.518	.749	−.442	—		
Red cell protoporphyrin (μg/100 ml RBC)	−.548	−.321	.226	−.452	—	
Serum ferritin (ng/100 ml)*	.495	.305	−.384	.552	−.302	—

Values > .202 significant at 5% level. Values > .332 significant at .1% level.

* Calculated on logarithmic scale.

were distributed from 1 to 15 ng/ml with increases of the remainder to a maximum of 127 ng/ml. Employing 95% confidence levels for the data obtained in the normal population at large, 24% of the subjects in this study were anemic (hemoglobin <11.8 g/100 ml), 55% had an elevated red cell protoporphyrin (>96 μg/100 ml) and 41% had a low serum ferritin concentration (<9 ng/ml).

Correlation coefficients between the different measurements employed in this study are shown in Table 2. Significant correlation coefficients were observed between transferrin saturation, ferritin and red cell protoporphyrin, and even higher

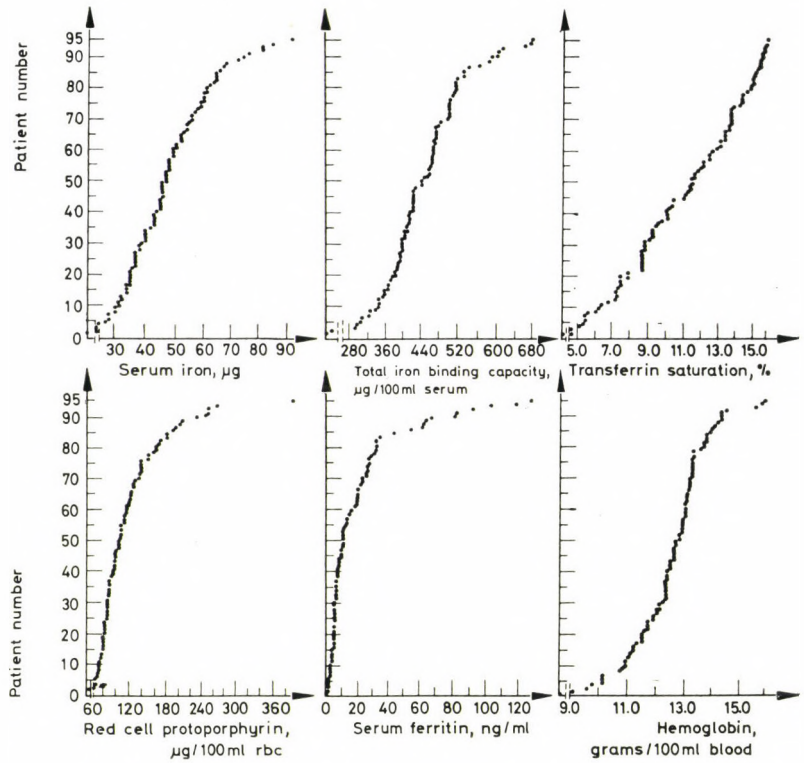

Fig. 1. The individual values for the 95 iron-deficient women are ordered numerically for each laboratory parameter

degrees of correlation between these indices and hemoglobin concentration. The frequency of abnormal values in 26 patients with anemia (defined according to WHO criteria as hemoglobin <12 g/100 ml) was considerably higher than in the overall group. In 92% of the anemic subjects protoporphyrin exceeded 100 µg/100 ml red cells and 88% had a serum ferritin <9 ng/ml serum.

Discussion

In the development of iron deficiency three stages have been described: 1. The depletion of iron stores associated with enhanced absorption; 2. iron-deficient erythropoiesis where plasma iron supply is suboptimal to meet erythron needs for iron; and 3. overt iron deficiency anemia. In the developmental sequence, variations may be imposed by the rate of development of iron deficiency, by alterations in the rate of red cell turnover, and by changes in the reticuloendothelial processing of catabolized iron. Thus, rapid blood loss can result in iron-deficient

erythropoiesis before iron stores are exhausted, and an iron supply adequate for basal rates of erythropoiesis may be inadequate at increased erythropoietic rates [11].

In this study the transferrin saturation was used to identify subjects with a restricted iron supply. Previous studies have indicated that a transferrin saturation of $<16\%$ is inadequate to support normal hemoglobin synthesis [12]. At the same time it was recognized that no single measurement of this type would be entirely accurate in its identification of iron deficiency.

In general, the other measurements performed supported the validity of transferrin saturation as a means of detecting iron deficiency. Significant correlations were observed between transferrin saturation and red cell protoporphyrin, serum ferritin and blood hemoglobin concentration. Equally striking, however, was the broad spectrum of values obtained with all of these measurements, overlapping with the normal range. The same overlap might well have applied to the transferrin saturation had some other measurement been employed for subject selection. Thus a group of subjects identified as iron deficient by one measurement was often not so identified by another measurement. It seemed likely that an important factor in explaining these inconsistencies were the different characteristics and meaning of each parameter. Thus plasma iron is subject to change within hours, whereas protoporphyrin and ferritin change over a period of weeks. Furthermore, while the plasma iron reflects the overall balance of input of iron into plasma and its removal by body tissues, the protoporphyrin integrates the iron supply of the individual erythroid cell with its requirements [3]. Neither of these measurements changes as long as sufficient iron is mobilized to meet the needs of the erythron. Ferritin, on the other hand, reflects a decrease in iron stores regardless of whether iron for erythropoiesis is adequate and is the only one of these measurements to differentiate iron deficiency from inflammation. Results obtained in this study would suggest that these differences are indeed significant and that the evaluation of iron status, whether in the individual subject or in a survey population, should rest on the measurement of several parameters. However, if the search begins with the more severely iron-deficient individual who has identifiable anemia, any of the methods employed appear highly reliable in identification of iron deficiency.

It is recognized that the survey conditions encountered in this study may not have been optimal. Blood was drawn in the afternoon at a time when plasma iron levels are lower than in the morning. Individual measurements were carried out in different laboratories and absolute standardization of certain methods may be questioned; for example, the normal values of red cell protoporphyrin and total iron-binding capacity obtained in the general survey population are somewhat high. However, there is no reason to question the relative values and thus the conclusions drawn would seem valid.

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Blood Groups and Diseases*

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The ABO blocd group system, and most other systems of hereditary blood factors, show wide differences in gene frequency between one population and another. Much of the variation found is probably due to random effects, but much also is almost certainly due to natural selection, certain phenotypes being more liable than others to suffer from certain diseases, with resulting loss of fertility. Most investigations have been based on determining the frequency of certain factors in those suffering from particular diseases. Thus, for instance, patients with carcinoma of the stomach have a raised frequency of blood group A, almost certainly because people of this group have a raised susceptibility to the disease. Several other established cases, and many other probable cases are known of such associations. Numerous examples have recently been discovered of associations of diseases with particular histocompatibility antigens. It is suggested that the gene frequencies in many if not most genetic polymorphisms are the result of an equilibrium, varying with locality, between the selective effects of different diseases and other environmental stresses on the various phenotypes of any given system.

When I first was asked to give this lecture, I thought of entitling it "Why blood groups?" but I decided that this was rather unfair since, while I am indeed posing this very question, I am not giving the answer.

I do not know the answer, and if anyone among you knows it he or she has kept very quiet about it. The answer is likely to be given one day as a result of your own researches, but meanwhile a number of clues to the answer are being found. I propose to speak to-day about some of the lines of investigation which are beginning to show how some of the systems of blood groups, and other inherited blood characters, are maintained in a state of balanced polymorphism; that is to say, why, under constant environmental conditions, the frequencies of the various blood groups or other phenotypes remain relatively constant in a given population. This may give us a clue as to how they evolved, and how their frequencies came to vary in populations living under a variety of conditions.

I shall not confine myself to the blood groups in the strict sense, because some of the most interesting pieces of evidence refer to other inherited blood characters such as the haemoglobins and the white-cell antigens. Indeed, some of the

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most important discoveries yet made in this field, in relation to the leucocyte antigens, have been published in the last few months, and came to my notice only after I had begun to prepare this lecture.

The most important connection between blood groups and disease relates, of course, to haemolytic disease of the newborn, and there are on record some cases of the disease arising from almost every theoretically possible incompatibility, within the major blood group systems, between foetus and mother. This subject has however been dealt with exhaustively in innumerable books and papers and it is one with which most of you are much more familiar than I, so that I do not propose to discuss it other than incidentally.

The ABO blood groups

The first suggestion that the blood groups might be involved in a disease process came surprisingly soon after the discovery of the blood groups themselves when, in 1905, Dienst [1] showed that in pregnancies where the foetus possessed an A or B antigen not present in the mother, the titre of the corresponding antibody in her serum tended to rise. It does not seem to have occurred to him that this might harm the foetus, or subsequent foetuses, but he thought that it might give rise to eclampsia in the mother. After this many authors published lists of frequencies of the ABO blood groups in sufferers from various diseases, but in nearly all cases it can now been seen that the numbers tested were too small to show significant divergences from the frequencies in healthy persons. The discovery by Levine, Katzin and Burnham [2] of the cause of haemolytic disease of the newborn does not seem greatly to have encouraged the search for involvement of the blood groups in the aetiology of other diseases and it was not until 1953 that a completely convincing example of an association between a particular phenotype and any other disease was found. This was the association between group A and carcinoma of the stomach, discovered by Aird et al. [3]. They compared 3632 cases of the disease with controls and found the ratio of group A to group O in the patients to be 23 per cent higher than in the controls. The probability of finding such an apparent association by chance in the absence of a true association is very small indeed in view of the large numbers tested. However, the consistency with which this particular association has continued to be found in scores of surveys over the past 20 years leaves no doubt at all as to its reality. The question of the reality of such associations in general has been argued in a number of papers including those of Wiener [4] and Manuila [5], but I do not want to spend a great deal of time to-day on this aspect of the subject. To my mind the paper of Roberts [6] leaves no doubt as to the reality of the effects described. Moreover, in a book which my colleagues and I are now preparing we propose to deal fully with such criticisms. Perhaps, however, I ought to say a few words about some of the objections. It is suggested, for instance, that the apparent effect (in carcinoma of the stomach) is due to an excess of about 20 per cent of group O in the blood donor

controls, due to selective recruitment. This may, indeed, occur in some places, but in Britain, from which a large part of the evidence for association comes, the work of Kopeć [7] leaves no doubt as to the representativeness of blood donor panels which cover total recruitment over a given period. With due precautions the same applies to most developed countries. Moreover, as we shall see, several diseases show a deficiency of group A, and this argument cannot be used for them.

If we accept the reality of the association, we still have to decide what it means. There are, *prima facie*, two main possibilities, one being that persons of group A are more likely than those of group O to contract the disease. The other is that the population is stratified, consisting of one sub-population with high frequencies of group O and of carcinoma and the other with low frequencies of both. On this hypothesis there would be no association within each of the sub-populations. There are indeed areas of Great Britain where such a blood group stratification may occur, but the association has been found not only in many different parts of Britain but throughout Western Europe; it has also been found in a number of Negro and Mongoloid populations. There can thus be no doubt that this is a world-wide phenomenon and thus it cannot be explained by local stratification, and it is almost certainly due to a susceptibility to the disease which is systematically higher in group A persons than in others.

A further possible source of spurious results is selective publication. Once a particular association has been recorded, possibly as a result of a chance association in a small series of observations, there is a possible temptation to publish results which show such an association and to leave unpublished those which do not. This is a real danger in the case of relatively rare diseases where individual series are bound to be small. The existence of this danger is an argument in favour of co-operative prospective surveys, where publication can hardly fail to take place, and of the meticulous publication of negative results. Such publication is partly a responsibility of editors, and it is one to which I try at every opportunity to draw their attention. Large series, involving a great deal of work, are most unlikely to remain unpublished even if the results are negative.

I hope that I have said enough for today about this, to me, rather dreary and sterile field of polemics. Behind it, however, lies something which I regard as much more interesting and fundamental, and that is the problem of how far gene frequencies of all kinds are the result of random processes and how far to natural selection with differing results in different environments. There has been a tendency in the past to treat this problem as though only one of the two alternatives could be valid, but more recently most human geneticists have agreed that both processes go on and the problem thus becomes one of assessing the relative contributions of the two processes.

There are a great many sets of observations on small isolated communities such as religious isolates and the populations of small islands, where blood group frequencies deviate very greatly from those found in the large populations from which they must have arisen. These deviations are certainly the result of chance effects acting either through the gene frequencies of the (usually few) founders,

or through subsequent genetic drift, or both. The question then arises as to whether the differences between populations in larger regions, or in the world as a whole, could have arisen, and whether they did arise, though much more slowly, as a result of similar chance fluctuations. There is little doubt that many of them considered solely from the mathematical point of view, could have arisen in this way, but they have not necessarily done so. The most satisfactory way of showing that differences are due at least in part to natural selection is to test patients suffering from diseases known to be partly due to the environment, and occurring before or during the reproductive period. If the blood groups, or other hereditary characters, have a different incidence in the patients and in healthy controls, this is at least *prima facie* evidence that natural selection is taking place.

To depart for a moment from the ABO blood group system, I hardly need to remind you that the classical example of natural selection in such a situation is that of the haemoglobins. It was for many years a puzzle why the gene for haemoglobin S, or sickle-cell haemoglobin, persisted with high frequencies in certain populations, especially in Africa, despite the fact that homozygotes for the *HbS* gene mostly died in infancy from sickle-cell anaemia. Allison [8] was the first to show convincingly that this was because the heterozygotes for this gene and the normal *HbA* gene were more resistant to malignant tertian *(falciparum)* malaria than were normal *AA* homozygotes. Thus *SS* homozygotes die of sickle-cell anaemia, *AA* homozygotes have a high death rate from malaria, but *AS* heterozygotes resist both diseases and pass both genes on to the next generation. This is the essence of the phenomenon, already mentioned, known as balanced polymorphism. It is thought to exist in relation to many other hereditary characters, but that of the haemoglobins remains the most clear-cut, and is one of the few cases where there is full concordance of the evidence from population genetics and that from the study of individual susceptibility to the disease. For β-thalassaemia the situation is almost identical with regard to the population genetics, but I know of no evidence for individual resistance to malaria in persons heterozygous for thalassaemia. It was, however, the example of thalassaemia that, in 1949, led Haldane [9] to put forward the hypothesis that has been so fully confirmed for sickling.

I now want to review rather rapidly those diseases other than carcinoma of the stomach, where there is, in my opinion, conclusive evidence of an association with the ABO blood groups, then to mention some less certain examples, and finally to look at some of the very varied examples of associations of other polymorphisms with diseases.

Some of the strongest and best confirmed associations of all are those of duodenal ulcer with group O and with hereditary non-secretion of the ABH antigens, yet they also illustrate some of the pitfalls of the work.

Aird et al. [3] found that peptic ulcers, considered as a single entity, showed a strong association with group O. Then Clarke et al. [17] found that the main association was between group O and duodenal ulcers, whereas gastric ulcers showed only a slight association. Duodenal ulcers had also a very strong

association with non-secretion of the antigens of the ABO system in the saliva. Yet when families were studied there was evidence of association within sibships with non-secretion but not with group O.

Some considerable time later Langman and Doll [10] investigated a very large number of cases of duodenal ulcer, elaborately classified, and concluded that there was indeed an association of duodenal ulceration as such with non-secretion (thus explaining the association within sibships) but that group O was associated specifically with bleeding and not with duodenal ulceration as a whole. It thus appears likely that the earlier investigators were really studying preponderantly those cases of duodenal ulcer which, because of bleeding, were brought into hospital.

The reverse side of the coin is probably shown by thrombo-embolic diseases. Numerous investigators, beginning with Gertler and White [11], had found some evidence for an association of group A with a variety of thrombo-embolic conditions. When my colleagues and I [12] brought together all the examples we could find in the literature we found overwhelming evidence for the association. When, however, all cases of coronary disease were brought together, though they showed a very marked association with group A, there was considerable statistical heterogeneity. This, however, was resolved when we separated the series into two sets, those referring to such conditions as "coronary insufficiency", without mention of thrombosis, on the one hand, and those clearly described as coronary thrombosis or cardiac infarction on the other. Each set was now statistically homogeneous, but the non-thrombotic cases did not differ significantly from the controls in their ABO frequencies, while the cases of thrombosis differed strongly from the controls.

There are thus two contrasting classes of conditions showing ABO associations: thrombosis with group A and haemorrhage with group O. Jick et al. [13] suggested that this was due to the known lower average level of anti-haemophilic-globulin in group O than in group A persons [14]. The differences are, of course, of a lower order altogether than that which separates the extreme deficiency characteristic of haemophilia from the normal. Yet there are genetically determined grades of haemophilia itself, and I should like, very tentatively, to suggest that the various haemorrhagic and thrombotic diatheses may be related to minor mutations of the structural gene for anti-haemophilic globulin. But this is a hypothesis that you, as haematologists, may be able to demolish at a blow!

There is strong evidence for other ABO associations such as that of group A with pernicious anaemia [15]. Then there are diseases where some workers have found an association and others have not. I agree emphatically with Roberts [6] in that these cases must not be regarded without further consideration as discrediting the whole subject, but that they merit further investigation. We have already seen that such an approach was able to suggest a reclassification of coronary diseases. Similarly for diabetes mellitus, there is, taking all cases together, a strong association with group A, but the series is highly heterogeneous. Classification by sex does not reduce the heterogeneity – nor apparently does classification by

age of onset, but here the data are still insufficient. Again in the case of multiple sclerosis, there are indications of an association with group O, yet surprisingly, when cases are classed by severity, it is the mild cases that show the association. Yet in diseases where the aetiology is still not understood, any clue as to aetiology, or as to the confusion of two diseases of different aetiologies, ought to be considered, however improbable such a situation may at first sight appear to be.

I have so far said little about infectious diseases. Most of the diseases that I have mentioned are diseases of middle age, and any mortality from them is unlikely greatly to affect the genetical composition of the next generation. Yet, apart from physical trauma and starvation, the main killing diseases of the young are the great epidemics. Moreover, there are considerable biochemical similarities between the antigens of some microorganisms and the blood group substances, and it is possible therefore that the blood group of an individual affects his or her ability to make antibodies to invading organisms.

There is good evidence for an association of blood group O with susceptibility to influenza of type A [16] while Clarke et al. [17] have found a marked association of rheumatic carditis (which is a late sequel of haemolytic streptococcal infection) with blood groups other than O, and with non-secretion of the ABH substances.

However, the most extensive investigations on associations between blood groups and infection are those of Vogel and his colleagues, who in numerous papers [18, 19] have given evidence of associations between group A and susceptibility to smallpox and between group O and susceptibility to cholera. There is, however, considerable controversy as to the validity of these results, especially in the case of smallpox. I have already summarized the situation in another paper and here will simply repeat my conclusion that while there is no reason to doubt the results of any of the investigators, it is desirable that others should carry out similar investigations under precisely defined conditions; it is important also that the extensive published results should be fully analyzed statistically to see how, if at all, they are compatible with one another, and whether the stated methods of investigation differ systematically between the different sets of results. It may be that here, as in other cases already cited, it will be found that once the "noise" has been filtered out, the pure tones carry a message of considerable importance.

Apart from the special case of haemolytic disease of the newborn which I do not propose to discuss except incidentally, the evidence for associations of blood groups of systems other than ABO, with diseases is rather slight. Moreover we do not know the function of the blood groups as a whole. For most of the other hereditary constituents of the blood, however, especially the red-cell enzymes and some plasma proteins, we do know their functions, and we know that different alleles give rise to different degrees of activity in performing these functions.

Thus the products of the haptoglobin alleles differ in the rate at which they remove haemoglobin from the plasma. It has been shown by Ritter and Hinkelmann [20] that the Hp^1 gene frequency in offspring tends to be higher in families where the father possesses an A or a B antigen not present in the mother than

where he does not. The probable explanation is that in cases where foetuses are affected by haemolytic disease of the newborn due to maternal immunization by foetal A or B antigens, a child of type Hp1 – 1 has the best and one of type Hp2 – 2 the worst chance of survival.

A number of clinical conditions have been attributed to homozygous deficiency of various enzymes. The best known and most important of these is X-linked glucose-6-phosphate dehydrogenase deficiency, which can give rise to severe haemolytic anaemia when a male monozygote or a female homozygote is exposed to the edible bean *Vicia faba*, or to certain drugs. A considerable number of alleles are known in this system, of which two at least give rise to very low enzyme activity, and potentially to haemolysis. Here again the question of maintenance of frequencies (sometimes as high as 60%) of such a potentially harmful gene arises. Population data show a clear association of high frequencies with the distribution of *falciparum* malaria, and there is now good evidence [21, 22, 23] from tests on individuals, of protection against malaria, not only by one of the deficiency genes but also by the gene Gd^{A+} which has a significant frequency in Africans but which gives rise to normal enzyme activity levels and is not known to predispose to haemolysis.

The aetiology of every disease includes hereditary and environmental components, and we have already looked at a variety of diseases in which one or the other is predominant. For instance, in carcinoma of the stomach the ABO blood group plays only a very small part in the causation, and environmental causes are almost certainly predominant. At the other extreme, sickle-cell anaemia is almost exclusively the product of the homozygous haemoglobin S genotype, and the environment, in the form of modern medicine, can only slightly mitigate the normal results of the genetical situation. But these are only the ends of an almost continuous spectrum, and I want now to draw your attention to some associations where the genotype is a major or even the principal aetiological factor, but not the only one.

One system involved in such association is that of the plasma α_1-trypsin inhibitors [24]. At least twenty alleles are known, mostly very rare. One moderately rare one gives rise to low and another rarer one to undetectable anti-trypsin activity, and these, at least when they occur in the homozygous state, are frequently associated with pulmonary emphysema and sometimes with infantile liver cirrhosis. A large amount of information on this system is included in contributions to a symposium, published under the editorship of Mittman [25].

Probably all homozygotes ultimately have some degree of lung damage, but it is not clear why, at a symptomatic level, some have emphysema, some cirrhosis, some both and some neither.

It is also likely that homozygotes and perhaps heterozygotes are predisposed to other lung diseases. Unfortunately, many published series are based on quantitative enzyme-inhibition tests, which cannot be interpreted with certainty in terms of the genotype. When more clinical series become available, tested by electropho-

resis which clearly defines the genotype, it will become possible to assess the total effect of the system on lung and liver pathogenesis.

Here, as with some of the other systems mentioned, we need to explain why deleterious genes persist with a substantial frequency in most populations. It has been suggested by Kueppers [26] that this is because males with deficiency are more fertile than normal, the low level of enzyme inhibitor facilitating the proteolytic action of the sperm head in penetrating the ovum.

The other class of close associations, those with the histocompatibility antigens, takes us back to something closely analogous with the red-cell antigen groups themselves. It has long been known that the leucocytes and platelets carry isoantigens against which repeatedly transfused patients make specific antibodies. Their practical importance, however, was revealed only when it was found that in each individual these antigens are shared with most of the tissues of the body, and that they are the main antigens which determine compatibility or incompatibility in tissue and organ grafting.

It is, however, mainly within the past year that it has been shown that these antigens have another aspect of great theoretical interest, and one which may even come to surpass in practical importance their relation to grafting operations.

The genetics of the histocompatibility or HL-A antigens are, broadly speaking, similar to those of the Rh red-cell groups or the Gm serum groups, but probably more complex than either of these. As with the Rh and Gm groups, however, there is intense argument as to the precise relationships of genes to antigens, and as to how far the observed complexities reside in the antigens and how far in the antisera. Fortunately all the leading workers throughout the world keep in close touch with one another – even closer touch than workers on red-cell groups – and they resolve their difficulties as they arise by means of workshop meetings at which they not only hold discussions but work together in the laboratory.

Mainly within the past year it has been found that a number of widespread diseases, the aetiology of which has hitherto proved baffling, have close and highly significant associations with particular HL-A antigens.

Even as recently as the last workshop meeting, at Evian, France, in May, 1972, which I attended, only one possible association was at all extensively discussed, that of Hodgkin's disease, and the final conclusion was that there was no firm proof of any association [27]. Since then, however, papers have appeared, admittedly each based on rather small numbers, which seem to show very close and highly significant associations with several other diseases.

Perhaps the most impressive is that found between ankylosing spondylitis and HL-A 27, where the antigen is present in about 90% of patients but only in about 6% of controls [28, 29]. Psoriasis shows a highly significant association with HL-A 13 and HL-A 17 [30] and coeliac disease with HL-A 1 and HL-A 8 [31]. Mackay and Morris [32] have shown a significant but less marked association between autoimmune active chronic hepatitis and the same antigens HL-A 1 and HL-A 8, while Gebhard et al. [33] have found an association between HL-A 8 and dermatitis herpetiformis, mainly in cases which also showed intestinal villous

atrophy. Jersild et al. [34] have found an association of multiple sclerosis with HL-A 3 and HL-A 7. Combining their data with those of others they find also a significant increase of W 18 and significant decreases of HL-A 2 and HL-A 12. Earlier work suggests, as already mentioned, some degree of association of this disease with red-cell group O.

It is difficult at this stage to review critically the data which are now pouring in weekly, but it seems that many diseases, hitherto of unknown aetiology but several of them suspected of being autoimmune, are closely associated with particular histocompatibility antigens. In every case it is desirable that independent workers should confirm these highly important findings. At least the laboriousness of the tests and the rarity of the reagents should ensure that all observations made are published, but Svejgaard [35] of the State University Hospital, Copenhagen, has taken special interest to ensure this, by inviting all investigators to send him their results. Moreover, the close connection between all histocompatibility workers will ensure that results are properly criticized. But at last it seems that many diseases which have long baffled investigators are being laid open to a full investigation of their genetics and their immunology, and thus one may hope that rational means of relieving or arresting if not completely curing some of them will be found in the course of the next few years.

It is rather more disturbing to realize that we may be able to identify in advance those individuals who are genetically highly susceptible to these crippling and hitherto incurable diseases. It may be, however, that we shall at the same time find out what are the environmental hazards which, combined with the genotype, decide whether a given disease shall occur, and we shall thus have, for the first time, a means of prophylaxis.

For the sake of completeness I ought perhaps to mention briefly one other association between polymorphism and disease, namely that between the hereditary phenylthiocarbamide taster and non-taster types and thyroid disease. Harris et al. [36] and Kitchin et al. [37] showed that there is a lowered frequency of tasters in nodular non-toxic goitre, and the latter authors also found a raised frequency in diffuse toxic goitre. These results, especially those for non-toxic goitre, have repeatedly been confirmed. The biochemical background to this is not in dispute, namely that phenylthiocarbamide is one of a group of antithyroid substances and drugs, all of which also are subject to the taster – non-taster phenomenon. Widström and Henschen [38] also found that in persons without goitre the level of protein-bound iodine in the blood tends to be higher in tasters than in non-tasters, thus suggesting that the two types of goitrous patients represent but the tips of a mainly submerged iceberg. Johnston et al. [39] have also shown that pubertal development tends to take place earlier in tasters than in non-tasters (which could well be a consequence of different degrees of thyroid activity).

Since an antithyroid substance subject to the taster – non-taster phenomenon occurs in cabbage and other cruciferous plants and has been shown to cause goitre, it seem possible that the frequencies of tasters and non-tasters are in the long run based on natural selection, with frequencies depending upon environ-

mental factors such as climate and the amounts of iodine and crucifer antithyroid substance in the diet, the consumption of the latter being less in tasters than in non-tasters. The antithyroid effect of the substituted thiocarbamides appears to be due to their displacing iodide (derived from food and drink) in competition to enter a chain of enzyme-catalyzed reactions including a peroxidase which liberates iodine from iodides [40, 41]. It is thus possible that the gene products which give rise to the taster polymorphism are a pair of isoenzymes, one being a fully active peroxidase and the other having reduced (or absent) activity.

Thus we come back to the question of why man, and indeed all vertebrates, show such a high degree of polymorphism, and such great differences between populations in the frequencies of those genes which govern the constituents of the blood and of other parts of the body. For enzymes and other proteins of known chemical activity it is easy to see that different levels of activity may be required in different environments, these requirements giving rise by mutation and natural selection to polymorphisms and to varying gene frequencies depending upon the environment. This need not exclude a great deal of random polymorphism between gene products not differing appreciably in activity.

However, it is less easy to see how the antigen polymorphisms of the red cells, white cells, platelets and tissue cells arose and are maintained. In general terms we might expect them to be related to resistance to microbial infection. However, in the case of the tissue immunity antigens Burnet [42] has recently put forward a hypothesis which is most attractive. He suggests that the HL-A polymorphism, which causes every individual of a species, such as man, to differ antigenically from almost every other individual he is likely to meet, is due to the long-term need for the prevention of spontaneous grafting of cell clones which might in some cases be capable of unlimited growth in their new hosts, thus giving rise to the universal occurrence of grafted cancers. I put this forward as the latest, though not necessarily the most acceptable, hypothesis. It is not susceptible to complete experimental verification, but one can envisage certain tests of its validity, at least in animals. It is not applicable directly to the red-cell antigens, since functional red cells do not multiply, but these antigens are almost certainly in some sense protective.

Finally, I hope I may be allowed to make a personal appeal. My colleagues and I are now attempting to bring together all the available data on associations between polymorphisms and disease. They will then be tested statistically, and where possible data on any given association will be combined by Woolf's method, which may in some cases show highly significant associations not obvious in the separate data. Equally, combining data may show that different sets are mutually contradictory and that some or all of them must be regarded with great reserve. I should therefore be most grateful for offprints or other records, whether negative or positive, of associations between any human polymorphisms and any diseases.

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Homologues of the Human A-B-O Blood Groups in Apes and Monkeys*+

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Red cells of all apes except the gorilla closely resemble human red cells in their reactions with anti-**A** and anti-**B**.** In chimpanzees only groups A and O occur, while in gibbons and orangutans groups A, B, and AB have been found but not group O. All gorillas tested have proved to be group B, but the agglutinogen B on gorilla red cells is extremely weak and demonstrable only after proteolytic-enzyme treatment. Monkey red cells do not react with anti-**A**, anti-**B** or anti-**H**, but their A-B-O groups are demonstrable by testing their saliva for the group-specific substances A, B and H, and their serum for anti-**A** and anti-**B**. Certain species of Old World monkeys have all four A-B-O groups, e.g., baboons and crab-eating macaques, though group O is rare in these species; in some species only group A occurs, while in other species only group B has been found. The situation in New World monkeys is complicated by the presence on the red cells of a B-like agglutinogen, irrespective of their A-B-O groups determined by saliva tests. All but one (an orangutan) of the hundreds of apes and monkeys tested by us to date have proved to be A-B-H secretors. The relationship of H to the A-B-O groups in apes and in monkeys is different from that in man.

Introduction

Of the many known human blood groups systems the A-B-O system remains the most important (for general review see [1, 2]). The four blood groups O, A, B and AB are determined by the presence or absence of two agglutinable substances (agglutinogens) A and B on the red cells. There are two corresponding isoagglutinins, anti-**A** and anti-**B**; according to Landsteiner's rule those and only those isoagglutinins are regularly present in the serum for which the corresponding agglutinogens are absent on the red cells.

This rule does not hold during the neonatal period when the isoagglutinins are not fully developed, and there are other rare exceptions, notably, in patients with agammaglobulinemia.

* This article is dedicated to Dr. Susan R. Hollán for her contributions to medicine in the field of hematology, on the occasion of the 25th anniversary of the National Institute of Haematology and Blood Transfusion, which she helped to found.

** To avoid ambiguity, symbols for serological specificities (blood factors) and their corresponding antibodies are printed in boldface type, symbols for genes and genotypes are printed in *italics*, and symbols for agglutinogens, phenotypes and blood group systems are printed in regular type.

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The regular occurrence of the isoagglutinins has been explained by the ubiquitous presence in nature of A-like and B-like antigenic substances, so that exposure to them is unavoidable during life from birth on [3]. This explains the importance of the A-B-O blood groups for blood transfusion, for which millions of tests are being done each year. Consequently, more is known about these blood groups than about any of the others. When nonhuman primates became available for experimentation, it was natural, therefore, that one of the first investigations was the search for homologues of the human A-B-O blood groups. In turn, studies in apes and monkeys have thrown further light on the nature of these blood groups in man.

Man

A few basic facts about the A-B-O groups in man are briefly reviewed here for comparison with the situation in apes and monkeys.

The agglutinogen A is not a uniform substance since by serological tests two main varieties can be distinguished, designated A_1 and A_2. Red cells of subgroup A_1 react not only with anti-**A** but also with a reagent called anti-$\mathbf{A_1}$; the latter can be prepared by absorption of anti-**A** serum with subgroup A_2 red cells, and also can be prepared from certain seed extracts (lectins). Subgroup $\mathbf{A_2}$ red cells are agglutinated by anti-**A** but not by anti-$\mathbf{A_1}$. There is, moreover, an agglutinogen H inherited independently of the A-B-O groups but closely associated with them, as shown, for example, by the fact that human group O and subgroup A_2 red cells react regularly and strongly with anti-**H** [4, 5]. The A-B-O blood groups and subgroups of A are genetically determined by a series of alleles, designated *O*, A^1, A^2 and *B*, respectively. The frequencies of the A-B-O blood groups in various populations show significant differences.

The blood group substances occur not only on the red cells but also in water-soluble form in body fluids and secretions, of which the saliva is the most convenient to use for testing. However, not all human beings have A-B-H blood group substances in their secretions. This is determined by a pair of allelic genes, *Se* and *se*, independent of the A-B-O blood group genes, so that homozygous persons of genotype *sese* are nonsecretors. Both secretors and nonsecretors have been found in virtually all races tested, nonsecretors reaching their highest frequency of about 40 percent in Negroes. In secretors, tests on saliva by the inhibition technique demonstrate the presence not only of the A-B blood group substances corresponding to the individual's blood group but also the regular presence of H. Substances A, B and H are all absent from the saliva of nonsecretors of any A-B-O group.

Apes

Chimpanzees. Since 1960 we have determined the A-B-O blood groups of more than 350 *Pan troglodytes* [8, 9], and of nine *Pan paniscus* [10, and unpublished data]. We have accurate records on all nine *Pan paniscus* (all group A), as well as

a random series of 288 *P. troglodytes*, of which 37 were group O and 251 group A. Thus, our results, as well as the more limited results of other investigators, indicate that of the four A-B-O blood groups, B and AB do not occur in chimpanzees. As in man, when serum of a group O chimpanzee is mixed with the red cells of a group A chimpanzee, isoagglutination regularly occurs, but not in the reverse combination. Landsteiner's rule holds in general since the sera of group O chimpanzees contain both anti-**A** and anti-**B**, while sera of group A chimpanzees have only anti-**B**. This can be demonstrated by testing chimpanzee serum against human red cells of group O, A and B. Tests on group A chimpanzee newborn and infants less than one year old showed no anti-**B** in their sera, indicating that, as in man, the isoagglutinins are not developed until some time after birth. Moreover, a few mature apparently normal group A chimpanzees were found lacking anti-**B**.

Among the chimpanzees in our series, the saliva has been tested of more than a hundred animals, all of which proved to be secretors. Thus, the nonsecretor type is absent or rare among chimpanzees. As in man, group O chimpanzee saliva contains H only, while group A saliva has both A and H.

The distribution of the A-B-O blood groups of different subspecies (or races) of *P. troglodytes* varies, comparable to the situation in various populations of man (cf. Table 1). Information regarding the subspecies has not been obtained for all the chimpanzees tested by us, but the data in Table 1 are sufficient to show that the frequency of group O in these animals can range from as low as 9.5 percent to as high as 39.4 percent. This may account for some of the discrepancies in the frequencies of the A-B-O blood groups of chimpanzees reported in the literature.

Table 1

The distribution of the human-type A-B-O blood groups in chimpanzees*

Subspecies	Group O	Group A	Total
Pan troglodytes troglodytes	2	12	14
Pan troglodytes schweinfurthi	13	20	33
Pan troglodytes verus	4	38	42
Pan troglodytes koola-kamba	1	1	2
Pan paniscus	0	9	9

*Modified from Wiener and Moor-Jankowski [6, 9].

When chimpanzee and human blood of the same blood group are cross-matched, human serum strongly agglutinates or hemolyzes chimpanzee red cells due to a regular presence of species-specific heteroantibodies. On the other hand, chimpanzee serum generally gives no or little agglutination of human red cells. Aside from these species-specific differences, it is difficult to distinguish serologically between group A chimpanzee and group A human blood or between O chimpanzee and group O human blood unless more refined tests are done. For example, in tests with anti-**H** lectin group O chimpanzee red cells are agglutinated

as in man but the reactions are often weaker and occasionally even negative, like for newborn human babies whose H agglutinogen is not completely developed [4]. Furthermore, anti-$\mathbf{A_1}$ reagents agglutinate chimpanzee group A red cells but the reactions are weaker than for human subgroup A_1. Moreover, in chimpanzees, as in man, subgroups of A appear to exist since about 1/10 of group A chimpanzee red cells give weaker reactions with anti-$\mathbf{A_1}$ though not negative as human subgroup A_2. Thus, the relative reactivity of the human and chimpanzee group A red cells may be arranged as follows:

$$\text{Human } A_1 > \text{Chimpanzee } A_1 > \text{Chimpanzee } A_2 > \text{Human } A_2$$

We recently tested newborn chimpanzee twins (unpublished data), both of group A. Potent anti-**A** serum produced weak agglutination involving only about half the red cells and resembling the weak reactions of red cells of newborn human babies of subgroup A_2. This indicates that in chimpanzees, as in man, the A-B agglutinogens are incompletely developed at birth.

Another difference between human and chimpanzee group A red cells is that, in contrast to man, red cells from chimpanzees of group A, irrespective of subgroup [6] almost invariably fail to agglutinate with anti-**H** lectin. However, saliva from group A as well as group O chimpanzees generally strongly inhibit anti-**H** lectin, not infrequently in even higher titers than human O salivas. These results do not support the frequently cited concept of H as precursor of A and B [41] and support instead our suggestion that the serological reactions observed depend upon steric interference between the determinant groups for H and for A-B [4, 5]. Presumably, the relative positions of these groups on the agglutinogen molecule in chimpanzees and man are not the same; in fact, differences have even been found among human races in this respect [5].

Evidence has been obtained that the blood group A specificity is due to the determinant group N-acetyl-D-galactosamine, but until recently [43] no chemical difference was found to account for the subgroup difference, A_1 and A_2. We [12] have suggested that the determinant group in these two subgroups is identical, and that the serological differences are due to differences in the length of subjacent oligosaccharide chains. The findings in chimpanzees support this idea, which makes the graded differences among the A agglutinogens of man and chimpanzee more readily understandable.

The rare pygmy chimpanzee, *Pan paniscus*, is considered to be a distinct species rather than a subspecies of chimpanzees. So far we have tested only nine dwarf chimpanzees of which three have been reported in detail and all were of group A [10]. Schmitt [11] has also tested a number of pygmy chimpanzees and found that they, like *Pan troglodytes*, have only the two blood groups O and A. We have found, like Schmitt, that the group A red cells of pygmy chimpanzees give reactions indistinguishable from human subgroup A_1 with various anti-**A** reagents, so that, in this respect, they differ sharply from *Pan troglodytes*. This result and our other blood group findings [10] support the concept of *Pan paniscus* as a separate species of chimpanzees.

Table 2

Results of titrations of sera of 43 chimpanzees against human red cells*

Blood group of chimpanzees**	Number of animals tested	Average titers in saline for				Average titers in acacia for			
		O	A_1	A_2	B	O	A_1	A_2	B
A	39	0	0	0	5	0	0	0	16
O	4	0	8	4	7	0	54	17	35

* After Wiener and Gordon [7].

** Four of the 39 group A chimpanzees had no demonstrable anti-**B** in their sera.

Table 2 gives the results of titrations of the anti-**A** and anti-**B** agglutinins in the sera of 43 chimpanzees, of which 39 were group A and 4 group O. It is noteworthy that the average anti-**B** titer was higher in the group O chimpanzees than in group A chimpanzees. No doubt the reason is that the group O sera in chimpanzees, as in man, contain not only the isoagglutinins anti-**A** and anti-**B** but also isoagglutinins designated anti-**C**, for a specificity shared by group A and group B red cells but lacking from group O [13, 14].

Gibbons and siamangs. In gibbons, isoagglutination defines three blood groups, homologous to the human blood groups A, B and AB; group O was not found among more than 140 animals tested to date.

As shown in Table 3, the studies on gibbons up to 1952 comprised only 18 animals, of which four could not be typed. In retrospect, the inability to type the gibbons must have been due to the use of anti-**A** and anti-**B** sera containing nonspecific heteroagglutinins, not removed by absorption. Our own studies on gibbons began in 1963 and our findings on a total of 57 gibbons are shown in Table 3. It is of interest that the distribution of the A-B-O blood groups recently reported by Nakajima and Prychodko [18] closely approximates our own findings in this species. In both series, moreover, the distribution of the A-B-O groups in *Hylobates lar lar*, who make up the bulk of the gibbons tested so far, does not differ significantly from that required by the Hardy-Weinberg rule. Thus, the results to date for the A-B-O groups in gibbons can be explained on the basis of two allelic genes, *A* and *B*, with gene *O* absent or, at most, extremely rare.

With regard to the other species (or subspecies) of gibbons, *Hylobates lar pileatus*, *H. hoolock* and *H. concolor*, only a few animals of each have so far been tested, as shown in Table 3. However, there is enough data to indicate a significant difference in the distributions of A-B-O blood groups in *H. l. pileatus*, which were all group B, as compared with *H. l. lar*. Only two siamangs, *Symphalangus brachytanites*, have been tested – both were group B.

The salivas from the 57 gibbons blood grouped by us have also been tested, and all the animals were found to be A-B-H secretors. All secreted the substance H in high titer together with the A-B blood group substances corresponding to their blood group. Tests on their serum showed no exception to Landsteiner's rule, and

Table 3

A-B-O blood groups in gibbons and siamangs

Species and subspecies	Investigators	Number of animals of group				Total
		O	A	B	AB	
Gibbons						
*Hylobates lar lar****	Wiener, Moor-Jankowski and Gordon	0	10	20	22	52
H. l. lar	Nakajima and Prychodko	0	10	23	30	63
*H. l. pileatus****	Wiener, Moor-Jankowski and Gordon	0	0	4	0	4
H. l. pileatus	Nakajima and Prychodko	0	0	2	0	2
*H. hoolock****	Wiener, Moor-Jankowski and Gordon	0	1	0	0	1
H. concolor	Nakajima and Prychodko	0	4	0	0	4
Hylobates (unspecified species)	Various authors* (1925—1952)	0	2	10	2	18**
Siamangs						
*Symphalangus brachytanites****	Wiener, Moor-Jankowski and Gordon	0	0	2	0	2

* After Schmitt [11].

** This includes four gibbons whose A-B-O blood groups could not be determined, probably because of the use of blood grouping sera containing nonspecific heteroagglutinins [15].

*** Species determined by Dr. Colin Groves.

cross matching of serum and red cells of gibbons gave the reactions expected from their blood groups.

Tests for the subgroups of A have also been done on our own series of gibbons. Among the 52 *H. l. lar*, the distribution proved to be: $A_1 = 10$; $A_2 = 0$; $B = 20$; $A_1B = 20$ and $A_2B = 2$. The reason why no animals of subgroup A_2 were found even though A_2B was found is that when group O is absent, the proportion of subgroup A_2 among group A is the square of the proportion of A_2B among AB's [19]. Since only one-eleventh of our group AB gibbons were subgroup A_2B, only one among 121 group A gibbons could reasonably be expected to be subgroup A_2, but so far only 10 group A gibbons have been tested by us.

The reactions of the red cells of the group A_1 and group A_1B gibbons with anti-**A** reagents were comparable to those of human red cells of the corresponding subgroups. Moreover, the reaction of anti-**A** sera with gibbon red cells of subgroup A_2B gave the picture of clumped cells on the background of unagglutinated cells, which is characteristic also of human A_2B red cells. Thus, the subgroups of A in gibbons resemble the subgroups of A in man, and in that respect differ from group A of *P. troglodytes* as already described.

Gibbon red cells differ from human red cells, however, in that group B gibbon red cells are strongly agglutinated by anti-**H** lectin *(Ulex europeus)*, the intensity and titer of the reactions being comparable to that of human group O cells. On the other hand, gibbon red cells of group A and group AB give negative or only weak reactions with anti-**H**.

Orangutans. Isoagglutination occurs in orangutans as in man, chimpanzees and gibbons, but tests to date have shown the presence of only three blood groups, homologous to the human blood groups A, B and AB, so that group O appears to be absent in orangutans as in gibbons. We have tested two series of orangutans (Table 4). The first series of 26 animals showed 22 group A, 1 group B and 3 group AB [20]. Salivas from these 26 animals were tested and all but one of them proved to be secretors. There were no exceptions to Landsteiner's rule of reciprocal rela-

Table 4

A-B-O blood groups in orangutans *(Pongo pygmaeus)*

Investigators	Number of animals of group				Total
	O	A	B	AB	
Wiener, Moor-Jankowski and Gordon [20]*					
1st series	0	22	1	3	26
2nd series	0	7	2	2	11
Combined	0	29	3	5	37
Other investigators (1925–1963)**	0	12	11	11	34
Total	0	41	14	16	71

* This original report dealt with only 26 orangutans. The additional 11 apes listed have not been published previously.

** After Schmitt [11].

tionship between agglutinogens and agglutinins in this series of 26 orangutans. For our second series of 11 orangutans (unpublished data), saliva and serum samples were received but no red cells (cf. Table 4). Nevertheless, the blood groups were readily determined; 7 animals were group A, 2 group B, and 2 group AB. The red cells of the group A and group AB orangutans of our first series of 26 animals were tested with anti-$\mathbf{A_1}$ reagents and all gave reactions corresponding to human subgroup A_1. The red cells of these animals were also tested with anti-**H** lectin *(Ulex europeus)* and all gave negative reactions, in contrast to our findings for gibbon red cells.

As shown in Table 4, when our findings are combined with those published by other investigators, the total number of orangutans tested is 71: namely, 41 group A, 14 group B, and 16 group AB. Application of the Hardy-Weinberg formula shows a marked deficiency of group AB animals. (The expected numbers

are 34 group A, 7 group B and 30 group AB.) Such a deficiency of heterozygotes could occur when two or more different isolates are combined, one with a high frequency of group A and low of group B, and the other with a low frequency of group A and high frequency of group B. Another possible explanation for the deficit of group AB could be the existence of group O orangutans, which, by chance, have not yet been encountered. Since it is known that the 71 orangutans do comprise several different series of animals we favor the former explanation.

Gorillas. Gorillas have been the least investigated apes because of their rarity. In the original study of Landsteiner [21] only a single gorilla was tested but it was not possible to determine its blood group. It occurred to one of us (A. S. W.) that the reason could be that the red cells of gorillas, as we had shown for monkeys, were devoid of A-B-H agglutinogens, while, at the same time, the tests on the serum for agglutinins was complicated by the presence of non-specific heteroagglutinins for the human red cells. As will be shown, the red cells of gorillas indeed fail to agglutinate with anti-**A**, anti-**B** and anti-**H** reagents, but the A-B-O

Table 5

Results of tests for A, B and H blood group substances in the saliva of 14 gorillas *(G. gorilla gorilla)**

Saliva of	Inhibition titers of saliva for			Indicated A-B-O blood groups**
	A	B	H	
Gorillas				
1. Anka	0	4	64	B_2
2. Banga	0	16	64	B_1
3. Chad	0	4	256	B_2
4. Calabar	0	1	128	B_2
5. Katoomba	0	16	128	B_1
6. Oban	0	16	256	B_1
7. Ozoom	0	2	32	B_2
8. Paki	0	4	32	B_2
9. Rann	0	4	32	B_2
10. Segon	0	4	64	B_2
11. Jive	0	16	64	B_1
12. Shamba	0	64	64	B_1
13. Oki	0	64	256	B_1
14. Choomba	0	16	256	B_1
Human controls				
Secretor O	0	0	32	
Secretor A	256	0	32	
Secretor B	0	32	2	
Nonsecretor	0	0	0	

* After Wiener et al. [23].

** The sera (after absorption with human group O red cells) from all the gorillas agglutinated human red cells of groups A_1 and A_2, but not red cells of groups O or B.

groups can nevertheless be determined easily by testing the saliva for the A-B-H blood group substances and by testing the serum, after absorption with human group O cells, for anti-**A** and anti-**B** agglutinins. It is of historical interest also that the first gorilla to be successfully grouped was a dead one [22] which was tested by extracting its salivary glands with hot saline solution.

Two species of gorillas exist, namely, the lowland gorilla, *Gorilla gorilla gorilla*, and the mountain gorilla, *Gorilla g. berengei*. All 23 lowland gorillas studied by us so far have proved to be group B, viz. the presence of B and H was demonstrated in the saliva and of anti-**A** in the serum. Because gorillas are rare animals, at first only material from one or two animals at a time could be tested, so that satisfactory comparative studies were not possible. Recently, however, blood and saliva from 14 lowland gorillas became available to us [23], all at the same time (these are part of the series of 23 animals cited above). In inhibition tests on their salivas carried out by the titration method, differences in inhibition titer for anti-**B** were observed, indicating the existence of subgroups; in this way, 7 of the gorillas were classified as B_1, and 7 as subgroup B_2 (see Table 5).

More recently, blood specimens were obtained and tested from two mountain gorillas, and saliva from one of these two animals. Both gorillas proved to be group B, like all the lowland gorillas we have tested [25]. These results are different from those obtained in 1940 by Candela et al. [24] who had reported two mountain gorillas to be group A. It must be borne in mind, however, that Candela's tests were done not on saliva but on urine, and blood grouping tests by the inhibition tests on urine are subject to error; e.g., using this method, Candela at first reported two of his lowland gorillas to be group AB but later found them to be group B.

Further tests on gorilla red cells have demonstrated that after treatment of red cells with ficin, a proteolytic enzyme [25], the red cells are specifically agglutinable by anti-**B** sera, but in much lower titer and avidity than human group B cells (tests on ficinated gorilla red cells with anti-**H** lectin, however, gave no clumping). Thus, the gorillas tested by us all appeared to have an extremely weak B-like agglutinogen on their red cells, unlike Old World monkeys of group B whose red cells fail to agglutinate with anti-**B** reagents, even after enzyme treatment.

Old World Monkeys

Baboons. Among the Old World monkeys, the most extensive studies have been carried out by us on baboons [26], because of their use in surgical experimentation, for which blood grouping is of importance. By now we have typed more than 1,100 baboons, and in Table 6 are listed the findings for those animals for which accurate records have been kept. As can be seen, of the five major species of baboons, we have tested significant numbers for all except the sacred baboon *(Papio hamadryas)* which are therefore not included in the table.

The red cells of Old World monkeys are not agglutinable by anti-**A**, anti-**B** or anti-**H** reagents. Therefore, the A-B-O blood grouping tests on baboons are

Table 6

Human type A-B-O blood groups of baboons

Species of baboons		Blood group distribution in the animals tested					Estimated gene frequencies			$\chi^2_{(1)}$	P
		O	A	B	AB	Total	*O*	*A*	*B*		
Previously reported [26]											
Yellow baboon	No.	0	18	20	22	60					
Papio cynocephalus	Percent	0	30.0	33.3	36.7	100	8.9	44.0	47.1	1.1	0.58
Olive baboon	No.	0	53	56	65	174					
P. anubis	Percent	0	30.5	32.2	37.4	100	8.4	45.0	46.6	2.9	0.27
Chacma baboon	No.	0	4	59	26	89					
P. ursinus	Percent	0	4.5	66.3	29.2	100	1.8	18.8	79.3	0.06	0.97
Guinea baboon	No.	2	27	93	66	188					
P. papio	Percent	1.1	14.4	49.5	35.1	100	10.6	29.9	59.5	0.02	0.99
Species undetermined	No.	1	42	65	65	173					
	Percent	0.6	24.3	37.6	37.6	100	10	39	51	0.7	0.70
New series*											
P. anubis	No.	0	13	233	98	344					
(Ethiopia)	Percent §	0	3.8	67.7	28.5		0	18.1	81.9	0.43	0.60
Other investigators											
P. cynocephalus	No.	0	32	50	63	145					
and *P. anubis*+	Percent	0	22.1	34.5	43.4		0.06	43.8	56.2	0.53	0.50
P. ursinus ursinus‡	No.	0	27	6	18	51					
	Percent §	0	53.0	11.8	35.3		0	70.6	29.4	1.17	0.25

* Unpublished observations.
+ Podliachouck and Dubouch [27].
‡ Downing et al. [28].
§ Gene frequencies estimated by direct count, assuming non existence of gene *O* in these two series.

carried out not as for man and most apes, but by testing saliva by the inhibition technique for the A-B-H blood group substances and the serum for the agglutinins anti-**A** and anti-**B**, after removal of non-specific heteroagglutinins from the serum by absorption with human group O cells. Among the 1,028 baboons tested by us and shown in Table 6 there were only three group O; the remainder were group A, group B or group AB. Among the baboons of known species, group O has so far been found only in the Guinea baboon. In our series of 188 Guinea baboons, the gene frequencies, estimated by the maximum likelihood method, proved to be $O = 10.6\%$, $A = 29.9\%$ and $B = 59.5\%$ (Table 6). The chi-square test showed an excellent fit between the observed distribution and the expected distribution of A-B-O blood groups, based on the theory of inheritance by triple allelic genes, as in man. In the other three species of baboons tested, yellow baboons, olive baboons and chacma baboons, we have not found any group O animals; however, we believe that group O also exists in those species but is rare. In fact, gene frequency analysis gives the best fit with the theoretical expectations when the presence of gene *O* is assumed, as shown in Table 6. The same holds true for a series of 145 yellow and olive baboons by Podliachouk and Dubouch [27] among which they found 32 group A, 50 group B and 63 group AB. It is of historical interest that in our very first study on the A-B-O blood groups of baboons [29] we did some family studies in which we encountered group A baboons with group B offspring and group B baboons with group A offspring. This led us to postulate the existence in these animals of gene *O*, even though no group O baboons had been found at that time.

Podliachouk and Dubouch [27] have tested blood and saliva from newborn baboons. They found that while the A-B-H substances could be demonstrated by inhibition test in the saliva of newborn baboons, the agglutinins anti-**A** and anti-**B** were not demonstrable in their serum. For this reason they have advised that the A-B-O grouping of baboons be postponed until the animals are at least 3 to 6 months old.

Landsteiner's rule holds also for baboons except that, as has been pointed out, the reciprocal relationship is between agglutinins in serum and blood group substances in the saliva rather than on red cells. Some apparent exceptions, in which no anti-**A** nor anti-**B** was found, seemingly contrary to expectation, could be resolved by the demonstration in the saliva of very weakly reactive A or B group substances, by using a more sensitive inhibition technique [29]. Since not a single animal among more than a thousand baboons tested by us has proved to be a non-secretor, it has always been possible up to now to determine the A-B-O blood groups of baboons from tests on the saliva. However, a few animals have been found with anti-**A** in the serum despite the presence of the blood group substance A in their salivas. The anti-**A** in these group A and group AB animals is peculiar in that it is not inhibitable by secretor saliva from group A baboons and group A human beings in contrast to the anti-**A** from group B baboons and group B human beings (see Table 7). These results establish the existence of anti-**A** agglutinins of two kinds, one designated by us anti-$\mathbf{A}^c$ because it is reactive exclusively with

Table 7

Results of inhibition tests using anti-**A** reagents of human origin and anti-**A** reagents derived from baboons of groups B and A*

Donors of saliva	Blood group of saliva donors	Anti-A sera tested with saliva dilutions												
		Human anti-**A** from a group B blood donor					Baboon anti-**A** from a group B animal (A # 267)					Baboon anti-**A** from a group A animal (A # 325)		
		1:1	1:4	1:6	1:64	1:256	1:1	1:4	1:16	1:64	1:256	1:1	1:2	1:4
Baboons														
A 267	B	++	++	++±	++±	++±	++	++	++	++	++	++	++	++
D 288	AB	−	−	−	−	+±	−	−	−	−	tr.	++	++	++
A 321	AB	−	−	−	−	+±	−	−	−	−	tr.	++	++	++
A 325	A	−	−	−	+	+±	−	−	−	−	−	++	++	++
Human														
E.B.G.	A	−	−	−	tr.	±	−	−	−	−	−	++	++	++
S.G.	B	++	++±	++±	++±	++±	+±	+±	++	++	++	++	++	++
D.S.	O	++±	++±	++±	++±	++±	++	++	++	++	++	++	++	++

* From Moor-Jankowski, Wiener and Gordon [29].

the A substance on the red cells and not in secretions, while the other, designated anti-$\mathbf{A}^s$, is reactive for the group substances both in secretions and on red cells. Obviously, a group A baboon would hardly be likely to form anti-$\mathbf{A}^s$ but could produce anti-$\mathbf{A}^c$ which would not react with soluble group A substances in its own secretions, and therefore would not be inhibitable by group A secretor saliva. Anti-**H** has also been shown to be of two kinds, anti-$\mathbf{H}^c$ and anti-$\mathbf{H}^s$ [4], and the same presumably holds for anti-**B**.

As has already been pointed out, baboon red cells fail to agglutinate in tests with human anti-**A** serum, human anti-**B** serum and anti-**H** lectin *(Ulex europeus)* even when reagents of the highest available titers and avidity are used. However, when tests were carried out with anti-**A** lectin (lima bean) agglutination resulted though only in very low titer [30]. The weak reactivity of baboon red cells with anti-**A** lectin was about the same for animals of groups A, B and AB. That the reactions of the lima bean lectin with baboon red cells was nevertheless due to the A-like specificity of the reagent was proved by the observation that the agglutination of baboon red cells could be inhibited by group A and group AB secretor saliva from man and baboon but not by saliva of group O, or group B, or saliva from human nonsecretors. Thus, all baboons have on their red cells an agglutinogen with a weak A-like specificity quite apart from their human-type A-B-O groups. Moreover, as has been pointed out, baboons lacking A substances in their saliva regularly have anti-**A** in their serum, and the new finding of an A-like agglutinogen on red cells of *all* baboons evidently does not interfere with this rule.

As shown in Table 6, there are wide variations in the distribution of the A-B-O blood groups among the various series of animals, e.g., the frequency of group A ranges from 3.8 percent to 53.0 percent. Surprisingly, this applies not only to baboons of different species but also to different series of the same species. Thus, a series of 344 *P. anubis* from which specimens were collected in the field in Ethiopia, had only 3.8 percent group A, while in a series of 174 *P. anubis* of unknown origin and obtained from animal dealers there were as many as 30.5 percent group A. It should be noted that the taxonomy of both series of *P. anubis* was verified by Professor Clifford Jolly of New York University. The most likely explanation for this difference in blood groups appears to be the geographical difference between the sites of origin in Africa of these two series of olive baboons, with resulting isolation and gene drift.

When the sera and red cells of baboons of the same species are cross tested, agglutination usually does not occur, aside from occasional reactions unrelated to A-B-O groups. This is as expected since the group substances which determine the animals' A-B-O groups are present only in the secretions and not on the red cells. Moreover, when red cells and sera from different species of baboons are cross tested, no agglutination is observed. This absence of natural heteroagglutinins is further evidence of the close serologic and probably also taxonomic relationship among these species.

Macaques. Among the Old World monkeys other than baboons extensive studies on the A-B-O groups have so far been carried out only for macaques

(cf. Table 8), because of their common use for medical experimentation. In their handbook, Napier and Napier [31] list as many as 12 species of macaques having numerous subspecies. Among these, so far, only a few species have been tested; our own studies have dealt principally with rhesus monkeys *(Macaca mulatta)* and crab-eating macaques *(M. fascicularis)*. In macaques as in baboons and all other Old World monkeys, the red cells have no demonstrable A-B-H agglutinogens, so that the A-B-O grouping has to be carried out on saliva and serum.

All the rhesus monkeys tested by us have reacted as group B, i.e., their saliva gave positive inhibition for B and H but not for A, while the serum regularly contained anti-**A** but not anti-**B**. Differences have been observed in that salivas of some rhesus monkeys inhibited anti-**B** in high dilutions while salivas from other monkeys gave only weak inhibition. This suggests the existence of subgroups of B in macaques similar to those we have found in gorillas.

Table 8

Human-type A-B-O blood groups of Old World monkeys excluding baboons*

Species	Number of animals of group				Total
	O	A	B	AB	
Drills					
Mandrillus leucophaeus	0	4	0	0	4
Geladas					
Theropithecus gelada	18	0	0	0	18
Celebes black apes					
Cynopithecus niger	1(?)	23	2	0	26
Patas monkeys					
Erythrocebus patas	0	26	0	0	26
Vervet monkeys					
Cercopithecus pygerythrus	0	7	1	0	8
Downing et al. [35]	0	39	10	10	59
Macaques					
*Macaca mulatta***	0	0	80	0	80
*Macaca fascicularis****					
Previously reported	1	10	5	4	20
New series	0	13	14	15	42
Total	1	23	19	19	62

* After Moor-Jankowski and Wiener [9]. For baboons, see Table 6.

** Rhesus monkeys.

*** Crab-eating macaques, or Java monkeys formerly designated Cynomolgus monkeys and *Macaca irus*.

In contrast to rhesus monkeys, crab-eating macaques have all four A-B-O groups, though, as in baboons, group O is rare (cf. Table 8). Thus far we have found no exceptions to Landsteiner's rule in this species. In crab-eating macaques as well as in rhesus monkeys, cross tests of serum and cells between animals of the same species show no agglutination, i.e., isoagglutination does not occur aside from rare weak reactions unrelated to the A-B-O groups. In addition, hetero-agglutinations does not occur between these two species, as could be expected from their close relationship.

Of the other macaque species we have tested only a few animals not listed in Table 8. Four stump-tailed macaques *(M. speciosa)* tested reacted as group B, i.e., the salivas had B and H, and the serum anti-**A**. Salivas of all five pig-tailed monkeys *(M. nemestrina)* inhibited anti-**H** but not anti-**A** nor anti-**B**; however, only three of these monkeys had the expected anti-**A** and anti-**B** isoagglutinins in their sera, while two had anti-**A** without anti-**B** [32, 33].

The most extensive studies on the A-B-O groups in macaques, as far as the number of animals and variety of species tested, have been carried out by Nakajima et al. [34]. However, the methods used by those investigators differ from those used by us. With few exceptions salivas were not tested for A, B and H. Instead, the red cells were treated with anti-**A** prepared in rabbits by injection with human cells, and anti-**B** prepared in a goat by immunization with tortoise red cells, and the eluates from the cells then tested. (In our own hands, tests by the elution technique have not given dependable results.) In addition, the macaque sera, after absorption with human O cells, were tested for the presence of anti-**A** and anti-**B** agglutinins. The results obtained by these investigators by serum tests are

Table 9

Results of A-B-O grouping of macaques; based on tests on sera for anti-**A** and anti-**B** agglutinins only*

Species	Locality	Number tested	Anti-**A** + **B** (O)+ %	Anti-**B** (A) %	Anti-**A** (B) %	(AB) %
M. fuscata fuscata	Japan	201	22.9	0	77.1	0
M. fuscata yakui	Japan	50	2.0	0	98.0	0
M. cyclopis	Taiwan	102	4.9	0	90.2	4.9
M. irus	Thailand	146	34.9	26.0	26.7	12.3
	Malaysia	232	30.2	32.3	24.6	12.9
	Philippine	60	5.0	5.0	88.3	1.7
M. mulatta	Thailand, Malaysia and Pakistan	70	2.9	0	92.9	4.3
M. speciosa	Thailand	173	5.2	0.6	93.6	0.6
M. nemestrina	Thailand	82	24.4	1.2	74.4	0
	Malaysia	144	88.9	2.1	9.0	0

* After Nakajima et al. [34]. Saliva not tested.
\+ Presumed blood group is given in parentheses.

shown in Table 9, together with their presumed A-B-O blood groups. It will be of interest to see whether these findings are later confirmed when salivas from macaques of these species are tested. It is noteworthy, however, that the frequency of group O reported by Nakajima et al. for crab-eating macaques was quite high whereas we found hardly any group O in this species (cf. Table 8). Also Nakajima et al. found 92.9 percent group B among rhesus monkeys *(M. mulatta)*, while we have found group B exclusively.

Other Old World monkeys [32, 33]. As shown in Table 8, 18 geladas *(Theropithecus gelada)* tested by us had saliva which inhibited anti-**H** but not anti-**A** or anti-**B**, indicating that they belonged to group O. However, Landsteiner's rule does not hold in this species, since most of the animals did not have the expected anti-**A** and anti-**B** agglutinins in their sera; instead, some animals had anti-**B** alone, others had anti-**A** alone, while still others had neither. This strongly suggests that the geladas tested actually had the blood groups A, B and AB but for some still undetermined reason the group substances A and B could not be detected in their saliva.

Patas monkeys *(Erythrocebus patas)*, on the other hand, appear to be all group A, since their saliva regularly inhibit anti-**A** and anti-**H** but not anti-**B**. In this species, moreover, Landsteiner's rule holds since the serum absorbed with human O cells regularly agglutinates human group B but not group A cells. It should be noted, however, that striking differences have been observed in the anti-**A** inhibition titers (but not for anti-**H**) indicating the presence of subgroups; about half of the animals reacted as A_1 and about half as A_2.

Among 26 Celebes black apes *(Cynopithecus niger* also called *Macaca maura)*, all but three reacted as group A. However, Landsteiner's rule did not hold in this species since among the group A animals only a minority had the expected anti-**B** agglutinins while the remainder had no group-specific agglutinins in their sera.

Only 8 vervet monkeys *(Cercopithecus pygerythrus)* have been tested by us of which 7 proved to be group A and one group B. Landsteiner's rule held in all cases. A larger series of these monkeys has been tested by Downing et al. [35]. In the total of 67 animals, there were groups A, B and AB, but no group O.

Only four drills *(Mandrillus leucophaeus)* have been tested by us; all of them reacted as group A, but only two of them had the expected anti-**B** in their sera.

New World Monkeys [36, 37]. In early studies on human-type blood factors in apes and monkeys Landsteiner and Miller [38] tested red cells of 16 New World monkeys (7 capuchin monkeys, 3 woolly monkeys, 3 spider monkeys and 3 marmosets). Anti-**B** reagents prepared by elution from human group B cells clearly agglutinated the red cells of all those monkeys, but anti-**A** eluates failed to react. Landsteiner and Miller concluded that all New World monkeys have on their red cells a B-like agglutinogen. These observations have been confirmed by our own limited more recent observations on such monkeys.

Our experiments on New World monkeys have to date included 54 animals, of which 31 were marmosets [36, 37]. Although these marmosets were represen-

tatives from four different species, namely *Saguinus oedipus*, *S. mystax*, *S. nigricollis* and *S. fuscicollis*, saliva and blood from all of them gave similar reactions. Firstly, we were able to confirm the presence of a B-like antigen on the red cells of all the animals. Secondly, all the animals were secretors and their saliva strongly inhibited anti-**A** but not anti-**B** reagents; but only very weak or no inhibition was observed in tests with anti-**H** lectin. Thus, according to the saliva reactions the animals were all group A, while the red cells gave B-like reactions. Tests on the sera for agglutinins, after absorption with human group O red cells, in most animals gave no reactions but in some marmosets, a weakly or moderately reacting anti-**B** for human group B cells was demonstrated which was not reactive for the animal's own red cells. This was further evidence that the B-like antigen on marmoset red cells was not identical with though similar to the human agglutinogen B. Tests on the red cells of marmosets with a variety of lectins including anti-$\mathbf{A}_1$ *(Dolichos biflorus)*, anti-**H** *(Ulex europeus)* and anti-$\mathbf{N}^V$ *(Vicia graminea)* resulted in negative reactions only. Similar findings had been reported by Gengozian [39].

Fifteen spider monkeys *(Ateles sp.)* from four different species (Table 10) have been tested by us [36]. Ten animals belonging to 3 different species had saliva which reacted as group A and their sera had the expected anti-**B** despite the presence on their red cells of a B-like agglutinogen. In the fourth species *Ateles cucullatus*, among five animals there were four group B and one group O, based on the reactions of their saliva.

The saliva from three squirrel monkeys *(Saimiri sciurea)* gave strong inhibition for A and H but none for B, while the saliva of the fourth animal inhibited

Table 10

Human-type A-B-O blood groups of New World monkeys*

Species	Number of animals of group				Total
	O	A	B	AB	
Spider monkeys					
Ateles marginatus	0	3	0	0	3
Ateles paniscus	0	1	0	0	1
Ateles ater	0	6	0	0	6
Ateles cucullatus	1	0	4	0	5
Capuchin monkeys					
Cebus albifrons	1	0	3	0	4
Squirrel monkeys					
Saimiri sciurea	1	3	0	0	4
Marmosets					
Various species	0	31	0	0	31

* After Moor-Jankowski and Wiener [9].

anti-**H** only (Table 10). However, Landsteiner's rule did not hold since the expected agglutinins were not demonstrable in the sera. This may have been due in part to the presence of B-like antigens on the red cells.

Among four capuchin monkeys *(Cebus albifrons)* three had B and H in their saliva while one had only H, indicating three group B and one group O. Anti-**A** was present in the sera of all four animals but the expected anti-**B** was missing from the serum of the group O monkey.

Obviously, it will be of interest to test many more animals from a greater variety of monkey species.

Discussion

As has been demonstrated in this review, all apes and monkeys tested to date have homologues of the human A-B-O blood groups, which is further evidence supporting their classification in the Order of Primates. To be sure, A-like and B-like antigens are distributed throughout the animal kingdom, but their occurrence as clear-cut blood groups exhibiting a reciprocal relationship between agglutinins and antigens (Landsteiner's rule) appears to be characteristic mainly of apes and most Old World monkeys. The findings described here are of interest not only for immunogeneticists but also to taxonomists, since they throw further light on the serology, genetics and immunochemistry of the A-B-O blood groups in man.

Apparently, in nonhuman primates as well as in man, each allelic A-B-O gene determines a corresponding glycosyl transferase which transfers the appropriate simple sugar determinant from a donor molecule to the blood group precursor molecule. Biochemical studies indicate that the blood group substances on the red cells are lipopolysaccharides, while the soluble group substances in secretions are mucopolysaccharides. Therefore, even though in both cases the same blood group genes appear to be involved, different precursor substances must act as acceptors for the appropriate determinant sugar. This implies separate pathways for the development of the group substances on the red cells and in secretions. In fact, in man, non-secretors have no clearly demonstrable group substances in their secretions even though their red cells give reactions typical for their blood groups, indicating that in individuals of genotype *sese* the pathway for development of group substances in secretions has been blocked or failed to evolve. In contrast, as has been pointed out in this review, in monkeys the group-specific substances are regularly demonstrable in the secretions but not on the red cells. Moreover, in their earliest stages of development the group substances of red cells and secretions share a common pathway, since in human individuals of the so-called Bombay type the group-specific substances expected from their A-B-O and H genotypes are not demonstrable either in secretions or on the red cells [40].

The studies on apes and monkeys have been particularly helpful for clarifying the relationship of H to the A-B group substances. According to one view,

H is considered a precursor of A and B,and the fact that in man group O individuals who are homozygous for the amorphic gene *O* have red cells and secretions most strongly reactive for H appears to support this idea [41]. However, in apes and monkeys the situation is quite different, and the reciprocal relationship between H and A-B does not hold. For example, in marmosets the saliva strongly inhibits anti-**A** and hardly anti-**H**, while in squirrel monkeys of group A the saliva strongly inhibits anti-**H** as well as anti-**A**. Moreover, in gibbons red cells of group B react with anti-**H** lectin to titers as high as human group O red cells, while in orangutans red cells of group B fail to react with anti-**H**. Perhaps the most striking argument is the recent report that the reactivity with the anti-**H** lectin of red cells of the various A-B-O groups and subgroups is not the same in Whites, Negroes and Chinese [5]. Thus, instead of H being a precursor of A and B it appears more likely that H and A-B develop in parallel, and the apparent reciprocal relationship between the strengths of the reactions for H and A-B observed in man may be due to steric interference caused by the proximity between the determinant groups which are responsible for the various specificities. In apes and monkeys where the reciprocal relationship between H and A-B is absent or far less apparent, the determinant groups for H and A-B may be more widely separated.

The observations on the A-B-O blood groups of chimpanzees have thrown further light on the nature of the human A_1-A_2 subgroups. Biochemical studies indicate that the identical determinant sugar molecule, N-acetyl-D-galactosamine, is responsible for the A specificity irrespective of subgroup [42]. According to one view, the difference between subgroups is a quantitative one, determined by differences in the number of the antigenic combining sites on the red cell envelope. We have suggested instead [12] that the serological differences between the subgroups may be due to the differences in the length of the subjacent oligosaccharide chains to which the determinant group is attached. The fact that in chimpanzees red cells of group A give reactions intermediate between those of human A_1 and A_2 is also readily understandable according to this concept, by postulating corresponding differences in the length of the subjacent sugar chain [46] (cf. Moreno et al. [43]).

It has already been pointed out that the anti-**A** and anti-**B** agglutinins in man probably result from exposure to microorganisms and foods having A-like and B-like specificities. The response to the exposure is canalized by the genetically determined group-specific substances present in the individual's body, so that when, for example, group-specific substance A is present, there will be no reaction to the introduction of A-like antigenic substances, while anti-**B** is readily produced. The fact that the Landsteiner rule holds so well in apes and most species of monkeys adds further support to this concept. There are certain apparent exceptions in New World monkeys which serve to confirm instead of refuting the concept. For example marmosets, which are group A according to the reactions of their secretions, usually lack the expected anti-**B** in their sera. However, it turns out that all the marmosets have a B-like agglutinogen on their red cells, similar to though not identical with the human agglutinogen B, so that anti-**B**, when it is

formed at all, is necessarily different in specificity from the anti-**B** present in human group A serum. An outstanding exception has been found in geladas, which have secretions reacting as group O but have sera often devoid of anti-**A**, anti-**B** or both. This puzzling exception is still under investigation.

With respect to taxonomy the gorillas are unique among apes in that their A-B-O groups resemble those of monkeys more closely than the A-B-O groups of other apes and man. It is significant, however, that with respect to other blood group systems, such as M-N and Rh-Hr, gorillas have proved to resemble man more closely than the other apes [44, 45]. These observations underline the danger of basing taxonomic classifications on any single trait. The position of a species in the taxonomic scale obviously should be decided on the basis of a great multiplicity of characteristics rather than any single property, even one as basic as the A-B-O groups.

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HL-A Phenotype and Anti-Rh_0(D) Immunization

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Ninety-three Rh-negative subjects were submitted to a planned schedule of anti-D immunization. The 62 immunologically responding and 31 non-responding individuals were tested for 25 HL-A antigens. No significant difference in phenotype frequency was observed between the groups.

Introduction

It is now a well-established fact that the major histocompatibility system (MHS) in mice [1] and in guinea-pigs [2] is closely associated with the genetic regulation of a number of immune responses.

In man, many associations between HL-A and susceptibility to diseases have been revealed [3] which could be attributed to some defect in a specific immune response, although other mechanisms might have also been involved.

One of the most logical ways to establish a correlation between humoral response in man and HL-A types is to analyze Rh-negative volunteers hyper-immunized against the antigen D. An approach of this type was part of the "HL-A and Immune Response" Workshop, very well organized by Susan R. Hollán, held in Budapest in 1972 [4]. This paper reports on a similar and concomitant study performed in France.

Methods

Anti-D immunization schedule

The Rh-negative volunteers for immunization were previously not transfused, D and D^u negative, unrelated healthy Caucasoïd males, of 43.6 years mean age (range, 21 to 62 years). Blood for stimulation was obtained from three group O Rh_0(D) positive donors, who fulfilled the criteria for whole blood donation as recommended by the WHO [5]. Blood was drawn on ACD solution, sedimented, and the plasma was trapped.

The volunteers were injected intravenously with 2 ml unwashed red cells at four-week intervals. The rise in anti-D antibody concentration was carefully followed by monthly titration of "saline agglutinins" and "albumin agglutinins", with fresh, trypsinized or papainized erythrocytes in serial serum dilutions. In addition, the indirect Coombs test was performed systematically. The results were read macroscopically.

After four injections of blood, the immune responders were further injected until the titre had risen to 1/1028 in the indirect antiglobulin test, and submitted to plasmapheresis in order to obtain batches of prophylactic anti-D immunoglobulin. The volunteers whose sera showed no detectable antibody, received, after a rest of three to five months, further four consecutive monthly injections. After a total of eight injections, no antibody production was detected by the indirect antiglobulin test. This was considered a low immune ability to develop anti-D antibodies.

HL-A typing

Ten antigens at the first locus and 15 at the second locus of the HL-A system [6] were tested by a two-stage microlymphocytotoxicity test performed with unabsorbed rabbit complement at 37°C [7]. In order to ensure serological homogeneity, samples of lymphocytes were previously frozen in liquid nitrogen [8] and typed with the same battery of 60 sera. Platelet complement fixation [9], was also performed parallel.

Statistical comparisons were made between the two categories of Rh negative subjects submitted to immunization, responders and non-responders, as well as a normal control population of 152 individuals taken from a reference panel.

The frequency of each HL-A allele was compared in the three categories using the χ^2 test with correction of the p value, multiplied by the number of alleles tested.

The sera of 67 volunteers were tested retrospectively for anti-HL-A antibodies against the lymphocytes of 20 subjects from a normal panel displaying all the HL-A specificities described as well as some "blanks".

Other markers

The subjects were also typed for serum allotypes (Gm, InV and complete erythrocyte phenotypes). An analysis of this study will be published later [10].

Results

Anti-D response

Antibody production was obtained in 62 cases (67%). Most subjects reached a peak agglutinin level after six injections. Some could be considered high responders, displaying a titre of 1/512 after four injections, but their number was too small (7 cases) to be analyzed separately.

Anti-HL-A response

Anti-HL-A antibodies were produced by 16% of the 48 responders and by 10% of the 19 non-responders tested. The difference was not significant statistically.

HL-A phenotypes

No significant difference in the frequency of the HL-A alleles tested in the anti-D responders and non-responders was observed, as shown in Table 1. However, HL-A10, W28 and W14 were low and W27 and Da31 were high in the non-responders.

Table 1

HL-A antigens tested	Antigen frequency in 62 anti-D responders, per cent	Antigen frequency in 31 anti-D non-responders, per cent	Antigen frequency in 152 normal controls, per cent
HL-A1	32	29	21
HL-A2	50	58	51
HL-A3	26	39	24
HL-A9	18	19	21
HL-A10	14	3	13
HL-A11	12	16	11
W28	10	3	8
W29	10	10	12
W32	5	3	6
Da25 (W30 + W31)	14	10	11
HL-A5	13	13	13
HL-A7	26	13	21
HL-A8	16	16	11
HL-A12	31	23	36
HL-A13	5	3	5
W5	16	23	19
W10	11	10	15
W14	13	0	9
W15	18	10	12
W17	5	10	6
W18	11	16	14
W21	3	7	5
W22	8	3	7
W27	2	16	4
Da31 (<W16)	3	13	3

No difference of frequency may be considered statistically significant using the χ^2 test corrected p probability multiplied by the number of alleles tested (25).

Other parameters

Mean age of the responders (42.3 years) was slightly lower than that of the non-responders (46.2 years), but this difference was not significant statistically ($p < 0.08$). A border line significant difference was observed in ABO phenotype between responders and non-responders: there was a lower percentage of A (29%) and a higher percentage of O (68%) among the non-responders ($p < 0.05$).

Discussion

The present data agree with the results of Petrányi et al. [4] who studied 40 Rh negative responders and 40 Rh positive non-responding individuals immunized against antigen D. No significant correlation with the HL-A phenotype was observed. Nevertheless, the number of cases is obviously too small. Further studies are needed to establish whether or not the difference in W14 (13 vs. 0 in non-responders), W27 (2 vs. 16) and Da31 (2 vs. 13) will be confirmed.

As all volunteers were male, sex studies could not be made.

These data, taken together with the Hungarian series only allow the conclusion that there is no striking association between any HL-A antigen and the ability to mount an immune response against the $Rh_0(D)$ antigen. However, the possibilities remain: (1) that a slight association will be found when more cases are analyzed; (2) that there is an association with several HL-A haplotypes which differ in each family, as is the case for hay fever [11]; (3) that the humoral response or its lack is a general genetic feature for all antigens as it is in the high and low mice strains of Biozzi et al. [12]. In this respect, it should be remembered that Petrányi et al. [4] found an inverse correlation between anti-D response and some bacterial response.

*

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The HL-A System
Genetics, (Patho)physiology and Clinical Relevance*

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The HL-A system remains one of the most fascinating and complex immunogenetic systems in man. Its role in determining the outcome of allograft survival is certain, but we have only begun to understand how to match between unrelated donor–recipient pairs. New findings have made this issue more realistic, as it appears that not only the serologically defined antigens play an important role in graft survival, but also the area which codes for the MLC determinants. Major emphasis in future histocompatibility studies will certainly concern the loci on the same chromosome which determine whether or not an individual is predisposed to a disease. Again, only a start has been made in unravelling the factors which determine this process.

In all mammals studied so far a highly polymorphic system has been found which is coded for by loci carried on an autosomal chromosome and which plays a major role in initiating the homograft reaction and determining graft survival. It is called the major histocompatibility system or complex (MHC), and is present on all nucleated cells. It was first detected in the mouse [1, 2], later in man [3, 4], the rhesus monkey [5], chimpanzee [6], dog [7], rabbit [8], rat [9], guinea pig [10], etc.

The system can be studied by methods *in vivo* as well as *in vitro*. The first include organ and tumour grafts or the transfusion of blood elements (platelets especially), the latter the use of serological and cellular techniques. In the first chapter I want to outline briefly the genetics and (patho)physiology of the human major histocompatibility system HL-A, as an introduction to its clinical relevance. In doing so I will especially deal with the question how a better understanding of the HL-A system and its physiology can be of help in improving the results of bone marrow transplantation. The ABO system in man is another important histocompatibility system [4, 11]. It might have an equivalent in animals but this has not yet been defined. Its role in bone marrow transplantation [12, 13] and blood component therapy [14] is thought to be of secondary importance and will not be discussed further.

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I. The genetics of the HL-A system

The genetic information for the HL-A system is in all probability carried on the autosomal chromosome C6. This has become clear thanks to hybridization studies, which have shown that the IPO-B locus, which is carried by C6, and the HL-A locus are on the same chromosome [15]. Hybridization studies also suggested that the blood groups P [16] and ME_1 [15] are on C6. Family studies clearly revealed the linkage between HL-A and PGM_3 [17] (Fig. 1). The HL-A region or

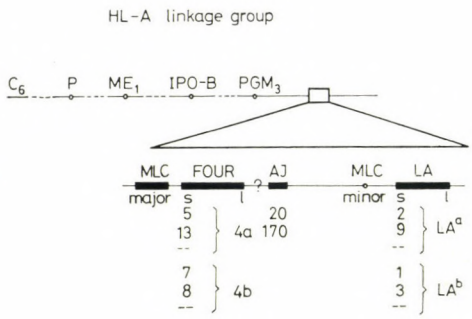

Fig. 1. Schematic representation of HL-A on chromosome C6. The sequence on the chromosome of P, ME_1 and IPO-B is unknown as is that of Four and AJ. The Ir genes and those predisposing for certain diseases (see text) are thought to lie in the region between the major MLC locus and AJ

supergene measures approximately 2 crossover units, and could thus enclose several thousands of genes. This approximation depends on the stability of this region, which is unknown. In man the HL-A supergene is enclosed at the left by the major MLC locus, at the right by the LA locus. It should, however, be understood that these are only interpretations of indirect measurements by serological and cellular techniques and that the exact boundaries of the HL-A supergene are unknown.

For instance preliminary data by Amos [18] suggest that to the left a locus might lie, which controls the morphogenesis of the tail end of the spinal column, while if the remarkable homology with the H-2 system would hold, at the right an equivalent of the mouse Tla locus could be expected to lie, a locus, that at least in the mouse partially controls MLC reactivity [19].

In man conclusive evidence for 3 and suggestive evidence for 2 other loci in the HL-A region are available. From left to right these are the MLC, Four, AJ, MLC minor and LA loci. The information for these loci carried by one chromosome is called a haplotype. These major genes code for proteins which are expressed largely on the cellmembrane, although recent studies suggest that they might also be present on the membrane of the nucleus [20]. Only the products of

the Four and LA loci have been analyzed to some extent [21]. The HL-A antigens are envisaged as protein icebergs floating in a sea of lipoproteins [22]. The molecule might carry at most two carbohydrate moieties but these have probably no haptene function [21]. The molecular weight is around 38,000, together with a β_2-microglobulin-like moiety over 50,000 [23].

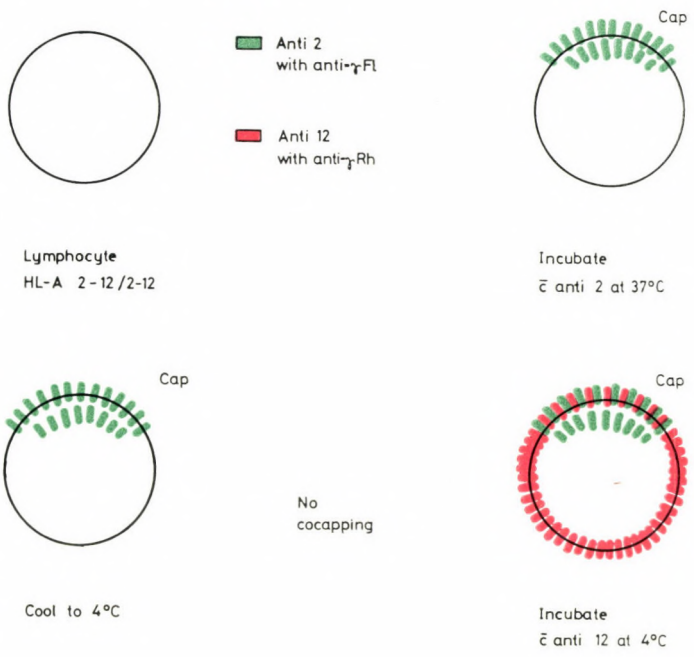

Fig. 2. The circle depicts a lymphocyte sample with the HL-A genotype 2—12/2—12. The cells are first incubated at 37°C with anti HL-A 2, washed, and then incubated with anti-human γ globulin, labelled with isothiocyanate. The molecules carrying the HL-A 2 antigen will agglutinate and cap above the Golgi apparatus. Next the cells are cooled to 4°C and the procedure is now repeated with anti HL-A 12 and rhodamine-labelled anti-γ globulin. The rhodamine is evenly distributed, which implicates that HL-A 2 and HL-A 12 are carried by two separate molecules (Kourilsky et al. [30], Bernoco et al. [31]).

The products of the Four, AJ and LA loci are recognized primarily by serology and are thus called serologically defined or SD. The products of the MLC loci were primarily recognized in the MLC reactions and are for that reason classified as lymphocyte defined or LD [24]. It should be understood that these terms are only introduced for heuristic reasons and do not imply that SD antigens cannot be recognized by cellular techniques or LD antigens by serology.

Crossovers have been frequently found and described between LA and Four and it is now well established that the crossover frequency is around 0.8% [25, 26].

Table 1

HL-A antigens and their gene frequencies

	Four (2nd) locus									Chimpanzees Φ	
	Caucasians Φ			Mongoloids Φ			Negroids Φ				
	DUT	GER	DAN	SMO	AYM	CHI	ZAM	KI	HK		
HL-A 5	6	9	4	4	16	6	1	2	12	0	
HL-A 13	3	4	2	1	0	12	1	1	2	0	
W17	4	3	5	0	0	9	17	37	24	5	4a (W4)
W27	4	5	5	6	0	2	0	0	0	2	
HL-A 12	13	13	13	2	0	1	19	6	7	0	
W5	9	11	7	25	1	3	8	2	2	0	
W15	8	6	9	19	43	19	2	4	9	1	
W16	3	3	0	0	10	7	2	1	2	0	
W21	1	3	n.t.	0	0	0	2	1	1	0	
Blank	7	6	15	24	12	15	27	6	7	68	
HL-A 7	15	13	15	2	0	1	10	9	13	0	4b (W6)
HL-A 8	11	8	12	0	0	0	2	13	3	0	
W10	10	7	12	8	18	19	0	12	12	0	
W14	2	2	n.t.	0	0	0	5	3	0	0	
W18	1	5	n.t.	8	0	1	4	2	4	0	
W22	3	2	1	1	0	5	0	1	2	24	

Crossovers between MLC and Four have also been reported but good statistics are not available [27, 28, 29]. This makes it impossible to measure the distance between MLC and Four accurately but it is generally assumed to be of the same order as between Four and LA. The existence of the LA, Four and MLC loci can be considered to be established beyond doubt, proof for the remaining two loci AJ and the minor MLC locus is less firm. Because some crossovers will stimulate weakly a sibling differing only for a LA locus antigen, it is assumed that a minor MLC locus might lie between LA and Four [29]. The possibility has, however, not been excluded that the minor MLC locus lies in fact to the right of LA or, although less likely, that the weak stimulation is due to a locus not linked to HL-A. Crossovers between Four and AJ have not been published, but it is assumed that they are separate loci on the strength of what we would like to call molecular serology [30, 31]. Figure 2 explains the technique. To simplify matters we have taken as test cell one which is double homozygous, i.e. it received the same information for LA and Four from both parents, the antigens HL-A 2 and HL-A 12. We will disregard the products of the other loci for the moment. The test cell is incubated with anti HL-A 2 at 37°C. The antibody will attach to the antigens and agglutinate them. The antigens form a cap which can be made visible by rhodamine-labelled anti-γ globulin. Next the cell is cooled to 4°C, washed and incubated with anti-HL-A 12, and thereafter incubated with iso-

in Caucasians, Mongoloids, Negroids and Chimpanzees

AJ (3rd) locus				LA (1st) locus									Chimpanzees Φ
Caucasians Φ				Caucasians Φ			Mongoloids Φ			Negroids Φ			
SWI				DUT	GER	DAN	SMO	AYM	CHI	ZAM	KI	HK	
W20 (SA-AJ)	(T1)	3	HL-A 1	16	14	16	0	1	1	2	1	5	13
FJH* (170)	(T2)	7	HL-A 2	28	29	32	12	41	31	14	22	14	0
UPS	(T3)	19	HL-A 3	16	16	15	0	3	1	5	15	5	0
"W5"	(T4)	14	HL-A 9	11	11	11	54	12	17	11	17	17	0
TW	(T5)	6	HL-A 10	4	6	4	3	0	5	5	8	2	0
Pl.16	(T6)	7	HL-A 11	7	5	5	6	0	28	0	0	0	16
Blank		45	W19	7	12	11	16	39	16	43	32	46	24
			W28	6	4	4	0	4	0	9	5	8	1
			Blank	5	3	2	9	0	1	11	0	3	46

DUT = Dutch [34], GER = Germans [35], DAN = Danes [36], SMO = South Moluccans [37], AYM = Aymaras [38], CHI = Chinese [39], ZAM = Zambians [40], KI = Kung 1 [41], HK = Heikum [41], Chimpanzees [42, 43], SWI = Swiss [32].

Most of the data presented in this table were collected during the 5th Histocompatibility Workshop. The three series are presented in the same order as they are depicted in Fig. 1. In the Four series all "official" specificities are given but several antigens such as HL-A 12 and W15 can be split by appropriate sera. The antigen HL-A 9 can be split in W23 and W24, HL-A 10 in W25 and W26, and W19 in W29, W30 and W32.

thiocyanate-labelled anti-γ globulin. At 4°C the cell membrane is "frozen", and although antibody will attach to the antigen, capping will not occur. In contrast to the capped green HL-A 2, the red HL-A 12 is evenly dispersed over the cell membrane. The conclusion from this experiment is that the HL-A 2 and HL-A 12 specificities are carried by separate molecules and thus in all probability they are coded for by separate loci. In exactly the same manner this has been proven to be the case for the AJ and Four locus antigens [32]. It has been suggested that the different HL-A genes arose by gene-doubling although there exists no phylogenetic proof for this [33]. If this would be the case one would expect that there exists an equivalent of AJ near LA, but this has so far not been found.

The HL-A system is the most complex polymorphism known in man and Table 1 bears witness of that [32, 34–43]. As expected there exists a wide variation in gene frequencies between the Caucasian, Negroid and Mongoloid races, in that the blank gene, i.e. the gene which product cannot be detected serologically, is low in Caucasians for the LA and Four series (< 0.10) and not so high in the other racial groups especially if one takes into account the fact that only Caucasian antisera were used. Next to the short [44], subtypic [45] (or private [46]) antigens the HL-A molecule carries long, supertypic (or public) specificities of which 4a and 4b are the best known. It is likely that the 4a and 4b specificities are coded for by a mutational site in the Four locus which differs from the one which codes for the short specificities (Fig. 1).

Another explanation of the 4a and 4b data (which we do not favour) is that they are an extreme example of cross-reactivity.

Cross-reactivity occurs frequently in HL-A serology and complicates the interpretation of HL-A genetics. It implies that a monospecific antibody can react with cells which carry 2 or more different short specificities from the same series. The short specificities are included in the cross-reactive one. The first example was the inclusion of 7c in 6b [47]. Later many other examples were found, for instance it could be shown that 7c included HL-A 7, W22 and W27, while 6b included in addition to 7c, W10, and HL-A 13 [44, 48].

The recognition of the MLC locus determinants has recently become possible. In brief two approaches can be followed, in the first approach the basic principle is

Table 2

Typing for MLC (LD)
Typing cells homozygous at LA, Four and MLC (SD and LD)

Pattern No.	Responder	Stimulator	MLC reaction	MLC type of X
1.	X	a/a_m	neg.	a/a a/b or a/c etc.
2.	X	a/a_m	pos.	b/c etc.
3.	a/a	X_m	neg.	a/a
4.	a/a	X_m	pos.	a/b or a/c or b/c etc.

Result 1 + 3 X = a/a
2 + 3 should not occur
1 + 4 X = a/b or a/c etc.
2 + 4 X = b/c, d/e etc.
X = cell to be typed for the MLC determinants
a/a = homozygous typing cell
m = mitomycin treated
(van den Tweel et al. [51])

that if two (healthy) individuals do not stimulate each other in the MLC test, this implies that they carry the same MLC determinants. Together with Eijsvoogel we showed that in this manner it is possible to demonstrate the existence of MLC determinants also between unrelated individuals [49]. When cells homozygous for the entire haplotype are used, preferentially obtained from cousin-marriage offspring, the interpretation of these results becomes much simpler [50, 51] (Table 2). The number of alleles at the MLC loci is unknown. I have suggested

Table 3

Typing for MLC (LD)
MLC inhibition and immunofluorescence (Parous woman Sch.: HL-A 2, 3, 7, W10)

		AB serum cpm	Serum Sch. cpm	$\frac{\text{AB serum}}{\text{serum Sch}}$	Fluorescence pos. cells, per cent
Sch.+	P_m	3300	400	7.3	17
	Q_m	14000	8000	1.7	4
	N_m	9700	1100	9.5	17
	R_m	10000	6400	1.6	9
	S_m	4200	2800	1.7	7
	T_m	5200	700	7.4	7
	U_m	13500	1300	10.0	16
	V_m	26500	1100	24.1	17
	W_m	1800	200	10.0	n.d.

All stimulator cells were SD identical with the responder cells Sch.: HL-A 2, 3, 7, W10.
The column $\frac{\text{AB serum}}{\text{serum Sch}}$ depicts the inhibition index values obtained by dividing the cpm obtained by culturing the cells in AB serum by the cpm obtained when they were cultured in serum Sch. The significant inhibitions are underlined. See further text. (Van Leeuwen et al. [57])

previously that they might be low (4 – 6) and that they could be estimated from the fraction of negative MLC reactions between parent and child [52]. Eijsvoogel using a different approach comes to a similar conclusion [53]. So far the existence of 8 MLC determinants has been recorded, some of which might be identical [50, 51, 54 – 56]. In the second approach a sensitive serological technique (double sandwich fluorescence) is used. Sera are selected which are able to inhibit the MLC reaction between SD identical individuals of which the donor of the responder-lymphocytes is also the donor of the MLC inhibiting serum (Table 3) [57]. There exists a fair although not absolute correlation between MLC inhibition and the results of fluorescence. It is of interest that at most 25% of the lymphocytes from peripheral blood react with such antibodies. If only these cells are responsible for the MLC stimulation this may suggest that this stimulation was dependent upon the presence of a subpopulation of the mononucleated cells (S cells). This could be in agreement with findings in the mouse [58].

Although the unravelling of the polymorphism of the LD determinants thus seems to be possible, it is quite likely that the relationship between the different determinants will turn out to be a quite complex one, which is complicated further by the existence of pseudo-allelism.

Because crossover between the HL-A loci occurs rather frequently one would expect that over the generations the association of the alleles of these loci would have been randomized (or equilibrated). This, however, is not the case. On the contrary quite a few alleles of the LA and Four loci occur much more frequently together on the chromosome than could be expected by chance alone [59]. This is called linkage disequilibrium. In Caucasians the haplotypes HL-A 1 and 8, and 3 and 7 show the strongest linkage disequilibrium. In other races other alleles occur more frequently together on the chromosome. The mechanism by which linkage disequilibrium can be maintained is unknown. It could be a remnant of an early chromosome retained through phylogeny because it carries a selective advantage: the haplotype 1 – 8 is retained in the face of near 1% crossing-over between the loci coding for 1 and 8 because having the haplotype 1 – 8 entails an advantage in survival [60]. This in itself would be an indication that this genetic region must be of physiological importance.

II. The (patho)physiology of the HL-A system

An important difference between HL-A antigens and erythrocyte antigens is that the former are carried on cells with a nucleus and an active protein synthesis, which are lacking in the latter. As a consequence HL-A antigens (in all probability also *in vivo*) are continuously produced and shed in the serum where they can be serologically detected [61]. The best way to study this phenomenon is incorporation of radioactive amino acids *in vivo*, but most studies have been done using lymphocytes labelled with anti HL-A antibody, incubating them at 37°C, whereafter at different time intervals complement is added and the percentage of living cells is determined [62, 63]. Figure 3 shows that after 4 to 6 hours most cells will not be killed by the addition of complement alone [48]. Killing is, however, achieved if with the complement a fresh batch of antiserum is added to the cell.

Antibody elution seems to be an unlikely explanation of these findings and it is assumed that they are due partially to capping and pinocytosis and partially to a shedding of antigen-antibody complex while new antigen is formed. This receives further support from the observation that the supernatant of antibody-coated lymphocytes is able to inhibit MLC test possible through the above-mentioned antigen-antibody complex [64]. It is unknown whether the released antigen has a physiological role. Feldman et al. [65, 66] suggest that the antigen might have a function in blocking T lymphocytes possessing receptors for self-histocompatibility antigens, and thus prevent sensitization against self- and

auto-immune reactions against cells carrying self HL-A antigens. Although the biological function of the SD antigens and the LD determinants and the loci coding for them is still rather nebulous, important observations have been made concerning their function. Gorer and Schütze noted that some inbred strains of mice were far more susceptible to infections with Gross leukaemia virus than others [67]. Lilly et al. showed that this susceptibility was related to the presence, or absence, of a certain H-2 haplotype [68]. A haplotype is again defined as that part of the chromosome which codes for the major histocompatibility antigens, e.g. the H-2 system in the mouse. It is as yet not clear which factors link H-2 haplotypes to susceptibility to Gross leukaemia virus infections. This susceptibility is assumed to be under the control of the immune response or Ir loci.

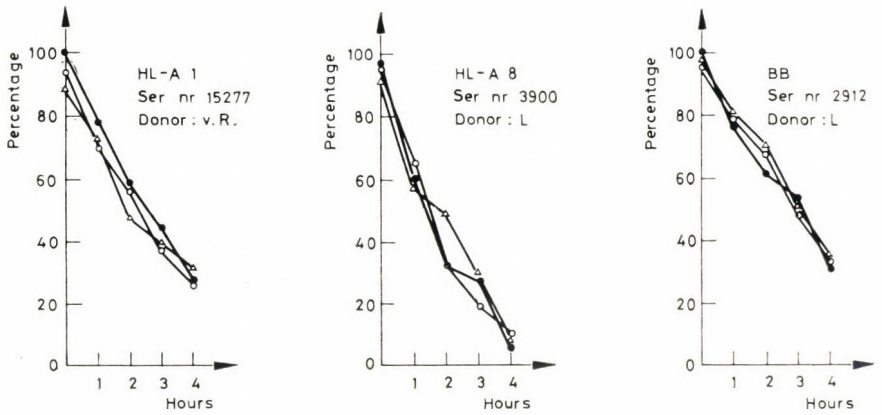

Fig. 3. Dynamic state of HL-A antigens on the lymphocyte membrane. Lymphocytes were coated with anti-HL-A antibodies, washed and incubated at 37°C. After 1, 2 and 4 hours, complement was added. Note that after 4 hours most cells were resistant to complement induced lysis. Reproducibility is excellent. BB equals W10 [48]

These Ir loci which were recognized by McDevitt and Benacerraf [69] and others determine the ability (or inability) of an individual to develop immunity against a number of well defined artificial and biological antigens. Some of the Ir loci lie in the direct neighbourhood of the loci coding for the SD antigens of the MHC and the *ad hoc* working hypothesis is that the human MLC loci (or loci very closely linked to them) are an equivalent of the immune response loci in the mouse. Most studies in the mouse have been done with artificial antigens and these have not yet been studied extensively in man. Preliminary findings in the rhesus monkey by Balner et al. [70] indicate that at least for (T, G)-A–L*

* A branched copolymer of polyalanine (A) and polylysine (L) with the addition of random sequences of tyrosine (T) and glutamic acid (G), a polypeptide with a molecular weight of ±150,000.

Table 4

Disease susceptibility and HL-A

Disease	HL-A antigen	N		Frequency in		Author
		pat.	contr.	pat.	contr.	
M. Hodgkin	4c Da5	41	44	.43	.24	Amiel [71]
	4c	35[1]	173	.56	.25	Forbes et al. [72]
		75		.45		
	W5	173	2000	.33	.18	Van der Does et al. [73]
	W5	158	273	.28	.09	Morris et al. [74]
SLE	W15	15	91	.46	.08	Waters et al. [75]
	W15	40	86	.40	.10	McDevitt et al. [76]
Hepatitis	HL-A 1	37	350	.60	.31	Mackay et al. [77]
	HL-A 8	37	350	.68	.18	
Gluten enteropathy	HL-A 8	49	268	.88	.29	Stokes et al.]78]
	HL-A 8	24	200	.88	.22	Falchuk et al. [79]
	HL-A 8	32	829	.84	.21	Van Hooff et al. [80]
Ragweed allergy	HL-A 7	18	77	.50	.20	Marsh et al. [81]
Dermatitis herpetiformis	HL-A 8	35	175	.60	.33	White et al. [82]
Psoriasis	HL-A 13	44	89	.27	.03	Russell et al. [83]
	W17	44	89	.23	.09	Russell et al. [83]
	HL-A 13	156[2]	386	.15	.05	White et al. [84]
	W17	156	386	.26	.08	White et al. [84[
	HL-A 13	102[3]	343	.17	.06	White et al. [84]
	W17	102	343	.30	.07	White et al. [84]
	HL-A 13	54[4]	43	.11	.02	White et al. [84]
	W17	54	43	.19	.16	White et al.]84]
Myasthenia gravis	HL-A 8	56	90	.38	.18	Fritze et al. [85]
	HL-A 8	100	533	.59	.19	Van den Berg et al. [86]
Ankylosing spondylitis	W27	75	75	.96	.04	Brewerton et al. [87]
	W27	40	906	.88	.08	Schlosstein et al. [88]
	W27	86	764	.86	.08	Keuning et al. [89]

[1] The total of 110 patients was investigated in two groups (35 = first series, 75 = second series)
[2] Caucasians
[3] Non-Jewish surnames
[4] Jewish surnames

the situation in primates might be comparable to that found in the mouse, i.e. that the capacity to respond to (T, G)-A – L is governed by a locus in the MHC region. Evidence that Ir loci might be operative in man does come from the observation that a wide variety of diseases occurs more frequently in individuals carrying certain HL-A, especially Four series antigens. Table 4 summarizes these findings.

Although Hodgkin's disease was the first condition in which a significant relation with an HL-A antigen (W5) was found [71–73], later studies have been able to confirm this only partly [74]. This may be due to the fact that susceptibility to Hodgkin's disease is not so much in linkage disequilibrium with the SD antigen W5 but to a LD determinant linked with W5 in some population. Now that LD typing seems to become possible it might be of interest to restudy these correlations. If the situation is still unsatisfactory for Hodgkin's disease and other malignancies, studied so far, HL-A association seems to be much more clear-cut for several illnesses which are either autoimmune (SLE, myasthenia gravis, gluten enteropathy) or allergic diseases such as ragweed allergy [75–89]. In the latter case the correlation might be again with the LD and not the SD determinants but can easily be detected because of the strong familial preponderance. In the first group the association with the SD determinants is in some instances so strong that one could wonder whether for instance the Bechterew-patients not carrying HL-A 27 are in fact suffering from a disease different from ankylosing spondylitis. The picture which emerges is that also in man the MHC loci are present which determine the immune response and through that the susceptibility to the disease. Although as yet unproven, one could envisage as a working hypothesis that (especially virus-induced) malignancies and allergies would tend to occur more frequently in individuals with certain LD phenotypes, while at least some autoimmune diseases are linked to SD phenotypes.

Elsewhere we have discussed homograft reaction and have proposed that the recognition of the foreign graft is primarily dependent on differences for the LD determinants but that graft destruction is primarily dependent on SD differences. Assuming the existence of a certain parallelism between the homograft reaction and the pathogenesis of these diseases, one may conclude that it would be of interest to investigate whether in autoimmune diseases, autoantibodies and/or autokiller cells specifically directed against the SD determinants are present [90].

If the striking homology between the HL-A and H-2 systems extends beyond serology, cellular interaction, role in the homograft reaction and immune response, in one word, susceptibility to disease, then it is to be expected that the same chromosomal region will also have its influence on morphogenesis. In the mouse, 15 crossover units to the left of H-2 is located the T-locus which controls the shape and/or presence of the tail [91, 92]. Amos and coworkers are studying the possibility that near to the HL-A supergene there might be a locus determining the occurrence of spina bifida occulta [18]. As yet unconfirmed findings suggest the occurrence of abnormal (over- or under-saturated) haplotypes in families in which cleft lip and palate occur [93]. In the mouse evidence has been presented linking this chromosomal region with the level of complement, testosterone, and even with the form of the mandibula [94].

III. Clinical relevance

A. *The HL-A system and the homograft reaction*

a) The problem definition

The overriding influence of the chromosomal region which codes for the MHC or HL-A in graft survival follows unequivocally from the better survival of grafts exchanged between HL-A identical siblings as compared to grafts exchanged between siblings differing for HL-A. This is true for all organs studied in man and in (random) bred animals, including bone marrow transplantation [95–101] (Table 5).

Table 5

The efficacy of HL-A matching in bone marrow grafting

	Bone marrow donors	
	HL-A identical	HL-A non-identical
Alive	6	2
Dead	5	14

In a world-wide survey it could be shown that bone marrow grafts from HL-A identical sibling donors are to be preferred when the immune potential in immune deficient children must be restored. Only cases in which a bone marrow take was certain or likely have been included. (From data collected and published by Buckley [95])

These findings constitute formal proof that compatibility for the chromosomal region which codes for the HL-A antigens is of decisive importance for graft survival. This implies also that it will be possible to solve the problem of donor–recipient matching between unrelated individuals. Now that there exists a clearer understanding of the genetic situation of the MHC in man it is at least possible to define a working hypothesis how the MHC influences graft survival, a working hypothesis which should be further studied and either confirmed or disproven. The problem can be defined as follows: The finding that HL-A identical sibling donor–recipient combinations enjoy the best graft prognosis does not imply that the serologically recognizable antigens are really the most important factors in determining graft survival.

It could well be that compatibility for another antigen, which is coded for by the same chromosomal region but which is different from the serologically definable or SD antigens, determines graft survival. As such could qualify the determinants which can be recognized in the mixed lymphocyte culture (MLC) test, the LD determinants. The question is then: *which antigens (the LD or the SD) trigger the homograft reaction and whether the same antigens which trigger this response are also the target through which graft rejection is effected.* Studies in

siblings will not be useful in answering this question because they are, as a rule, either identical for the four (or five) thus far recognized loci of the MHC or dissimilar for all four of them. Studies in unrelated individuals selected to be either identical at SD or at LD or both, may provide the answer.

In the analysis it will be necessary to differentiate between recipients who have been exposed to blood transfusions, pregnancy, etc. and those who have not. Such exposure can (but not necessarily will) alter the immune status of the recipient. If the immune apparatus of the recipient has been activated by previous blood transfusions or transplants etc., an organ graft will often be rejected more rapidly (second set). Such a recipient is called a "responder". The division between responders and non-responders in clinical organ transplantation is, as a rule, based on the presence or absence of lymphocytotoxic antibodies [102, 103].

This is, however, an oversimplification because it is certain that many recipients who did not form cytotoxic antibodies after immunization procedures, carried other types of antibodies such as those which can block the MLC test [104, 105]. It could be that such MLC blocking antibodies actually can enhance an organ graft. This has been discussed elsewhere [106, 107] but I want to stress that by using the presence or absence of lymphocytotoxic antibodies to differentiate between responders and non-responders I do not want to imply that these antibodies are responsible for actual graft rejection.

In the analysis it will thus be necessary to separate graft recipients who have received blood transfusions, have been pregnant, etc. and those who have not.

In the first group a distinction should be made between those who have formed antibodies and those who have not. Finally the effect of immunosuppression should be taken into account. I will not compare the homograft reaction with GVH disease in any detail. Whether the two phenomena are in fact related is largely unknown and disputed by many. This author is of the opinion that the major difference might be that in the first the reactive immune apparatus is large and the target small, while in GVH disease the reverse is true. For that reason, a better understanding of the the homograft reaction will amost certainly help us to understand and hopefully combat the GVH reaction.

b) Non-immunized recipients

Koch et al. [108] have shown that, if unrelated donor and recipient are SD identical but LD different (MLC-positive) skin graft survival is prolonged from 10 to 12 days (Table 6). When they are LD- and SD-identical skin graft survival is prolonged to 15.3 days. Sasportes et al. [109] and Ward and Seigler [110] found that, in the critical "SD-different, LD-identical" (MLC negative) group, skin graft survival was prolonged to about the same extent. In view of the small number of skin transplants performed in the two last combinations, these data should be regarded as preliminary, especially because the data by Sasportes et al. [109] and Ward and Seigler [110] were obtained not in unrelated donor–recipient pairs, but in parent–child and sib–sib combinations. If confirmed on a larger material they

Table 6

Skin graft survival and matching for LD and SD

	N	Mean skin graft survival	s.e.	Publication	Significance
LD ≠ SD ≠	24	9.96	17.8	Koch et al. [108]	0.2
LD ≠ SD =	14	11.86	.3	Koch et al. [108]	0.1
LD = SD ≠	6	17	12	Sasporte et al. [109]*	.04
				Ward Φ [110]	n.s. .03 .001
LD = SD =	4	15.25	2.3	Koch et al. [108]	

N = number of grafts, ≠ indicates non-identity; = identity. All skin grafts were exchanged between unrelated donor–recipient pairs except the (critical) LD = SD ≠ grafts which were exchanged between parent and child* and siblings Φ.

would suggest that in the non-immunized recipient skin graft surival is more prolonged by identity for LD than for SD. It could even be argued that the prolonged skin graft survival in the "LD-different, SD-identical" group was largely due to the fact that the LD differences were only slight and resulted in a MLC index of 3 to 8. A MLC combination taken at random would have an index up to 30. Ceppellini et al. [111], Jeannet [112] and Hamburger et al. [113] have also shown that skin, respectively, kidney graft prognosis in parent–child and sibling combinations correlated with MLC reactivity, i.e. the lower the MLC reactivity, the better was graft prognosis. Cochrum et al. [114] have confirmed and extended these findings in unrelated kidney donor–recipient pairs. Table 7 summarizes

Table 7

Kidney graft survival and matching for LD and SD
(unrelated donor–recipient pairs)

	N	Percentage of grafts functioning
MLC high, SD ≠	21	18
MLC high, SD =	3	(66)
MLC low, SD ≠	33	88
MLC low, SD =	2	(100)

N = number of grafts. MLC high implies a MLC stimulation index of 8 or higher, SD = implies identity for at least 3 HL-A antigens. (Modified from Cochrum et al. [114])

their observations. We have rearranged them in such a manner that they can be compared with the skin graft data.

Unfortunately, the MLC-high, SD-identical and the MLC-low, SD-identical groups are too small to allow meaningful conclusions but it is especially relevant that the MLC-low, SD-incompatible group shows such good survival. It can be concluded that SD identity, in many instances, is not necessary for good graft survival. Cases with lymphocyte antibodies were excluded from this material.

The most dramatic proof for the importance of matching for LD was obtained by a group of workers in Copenhagen who performed a successful bone marrow transplant in a child with combined immune deficiency [115]. The donor was an uncle who carried, thanks to a crossover event, the same LD determinants but different SD antigens as the child. The bone marrow graft took, the child recovered, there was no graft-versus-host disease. This case strongly suggests that in the face of true identity for the LD determinants between donor and recipient, differences for SD appear not to matter. On retrospect a similar case was also successfully transplanted in Minneapolis. Here SD non-identity was further complicated by ABO incompatibility [116].

c) Immunized recipients

Experiments in Rhesus monkeys [117] and in man [3] have shown that pre-immunization against a specific transplantation antigen will shorten the survival of subsequent skin grafts carrying that antigen. For instance, after intradermal immunization with buffycoat cells from a donor A, who carried the antigen

Table 8

Skin graft survival in HL-A 7 negative recipients after immunization with HL-A 7 positive leucocytes

Immunizing dose (i.d.)	Interval (days)	Survival of skin graft	
		HL-A 7 pos.	HL-A 7 neg.
1.2×10^8 leuc.	14	0	12
2.2×10^8	18	5	9
2×10^8	16	$5^1/_2$	$8^1/_2$
0.5×10^8	13	7	14
2×10^8	14	9	11
2.1×10^8	14	$9^1/_2$	$11^1/_2$
	Mean	6	11

Recipients were immunized by an injection of buffycoat cells from a donor A and after a 13—18-day interval challenged with a skin graft from an HL-A 7 positive and from an HL-A 7 negative donor B and C, unrelated both to donor A and the recipient (see further text) (van Rood et al. [3]).

HL-A 7 lacking in the recipient, skin grafts from an unrelated HL-A 7 positive donor would have a mean survival time of 6 days, whilst skin grafts from an unrelated HL-A 7 negative donor had a mean survival time of 11 days [3] (Table 8). Similar findings have been obtained for 4a, 4b, HL-A 2 [4] and HL-A 8 [118]. These results are the more striking because they were obtained in the early days

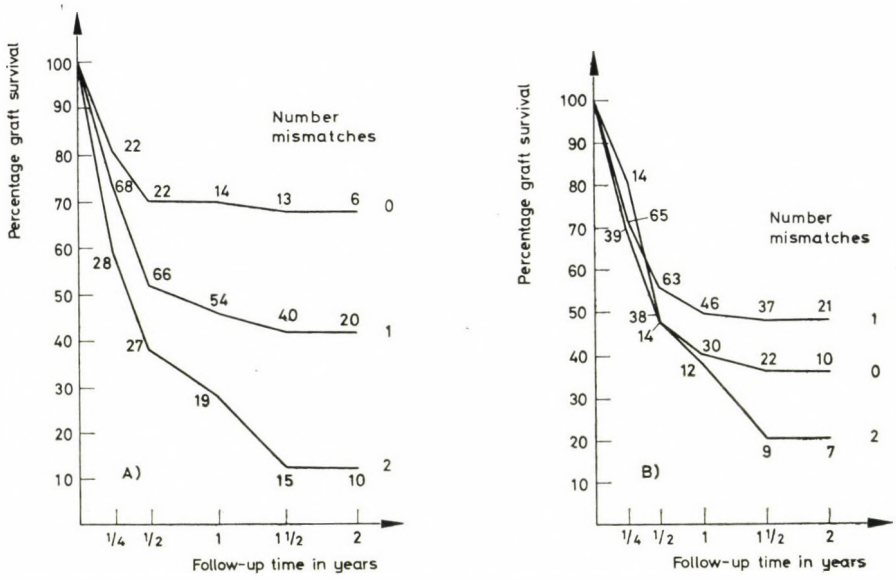

Fig. 4. Survival of 118 kidney grafts in recipients who had been immunized and had formed lymphocytotoxins in relation to the number of mismatched antigens of the Four (graph A) and LA (graph B) series. Note that there exists a highly significant influence if the grafts are matched for Four (70% survival at 2 years for the matched grafts versus 13% for grafts mismatched for 2 Four antigens). Matching for LA makes no significant difference [103]

of HL-A typing when we were able to recognize only part of the now-known HL-A antigens. Because, in almost each case, the graft carrying the antigen against which the recipient was immunized (HL-A 7, 4a, etc.) survived shorter than the control which did not carry that antigen, it seems likely that HL-A 7, 4a, 4b, etc. were the target for the skin graft rejection and not a structure which was in linkage disequilibrium with HL-A 7, 4a, 4b, etc.

It can be assumed that all combinations were LD-incompatible. These findings underline the importance of matching for SD in the immunized recipient. Data on the importance of LD matching in the immunized recipient are not yet available except for the HL-A identical siblings receiving a kidney graft in which survival is good, but this group cannot be compared to the unrelated donor–recipient combinations. In the immunized group of patients who received a graft with immunosuppression (kidney graft recipients with leucocyte antibodies) the

effect of matching especially for the Four locus antigens can be clearly demonstrated [103] (Fig. 4). Because of the linear correlation between the number of antigens mismatched and the percentage grafts surviving it is again highly likely that these antigens are the target through which the graft is attacked and not a structure coded for by a gene in linkage disequilibrium with the one coding for the SD antigens. For recipients immunized against SD and in need of a bone marrow transplant, matching for SD is also an absolute prerequisite because stem cells mismatched for SD will be destroyed before they can reach the bone marrow. Theoretically, one could try to define the specificity of the antibodies present in the recipient and attempt to select donors who carried SD antigens which do not react with these antibodies. In practice, this will be next to impossible because often these antibodies although quite effective in destroying transfused cells *in vivo* are weak and difficult to recognize *in vitro*.

d) LD–SD interaction in vivo and the allograft reaction

The data presented above can be summarized and interpreted as follows. In the non-immunized recipient or in the recipient who has received blood transfusions but did not form lymphocytotoxic antibodies, graft survival will be improved by matching for LD, which in this situation seems even more important than matching for SD. In other words, in the unprimed recipient or in the recipient who has been primed ineffectively (i.e. without the formation of cytotoxic antibodies or killer cells), matching for LD will improve graft survival, probably because the induction of homograft sensitivity develops more slowly or less effectively. This could imply that the presence of a foreign graft is primarily recognized by the non-primed immune apparatus of the recipient through differences for LD.

When such differences exist, the immune apparatus of the recipient will be activated and killer cells and/or antibodies will be formed. These killer cells and/or antibodies will attack the graft not primarily through differences for the LD determinants but through differences for SD which exist between graft and recipient.

This is borne out by the finding that, if the recipient has been immunized and donor and recipient are matched for the SD antigens, graft survival is significantly improved even if matching is only partial. In these considerations, the effect of non-HL-A systems has not been taken into account. It is possible that they form the target through which graft destruction takes place in SD identical donor–recipient combinations. In this context, it will be of interest to assess the role of the only non-HL-A system recognized so far in man, the group Five system [119].

The proposed mechanism for the homograft reaction shows a striking similarity with *in vitro* findings by Eijsvoogel et al. [53, 120] and Trinchieri et al. [121] in man and by Alter et al. [122] in the mouse. These authors studied the role of the LD and SD determinants in the cell-mediated lympholysis or CML test. They have concluded that activation of the killer cells takes place through

disparity at the LD loci, but that target cell destruction is only effectuated if killer and target cell differ for the SD antigens. The CML test might thus be a good *in vitro* model for the homograft reaction. Whether this LD–SD interaction reflects a T and B cell cooperation in the homograft reaction is unknown.

e) Role of LD–SD determinants in bone marrow transplantation

Recent publications bear witness to the fact that bone marrow transplantation can be quite successful in prolonging life in a significant manner or perhaps even curing the patient [123–126]. Nevertheless patients who received a bone marrow transplant because of a (combined) immune deficiency tend to do better than patients transplanted for aplastic anemia or leucaemia. This might be due to the fact that one problem in bone marrow transplantation does not enter in the transplantation of immune deficient children, i.e. giving an adequate but not too high dose of immunosuppression to permit the graft to take [123, 124]. A possibility which also could be considered is that such recipients have only few and almost certainly hypofunctional "passenger" lymphocytes and, for that reason, will activate the lymphocytes in the graft less effectively than would a recipient with a normal although suppressed immune apparatus. It might be worthwhile especially in the light of what has been said in the preceding chapter about the LD–SD interaction and because of the possibility that enhancement is due to anti-LD antibodies [107] to investigate whether passively transfused antibodies directed against the LD determinants of the recipient might mitigate the graft-versus-host (GVH) reaction. Preliminary observations by Buckley et al. using a serum that also contained SD antibodies suggest that this could be a worthwhile procedure [127].

It is clear that the late GVH reaction even when HL-A identical sibling donors are used is still a major problem. Of the many avenues which were recently suggested one should have high priority: the assessment of the role of non-HL-A systems. This point is especially relevant because one such system, the group Five system with two antigens (5a and 5b) which occurs on all nucleated cells studied thus far, can be recognized easily and adequately [119]. It is hoped that future bone marrow donors and recipients will be typed for these determinants.

That they cannot explain all GVH reactions in HL-A identical sibling situations is borne out by the fact that one of our patients died of chronic GVH after having received a transplant from an HL-A identical group Five identical sibling graft. Alloantigens present on epidermal cells only could be another system which would be worthwhile to match for [128, 129]. Even though there are still many problems when HL-A identical siblings are used as donors, this should not stop us to investigate whether unrelated donors can be used when HL-A identical siblings are not available. I am not referring to the use of HL-A mismatched donors under a regime of ALG as introduced by Mathé et al. [130], but to the LD-identical, and, if possible, SD-identical unrelated donor transplanted after cyclophosphamide immunosuppression. Identity for SD will be a prerequisite

in hypertransfused patients because, otherwise, the stem cells will be destroyed before they have a chance to settle in the marrow. For the time being, SD-identical unrelated donors will be used anyhow because only in this group one has an about 10% chance to find a donor who is MLC-negative and thus LD-identical with the recipient [49, 50]. One such bone marrow transplant has been performed, however, without success [131]. When LD typing becomes available as a routine procedure, large groups of potential bone marrow donors should be typed also for the MLC determinants. In analogy to what is done in kidney transplantation, this could probably best be realized under the auspices of an international organization (Europdonor) [132].

Experiments in primates will have to establish whether lethal GVH will not always occur in the presence of LD identity (even in the face of SD disparity) as suggested by the Copenhagen bone marrow transplant [115] and the mouse data reported by Bach and Bach [133]. The critical question is whether this will also be true if donor and recipient are unrelated.

B. *The HL-A system and blood component therapy*

Platelet and granulocyte transfusions are not much of a problem for a well organized bloodbank as long as the recipient is not immunized against the HL-A antigens. When the recipient is immunized against HL-A, only HL-A identical or compatible platelets will be efficacious. Fig. 5 illustrates this point [134, 135]. It depicts the case history of the first patient whose life was saved by HL-A matched platelets. She was suffering from life threatening thrombocytopenia due to chloramphenicol bone marrow aplasia and was treated by platelets from blood

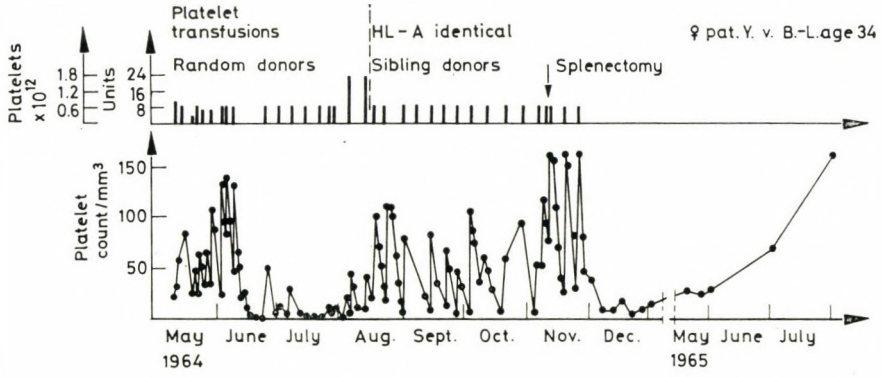

Fig. 5. Case history of a patient suffering from chloramphenicol-induced bone marrow aplasia. At first it was possible to elevate platelet count with platelet transfusions obtained from random donors (May to June). When the patient became immunized, the recovery of the transfused platelets was almost nil (June to August). Platelets from HL-A identical sibling donors showed again a good recovery. After splenectomy, recovery was almost twice as high when compared with the preoperative recovery [134].

transfusion donors taken at random. When the patient became immunized against HL-A, platelets from unselected donors were not effective any longer, but platelets from HL-A identical sibling donors were effective. After 6 months of supportive treatment with HL-A identical sibling donors, the patient recovered and is now living and well 9 years after her aplastic phase. This case history illustrates nicely one of the alternatives to bone marrow transplantation: supportive treatment with blood components. For life threatening thrombocytopenia, this is a realistic alternative, for neutropenia it is not. Progressive neutropenia (200–150 granulocytes per mm^3 and dropping) is thus also in our opinion an absolute indication for bone marrow transplantation.

The case history highlights also several of the problems in long-term blood component therapy. The most important ones are:

1. Donors, HL-A identical or compatible with the recipient, must be available; HL-A identical siblings are best [136], but HL-A identical unrelated donors can be used also although they are in our hands less satisfactory than the HL-A identical siblings [137]. This might be due to incompatibilities for only recently detected HL-A antigens such as those of the AJ series or non-HL-A antigens, especially those occurring only on platelets (such as P1A1 or Zw^a) [138]. For granulocyte transfusions granulocyte specific antigens are equally relevant [139].

2. Supportive treatment must often be continued for months, which is a heavy physical and psychological strain both on donor and bloodbank personnel. For that reason it would advantageous if frozen compatible platelets would be available [140].

3 Long-term supportive treatment can be realized much easier if donors, (triple) homozygous for the HL-A supergene (e.g. children from cousin marriages) who are compatible with all recipients, who carry the corresponding antigens, would be available. As universal donors do not exist for the HL-A system we have dubbed these (triple) homozygous donors "ideal donors". We have suggested that such donors should be registered in a central file (Europdonor) and their platelets could be regularly collected and frozen, solving in this manner at least one logistic problem in long-term supportive blood component therapy [132].

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Paternity Research Using the HL-A System

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Forty-seven cases of paternity research are reported. Paternity was excluded in 11 of these cases by the HL-A system (9 of the 11 were excluded also, on the basis of blood group systems). The technique of exclusion is discussed. In 36 cases, the paternity could not be excluded. We have presented a method for estimating the fraction of the population compatible with possible paternity so as to give an approximative evaluation of presumption of paternity. The limitation of such presumptive appraisals is discussed, as well as the genetic and serologic problems which have been encountered. Keeping these limitations in mind, the HL-A system appears to be a very precious tool in paternity research.

In a previous report, we have emphasized [9] the great interest of HL-A typing in 23 cases of disputed paternity. Since then we have studied 27 further cases. Of the total of 50, we shall now present and discuss 47 cases of paternity research.

Methods

The methods used for HL-A typing of the mother, the child and the presumptive father have been described in detail [9]. In brief, typing is done by a microlymphocytotoxicity method derived from the two-stage assay of Terasaki, using Falcon plates and pooled rabbit serum as source of complement.

The attention was given to the collection of blood samples, the identification of each individual and later of the lymphocytes which were obtained on Ficoll-triosil gradient and immediately frozen in liquid nitrogen.

Lymphocyte typings of the mother, child and presumptive father were studied at the same time, with the same antisera and the same batch of rabbit sera.

Reading of cytotoxicity was done between 30 and 60 minutes after complement had been added, independently by two highly trained technicians. A third reading was done after 18 hours, the plates being kept at +4°C.

The HL-A specificities which were tested with monospecific antisera were at least the following: HL-A 1, 2, 28, 3, 11, 9, 10, W.* 29, 32 and Da** 25 at

* W. = Workshop
** Da = Dausset

the first locus; and HL-A 5, 7, 8, 12, 13, 14, 17, 27, W. 5, 10, 15, 18, 21 and 22 at the second locus.

To the 10 specificities belonging to the first locus, another one must be added for taking into account a blank at this locus. We refer to it as x_1. To the 14 specificities of the second locus, we have also to add, for the same reason, another one; we refer to it as x_2. This implies that we can detect 165 haplotypes (11×15).

One blank means either homozygosity for the only detectable specificity at a given locus or the presence of an unknown specificity at the same locus.

Two blanks at a locus indicate either homozygosity for an unknown specificity or the presence of two unknown specificities.

In the cases studied recently, we have used a larger number of antisera allowing to detect additional specificities. This increases the selectivity of HL-A typing without modifying the general conditions.

We used well defined antisera and 1 to 3 of them for each HL-A specificity. The correlation between antisera of the same specificity was always very high (r between 0.85 and 1).

Interpretation of results

First, we shall consider the usual situation in which HL-A groups are studied. In a first step the phenotypes of mother and child are compared in order to deduce which specificity present in the child and absent in the mother were transmitted by the real father.

1. *The transmitted haplotype is known.* This is the case when mother and child have both 4 HL-A specificities expressed and when only 2 specificities are common; in such a case it is possible to ascertain which haplotype has been transmitted by the real father.

2. *Two kinds of haplotypes* could have been transmitted by the real father according to 2 situations:

First, when mother and child have three specificities in common, the fourth one being different.

Second, when the child has, besides two specificities shared with his mother, one not found in his mother and a blank. In this case, he may have received from his real father either the third specificity associated with an unknown specificity x, or the third specificity associated with one of the two specificities common with the mother.

For example: mother $a_1 n_1$, $b_2 n_2$
child $a_1 c_1$, $b_2 -$

The real father may then have transmitted either (c_1, x_2) or (c_1, b_2). In the first case, the child is heterozygous for b_2; in the second case, he is homozygous for b_2.

3. *Four haplotypes* may be transmissible in 3 situations.

First, when the child has three specificities in common with his mother and one blank. For example a_1b_1 and c_2 being present in the mother, the three specificities are detectable in the child. In such case, the true father may have transmitted either:

$$(a_1, c_2) \text{ or } (b_1, c_2) \text{ or } (a_1, x_2) \text{ or } (b_1, x_2).$$

Second, when the two specificities detectable in the child are those transmitted by the mother. For instance, the child is a_1-, b_2-, the mother being a_1n_1, b_2n_2.

The true father may have transmitted either (a_1, b_2) and the child is homozygous for this haplotype, or (a_1, x_2) (homozygous for a_1) or (x_1, b_2) (homozygous for b_2) or (x_1, x_2) the transmitted haplotype is made of undetectable specificities.

Third, a further possibility, still more exceptional, is the situation in which mother and child have four identical specificities, for instance a_1, c_1, b_2, d_2. The four haplotypes eventually transmitted by the real father are then (a_1, b_2) or (a_1, d_2) (c_1, b_2) or (c_1, d_2).

Knowing which haplotype or haplotypes are transmissible, one has to consider the specificities found in the presumptive father.

If only one haplotype is transmissble, the two specificities should be present in the phenotype of the presumptive father. Otherwise his paternity may be excluded.

If two or four haplotypes are transmissible, one of these haplotypes should fit with the phenotype of the presumptive father. Otherwise his paternity may be excluded.

The fact that a man has in his phenotype the specificities constituting the transmissible haplotype(s), does not mean that he is really the father. Thus, it is interesting to evaluate for the court the fraction of the population to which the man belongs and whose phenotypes are compatible with the hypothesis of paternity. The smaller the fraction of the population involved, the higher becomes the presumption of paternity for the man belonging to that fraction.

Calculation of the fraction of the population whose paternity may not be excluded

This calculation has to be performed for one or more transmissible haplotype(s). The method of calculation we have adopted to reach such an estimation has already been published [9].

Beginning with the simple case of only one transmissible haplotype, we have to keep in mind that if the two involved specificities are found in an individual, it does not necessarily mean that they are both on the same chromosome (in position *cis*); they may as well be on two chromosomes (in position *trans*). As it is

impossible to establish the genotype by serological reactions, the fraction of population to be taken into account for calculation is the totality of men having the transmissible specificities, including those having them in *cis* and those having them in *trans*.

It is preferable to use haplotype frequencies rather than phenotype frequencies, since there is an important linkage disequilibrium between many specificities; the informations given by haplotype frequencies are the more useful.

Haplotype frequencies may be found in tables as those published by Dausset et al. [2] or by Svejgaard et al. [14] or Mayr [4]. Some differences exist between these tables, but they are small and do not modify significantly the results of calculations which provide only an order of magnitude.

If the observed frequency of the transmitted haplotype is (a_1, b_2), the frequency of genotypes having it in *cis* is:

$$\text{F } cis = 2\,(a_1, b_2) - (a_1, b_2)^2.$$

The frequency of genotypes having a_1 and b_2 only in *trans* in the same population is:

$$\text{F } trans = 2\,(a_1, \bar{b}_2) \times (b_2, \bar{a}_1)$$

$(a_1, \bar{b}_2)$ corresponds to the totality of haplotypes containing a_1 without b_2, (a_1, x_2) being included. Similarly, $(b_2, \bar{a}_1)$ indicates the sum of all haplotypes containing b_2 without a_1, (x_1, b_2) being included.

The calculations are somewhat complicated if more than one haplotype is transmissible; however, the same type of calculation may be used:

For 2 haplotypes (3 specificities, ex. a_1, b_2, d_2)

$$\text{F cis} = 2[(a_1, b_2) + (a_1, d_2)] - [(a_1, b_2) + (a_1, d_2)]^2$$

$$\text{F trans} = 2(a_1, \bar{b}_2, \bar{d}_2) \times [(b_2, \bar{a}_1) + (d_2, \bar{a}_1)]$$

$(a_1, \bar{b}_2, \bar{d}_2)$ means the sum of all haplotypes having a_1 without b_2 or d_2.

For 4 haplotypes. For instance, the 4 following haplotypes may have been transmitted: (a_1, b_2); (a_1, x_2); (c_1, b_2); (c_1, x_2)

$$\text{F cis} = 2[(a_1, b_2) + (a_1, x_2) + (c_1, b_2) + (c_1, x_2)] - [a_1, b_2) + (a_1, x_2) + (c_1, b_2) + (c_1, x_2)]^2$$

$$\text{F trans} = 2[(a_1, \bar{b}_2, \bar{x}_2) + (c_1, \bar{b}_2, \bar{x}_2)] \times [(b_2, \bar{a}_1, \bar{c}_1) + (x_2, \bar{a}_1, \bar{c}_1)]$$

An example of real calculation for each of these three possibilities is given in the Appendix. Such calculations may sometimes be simplified if the mother's family has been typed; in such cases it is often possible to reduce the number of transmissible haplotypes.

On the other hand, it may be possible to strengthen the possibility of the presumptive father being the real one, if from the typing of his family we can demonstrate that he has the transmissible specificities in *cis* (see Table 6).

Within the whole population whose exclusion is impossible, only the fraction having the given specificities in *cis* can really be the father, as the fraction having them in *trans* cannot transmit these specificities in block to the offspring, with the exception of the very rare cases of recombination within HL-A loci (see Discussion).

Results

We shall successively consider 11 cases of paternity exclusion and 36 cases in which the paternity of the alleged father could not be excluded.

1. *Paternity exclusions*

In Table 1, 11 cases of paternity exclusion are summarized.

Nine of them are typical cases of exclusion of a man who could not be the father of the child. Two cases have to be considered separately.

Four of the first 9 cases were simple (Cases 22, 26, 31 and 36). Knowing the phenotypes of mother and child, only one haplotype was transmissible. This haplotype was defined by two specificities each at one locus. None of these two specificities were present in the presumptive father.

In Case 12, two specificities (HL-A 3 and 7) were coming from the true father. The phenotype of the presumptive father was 11, 9, 7, −, thus HL-A 3 was missing. This sufficed for excluding the paternity, but some doubt could have been raised due to the fact that HL-A 11 and HL-A 3 are cross-reacting specificities. In this case, however, the paternity could also be excluded by the erythrocyte groups.

In Cases 10 and 32, three specificities were involved, defining two transmissible haplotypes. The paternity could easily be excluded since none of these three specificities were present in the phenotype of the presumptive father.

In Cases 14 and 24, four specificities were involved (including x_1 or x_2). In both cases, three specificities among the four were lacking in the phenotype of the alleged father who could thus be excluded.

Cases 25 and 28 have to be considered apart.

In Case 25 (Table 1), we were asked to designate which one of two alleged fathers was the real father. Such appraisement is usually very easy with HL-A typing. The transmissible haplotype was *(W. 28, W. 5)*. One of these men had none of them and could safely be excluded. The other had these two specificities: his paternity could be considered likely.

In Case 28 (Table 1) we were asked to find out whether a man who wanted to marry one of his supposed daughters was in fact the father of the girl. Since the mother had died, we could not have any information about the haplotypes she has transmitted to her daughter. However, HL-A typing could establish that this man could not be the father either of this girl or of her sister; thus the marriage could not be forbidden due to paternity.

Table 1

Paternity exclusions

Case No.	Mother's phenotype		Child's phenotype		Haplotypes transmissible by the true father		Phenotype of the alleged father		
10	1,2	7,12	1, Da 25	12, —	*(Da 25, 12)*;	*(Da 25, x2)*	1,9	7,8	Excluded
12	11,Da 25	8,13	3, Da 25	7, 13	*(3, 7)*		11,9	7,—	Excluded
14	2,Da 25	Da 6,—	—,Da 25	Da 6,—	*(Da 25,x2)*;	*(Da 25,Da 6)*	1,9	8,—	Excluded
					(x1, Da 6);	*(x1, x2)*			
22	2,3	5,W.17	3,11	5,W.5		*(11,W.5)*	2,W.19	5,—	Excluded
24	1,3	5,W.10	—,3	5,W.10	*(3,5)*;	*(3, W.10)*	2,3	12,—	Excluded
					(x1, 5);	*(x1, W.10)*			
26	11,Da 22	12,W.27	1,11	7,W.27		*(1, 7)*	2,—	5,—	Excluded
31	2,3	W.15,—	1,3	W.15,W.17		*(1, W.17)*	3,9	W.5, 12	Excluded
32	1,9	12,13	1,—	8,12	*(1, 8)*;	*(x1, 8)*	2,9	W.15,W.10	Excluded
36	W.28,W.19	5, 12	W.28,3	5,Da 6		(3, Da 6)	9,10	8, W.10	Excluded
25	9,W.32	5,7	W.28,W.32	5,W.5		*(W.28,W.5)*	W.28,Da 22	12,W.5	Non excluded
							2, 9	W.15, W.10	Excluded
28		1st child .	.3,Da 25	7,W.18			—,—	W.5,W.15	Excluded
		2nd child .	.2,W.31	12,W.27					

Table 2

Non-exclusions: 1 transmissible haplotype

Case No.	Mother's phenotype		Child's phenotype		Haplotype transmitted by the true father	Phenotype of the alleged father		Frequency in the population of the involved specificities		
								total	*cis*	*trans*
34	2,9	12,–	2,10	12,W.14	*(10, W.14)*	2,10	13,W.14	0.0046	0.002	0.0026
2	10,–	W.27,–	10,W.28	W.14,–	*(W.28,W.14)*	9,W.28	5,W.14	0.006	0.002	0.004
43	10,2	5,W.22	W.31,10	5,W.10	*(W.31,W.10)*	W.31,9	W.10,W.15	0.0105	0.0088	0.0017
16	–,–	W.27,W.15	2,10	W.27,8	*(10, 8)*	3,10	7, 8	0.012	0.002	0.010
44	3,10	W.14,–	10,W.21	W.17,–	*(W.31,W.17)*	2,W.31	W.17,12	0.0132	0.0002	0.013
33	1,2	W.17,8	2,11	W.17,W.27	*(11, W.27)*	11,9	W.27,–	0.0138	0.008	0.0058
23	1,–	W.17,–	1,3	W.17,W.14	*(3, W.14)*	2,3	7,W.14	0.017	0.012	0.005
4	2,–	5,–	2,10	5,W.5	*(10,W.5)*	9,10	W.5,W.27	0.017	0.008	0.009
18	2,11	5,12	W.28,11	5,W.27	*(W.28,W.27)*	W.28,Da 25	W.27,–	0.021	0.016	0.005
17	11,Da 25	W.14,–	2,Da 25	W.22,–	*(2, W.22)*	1,2	W.17,W.22	0.022	0.006	0.016
3	3,9	Da 6,–	2,3	Da 6,W.27	*(2, W.27)*	2,–	W.27,–	0.05	0.034	0.016
42	9,–	7,12	2,9	12,W.15	*(2, W.15)*	2,W.19	W.15,–	0.052	0.04	0.012
21	1,2	5,–	1,3	W.5,–	*(3, W.5)*	3,–	W.5,–	0.061	0.042	0.019
30	1,W.28	W.15,W.17	1,2	W.5,W.17	*(2,W.5)*	2,3	W.5,–	0.075	0.0278	0.047
38	W.19,–	W.5,12	3,W.19	7,12	*(3, 7)*	3,9	7,12	0.096	0.0784	0.0176
6	10,W.28	W.18,W.27	2,10	12,W.18	*(2, 12)*	2,W.19	12,W.27	0.121	0.072	0.049
47	2,3	12,17	1,2	8,17	*(1, 8)*	1,10	8,–	0.134	0.130	0.004

Table 3

Non-exclusions: 2 transmissible haplotypes

Case No.	Mother's phenotype		Child's phenotype		Haplotypes transmissible by the true father		Phenotype of the alleged father		Frequency in the population of the involved specificities		
									total	*cis*	*trans*
46	W.29,Th.	14,12	W.29,Th.	W.5,14	*(W.5,W.29)*;	*(W.5,Th)*	W.29,—	W.5,12	0.03	0.007	0.023
13	1,—	8,—	1,Da 25	8,—	*(Da 25,x2)*	*(Da 25,8)*	1,Da 25	—,—	0.059	0.047	0.012
39	1,Da 22	5,8	1,10	5,—	*(10, 5)*;	*(10, x2)*	1,10	—,—	0.070	0.048	0.022
40	2,—	W.5,—	2,—	8,W.5	*(2, 8)*	*(x1, 8)*	2,—	8,W.5	0.101	0.028	0.0736
20	3,9	7,8	W.28,3	7,—	*(W.28, 7)*;	*(W.28,x2)*	1,W.28	5,—	0.112	0.069	0.043
35	W.31,—	—,—	W.31,—	12,—	*(W.31,12)*;	*(x1,12)*	W.31,—	12,—	0.135	0.10206	0.0327
15	1,3	W.17,7	1,9	W.17,—	*(9, W.17)*;	*(9, x2)*	3,9	—,—	0.171	0.130	0.041
1	1,10	5,8	9,10	5,—	*(9, 5)*;	*(9, x2)*	2,9	—,—	0.171	0.129	0.042
5	1,W.19	8,12	1,2	8,12	*(2, 8)*;	*(2, 12)*	1,2	12,—	0.177	0.082	0.095
8	1,11	W.17,7	1,3	W.17,—	*(3,W.17)*;	*(3, x2)*	2,3	—,—	0.184	0.120	0.064
7	1,—	8,—	—,—	8,—	*(x1, 8)*;	*(x1,x2)*	3,—	13,—	0.192	0.137	0.055
11	W.19,—	W.14,12	—,—	W.14,—	*(x1,W.14)*;	*(x1,x2)*	2,—	W.14,12	0.192	0.135	0.057
45	1,2	8,—	1,3	8,—	*(3, 8)*;	*(3, x2)*	3,W.19	12,—	0.193	0.120	0.073
19	1,3	W.14,W.15	2,3	W.15,—	*(2,W.15)*;	*(2, x2)*	2,W.28	—,—	0.366	0.283	0.083
29	2,9	W.15,—	2,—	—,—	*(2, x2)*;	*(x1,x2)*	2,9	W.5,—	0.407	0.352	0.055

Table 4

Non exclusions: 4 transmissible haplotypes

Case	Mother's phenotype		Child's phenotype		Haplotypes transmissible by the true father		Phenotype of the alleged father		Frequency in the population of the involved specificities		
									total	*cis*	*trans*
9	3,W.19	5,W.5	W.19,–	5,W.5	*(W.19, 5)*; *(x1, 5)*;	*(W.19, W.5)* *(x1, W.5)*	3,W.19	7,W.5	0.109	0.067	0.042
37	9,W.31	7,12	9,–	7,12	*(9, 7)*; *(x1, 7)*;	*(9, 12)* *(x1, 12)*	9,–	7,–	0.23	0.16	0.073
48	1,3	W.5,8	1,–	8,–	*(1, 8)*; *(x1, 8)*;	*(1, x2)* *(x1, x2)*	1,11	W.5,8	0.417	0.323	0.0936
27	2,10	5,W.10	1st child: 2,10	12,W.10	If the 2 children are of the same father, he may have transmitted *(2, 12)*, *(2,5)*, *(2, x2)* or *(x1, 5)* or *(x1, x2)*		2,–	5,12			
			2nd child: 2,–	5,–							

Among these 11 cases with paternity exclusion, the exclusion was confirmed in 9 cases by other blood group systems. But, in Cases 14 and 28, only HL-A typing could disprove the paternity (see Table 5).

Table 5

Case No.	Excluded by
10	ABO and HL-A
12	Duffy and HL-A
22	Rhesus, Duffy, MN, phosphoglucomutase and HL-A
24	MN. Ss and HL-A
25	ABO and HL-A
26	Rhesus, Gc and HL-A
31	Rhesus and HL-A
32	Haptoglobin and HL-A
36	Rhesus, haptoglobin, phosphoglucomutase and HL-A
14	only HL-A
28	only HL-A

11 cases of paternity exclusion
9 exclusions were made on the basis of both HL-A and blood group systems
2 exclusions were made on the basis of HL-A only

2. *Paternity non-excluded*

In 34 cases, the paternity of the presumptive father could not be excluded.

In 17 of these 34 cases, only one haplotype was transmissible (Table 2). We have calculated for each case the fraction of the population which, having the two involved specificities in its phenotype, could not be excluded. The 17 cases have therefore been listed in order of increasing frequency. These frequencies range from 0.0046 to 0.134.

It is obvious that the chance to find at random a man among the general population who could be the father is very small. In more than half of these cases, it was less than 0.022. In such cases, therefore, the presumption of paternity is very high. The presumption still remains high in the other cases and can be strengthened by the analyses of other blood systems which further reduce the fraction of population fitting with the paternity hypothesis. For instance, in Case 47, the fraction of the population which could not be excluded was relatively high due to the fact that the haplotype transmitted by the true father was a rather common haplotype (*HL-A 1, 8*), but the involved fraction of the population which was 0.134 using the HL-A system could be made smaller to 0.045, since only one third of the population was sharing the blood groups of the alleged father.

In 15 cases (Table 3), two haplotypes were transmissible; this of course increases the fraction of population which cannot be excluded. It ranges from

Table 6

	Father	Mother
Mother .... HL-A 1, —; HL-A 8, —	HL-A 3, —; HL-A 7, HL-A 13	HL-A 2, —; —, —
Child .... —, —; HL-A 8, —		
The real father may have transmitted (x_1, *HL-A 8*) or (x_1, x_2)	Presumptive father: HL-A 3, —; HL-A 13, —	His genotype is HL-A 3, HL-A 13 x_1, x_2.

Case 7

The study of the presumptive father's family strengthens the paternity of the presumptive father. He is not excluded and he has the haplotype (x_1, x_2).

0.03 to 0.407. These figures may also be reduced by combining the fraction of the population not excluded by erythrocytes, serum protein groups and HL-A groups.

In 4 cases (Table 4), four haplotypes were theoretically transmissible and the total fraction of the population which cannot be excluded is comprised between 0.109 and 0.417.

Discussion

We shall successively discuss

1. The serological reactions;
2. The genetic data (recombinations, probability to bear the specificities in *cis* or in *trans*, probability of homozygosity);
3. The frequency estimations of the involved specificities in the population;
4. The use of the HL-A system in disputed paternity (theoretical chance of exclusion and practical deductions).

1. *Serological reactions*

The assertion in paternity research by the HL-A system is that any antigens transmitted by the father may be recognized in the child. This implies that HL-A antigens are well-developed at the time of birth. This fact has been proved not only in mice [5, 6] but also in man: Seigler and Metzgar [7] have shown that HL-A antigens are detectable in the human foetus as early as the 6th week of intrauterine life and that the reactions are as strong during embryonic life as in adults.

A synergetic effect could be involved in HL-A typing when polyspecific sera are used, made functionally monospecific by dilution; thus one may postulate that the reaction could be different in father and in child for one same specificity due to the influence of the other haplotype coming from the mother. Such interferences are not found when using strong HL-A typing sera, and are further eliminated by using several sera from different sources for each specificity. Any discrepancy or doubt concerning one specificity in the alleged father and/or in the child requires the use of other test sera or excludes the possibility of conclusion.

In the course of 47 analyses we never had real difficulties. Of course, reactions with some antisera may be weak, or a positive reaction with an antiserum may be negative with another one of very close specificity. But such discrepancies between reagents were generally identical between lymphocytes of genitor and offspring indicating that these unusual reactivities were transmitted genetically; instead of weakening conclusions, the atypical reactions increase the probability of genetic transmission between people displaying these peculiarities.

Differences of reactivity between those homozygous and heterozygous for an antigen have been described by Ahrons and Thorsby [1], but they are rare and weak and do not interfere with the results.

Cross-reactions exist between some groups of antigens belonging to the same

locus but never between antigens of a different locus. For instance, they can be demonstrated between HL-A 3 and HL-A 11 or between HL-A 2 and HL-A 28; however, if the antisera have been chosen well, it will be easy to avoid this pitfall.

Some problems could arise in drawing blood of young or newborn infants. But due to the high lymphocyte ratio in childhood, 2 ml of blood is sufficient for typing. We have been obliged in only one case to use capillary blood, allowing a single typing without duplicate.

2. *Genetic background*

The genetic background is based on studies of an increasing number of families. Several thousands of Caucasian families have been tested allowing a precise genetic description of the HL-A system. The HL-A system has two closely linked loci each of them having a series of alternative codominant alleles. Due to the strong linkage between the two loci, the two specificities borne by the same chromosome are transmitted in block to the offspring. However, crossing-over within the two loci may occur, and their frequency is estimated at 0.008. These recombinations must not be forgotten when it is possible to demonstrate that a presumptive father has two involved specificities in trans.

The occurrence of a recombination must modify an exclusion based only on the localization in trans of the involved specificities.

At the time of our first publication, we were calculating the chance that a given presumptive father had to bear the two involved specificities rather in cis or rather in trans, by comparing the frequency of the different haplotype combinations in his phenotype as given by haplotype tables.

However, if the ratio of frequency of genotypes having specificities in cis over that having them in trans in the general population could be calculated easily, its reliability will be questionable for the appreciation in an individual case of the probability of paternity. The only safe way to know the genotype is to study the man's family.

We have also considered the usefulness of estimating the probability for a child with a blank at one locus to be homozygous for the single specificity expressed at this locus. Using the tables of haplotype or genotype frequencies, it is possible to calculate the chance of encountering such homozygous states. This incidence is high for some specificities of great gene frequencies (HL-A 2, 3, 12, etc.) and low for rare specificities (W. 16, W. 21, W. 22, etc.) but for most of them the probability is not far from 0.50 and so does not modify the calculation of the fraction of the population whose paternity cannot be excluded.

3. *Mode of calculation of the fraction of the population*

The mode of calculation of the fraction of the population bearing the specificities involved in each case of disputed paternity has to be discussed. If the haplotype frequencies are a far better estimation than the gene frequencies, we have

to keep in mind that only the haplotype frequencies of the given population to whom the alleged man belongs have to be taken into account. As already mentioned, the statistical differences between tables for populations of Caucasian stock are real, but they play a relatively minor role. Using two different tables, one may obtain a total frequency in the population of e.g. 0.2 with one table and 0.4 with the other one. But such a difference (1 *versus* 2) does not transform the order of magnitude with which the court has to deal. A more precise estimation has no real significance in an individual matter. The calculation established only the probability of exclusion of a man randomly chosen in a general population.

It is obvious that in an individual case the probability may be highly controversial, because the genetic presumption can be extended to the brothers, the father and the sons of an alleged father who have a high probability to bear the given haplotype. It may, however, be of interest for the court to know if the probability to find at random a man whose specificities are fitting with those of the child is 0.02 or 0.30; but even if this probability is only 0.001, it is important to point out that this probability is a genetic probability and that other kinds of information can formally exclude the paternity of the alleged father.

4. *Probability calculation in paternity exclusion*

We have not considered the chance of exclusion *a priori* for one or two specificities in the general population. Such calculations have been made by different authors for blood group exclusions and for HL-A exclusions [3, 4, 10, 11].

It is interesting to know the *a priori* ability of exclusion of one system compared to another one and to know that indeed the HL-A system alone gives an *a priori* chance of exclusion of at least 80% and that by combining all blood group systems, the chance to exclude *a priori* the paternity of a man taken at random exceeds 98%. But in a particular appraisement, these statistical considerations are of a limited value. What one has to appreciate in the knowledge of the HL-A groups of mother and child, is the presumption for a man (who is not excluded) to be the real father. In our series, the paternity of 11 among 47 men could be excluded. The value of such exclusion if serological reactions are clear-cut is the same as that of other blood group systems. The concordance was perfect and, in all cases but two, the exclusion was based also on erythrocyte groups. Of course, HL-A typing needs to be absolutely reliable. Obviously only few laboratories, highly specialized in HL-A typing and having a large battery of very well defined antisera, may give the necessary guarantee. When such conditions are fulfilled, the HL-A system appears to be a very precious tool in disputed paternity.

*

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APPENDIX

Examples of calculation of the fraction of population bearing the transmissible specificities.

1. ONE HAPLOTYPE TRANSMITTED

Case 44
Phenotype of the mother HL-A 3, HL-A 10; W.14, –
Phenotype of the child HL-A 10, W.31; W.17, –
The real father has transmitted: (*W.31*, *W.17*)
Phenotype of the presumptive father: HL-A 2, W.31; W.17, HL-A 12.
Having W.31 and W.17, his paternity cannot be excluded.
Fraction of the population having the haplotype (*W.31*, *W.17*).
Frequency of (*W.31*, *W.17*) :< 0.0001
F *cis* = $2 \times 0.0001 - (0.0001)^2 = 0.0002$
Frequency of haplotype (*W.31*, $\overline{W.17}$) = 0.1184
Frequency of haplotype (*W.17*, $\overline{W.31}$) = 0.0550
F *trans* = $2\,(0.1184 \times 0.055) = 0.013$

Total frequency of W.31, W.17 in the population = 0.0002 + 0.013 = 0.0132. Conclusions: the presumptive father belongs to 1.3% that of the general population which is statistically able to carry W.31 and W.17 in *cis* or *trans*.

2. TWO HAPLOTYPES TRANSMISSIBLE

Case 40
Phenotype of the mother HL-A 2, – ; W.5, –
Phenotype of the child HL-A 2, – ; HL-A 8, W.5
The real father may have transmitted (*HL-A 2*, *HL-A 8*) or (x_1, *HL-A 8*).
Phenotype of the presumptive father: HL-A 2, – ; HL-A 8, W.5.
His paternity cannot be excluded.
Calculation of the fraction of population which cannot be excluded having HL-A 2, HL-A 8 in *cis* or *trans*, or x_1, HL-A 8 in *cis* or *trans*.
Haplotype frequency (*HL-A 2*, *HL-A 8*) = 0.005
(x_1, *HL-A 8*) = 0.009
F *cis* = $2\,(0.005 + 0.009) - (0.005 + 0.009)^2 = 0.028$
Frequency of haplotypes (*HL-A 8*, $\overline{\text{HL-A 2}}$, $\overline{x_1}$) = 0.077
Frequency of haplotypes (x_1, $\overline{\text{HL-A 8}}$) = 0.171
Frequency of haplotypes (*HL-A 2*, $\overline{\text{HL-A 8}}$) = 0.307
F *trans* = $2\,[(0.077)\,(0.171 + 0.307)] = 0.0736$
Total frequency F *cis* + F *trans* = 0.028 + 0.0736 = 0.1016
Here the presumptive father belongs to 10% of the general population statistically able to carry HL-A 2, HL-A 8 and x_1.

3. FOUR HAPLOTYPES

Case 48
Phenotype of the mother HL-A 1, HL-A 3; W.5, HL-A 8.
Phenotype of the child HL-A 1, – ; HL-A 8, – .
The real father may have transmitted (*HL-A 1*, *HL-A 8*) (x_1, *HL-A 8*) (*HL-A 1*, x_2) or (x_1, x_2).

Phenotype of the presumptive father: HL-A 1, HL-A 11; W.5, HL-A 8.
Having HL-A 1 and 8, his paternity cannot be excluded.
Calculation of the fraction of population whose paternity cannot be excluded.
Haplotypes frequencies: $(HL\text{-}A\ 1,\ HL\text{-}A\ 8) = 0.067$
$(HL\text{-}A\ 1,\ x_2) = 0.039$
$(x_1,\ HL\text{-}A\ 8) = 0.009$
$(x_1,\ x_2) = 0.062$

$$F\ cis = 2\,(0.067 + 0.039 + 0.009 + 0.062) - (0.067 + 0.039 + 0.009 + 0.062)^2 = 0.323$$

Frequency of haplotypes $(HL\text{-}A\ 1,\ \overline{x_2},\ \overline{HL\text{-}A\ 8}) = 0.053$
Frequency of haplotypes $(x_1,\ \overline{x_2},\ \overline{HL\text{-}A\ 8}) = 0.109$
Frequency of haplotypes $(HL\text{-}A\ 8,\ \overline{x_1},\ \overline{HL\text{-}A\ 1}) = 0.015$
Frequency of haplotypes $(x_2,\ \overline{x_1},\ \overline{HL\text{-}A\ 1}) = 0.274$

$$F\ trans = 2\,[(0.053 + 0.109)\,(0.015 + 0.274)] = 0.0936.$$

Total frequency F *cis* + F *trans* = 0.0936 + 0.323 = 0.4166

The presumptive father belongs to 41.6% of the population which is able to carry HL-A 1, HL-A 8, x_1 or x_2 in 4 haplotype combinations.

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HL-A in Connection with Blood Transfusion

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The following aspects of HL-A, leucocyte- and platelet serology that may be of importance in blood transfusion practice are discussed: The preparation of leucocyte-free blood, in particular the new method by Diepenhorst et al. using filtration over cotton wool. Heteroimmunization in rabbits showed that in blood filtered immediately after withdrawal less leucocyte antigens are present than in leucocyte-poor blood prepared in any other way; the detection of non-agglutinating, non-cytotoxic antibodies; the full expression on the red cells of a normal donor of all the HL-A antigens of his genotype.

For many years our common interest in many aspects of haematology and especially immunohaematology, but also cordial ties of friendship, have brought us into frequent contact with Professor Susan R. Hollán. It is therefore not only an honour, but particularly a great pleasure for us to be able to participate in this special issue of Haematologia, dedicated to Professor Susan R. Hollán.

We have chosen the above topic for this paper, as HL-A and blood transfusion are among those subjects that are of common interest to Professor Hollán and ourselves. It is our intention to discuss some aspects of HL-A and leucocyte- and platelet serology in general, which in our opinion are of importance in the practice of blood transfusion.

I. The preparation of leucocyte-free blood

There are categories of patients in whom immunization against HL-A antigens or against leucocyte or platelet antigens, in general, must be avoided. Although Opelz et al. [11] have found that in transfused patients who have not formed cytotoxic leucocyte antibodies, kidney graft survival is significantly better than in non-transfused patients, they and others are of the opinion that immunization against HL-A antigens must be prevented in future recipients of kidney grafts. The two reasons are as follows. In the individuals who are immunized after blood transfusion, there is a chance that cytotoxic antibodies are formed with such a wide range of reactivity that no compatible kidney donor, i.e. a donor whose lymphocytes react negatively in the cross-match with the patient's serum,

can be found. Moreover, as further discussed under II, there are non-agglutinating, non-cytotoxic leucocyte antibodies which may be of importance for graft survival and which cannot be detected in the cross-match as it is done at present.

Immunization must also be avoided in candidates for bone-marrow transplants and in any patient of whom it may be expected that he will need platelet therapy during the course of his illness. These patients, should they need blood transfusions, should receive blood with as few leucocytes and platelets as possible. The following methods for the preparation of such blood have been in practice for some time.

Repeated centrifugation and after each centrifugation removal of the buffy coat by which 65–85% of the leucocytes are removed [2].

Dextran sedimentation which eliminates 70–95% of the white cells [10].

Freezing and thawing of glycerolized red cells; 85–100% of the white cells are removed, but leucocyte fragments may remain in the final product (*vide infra*) [9].

Recently, a new method has been developed in our Institute by Diepenhorst et al. [3a, b, c] using filtration through columns of cotton wool. This method already known in 1926 [6] is based on the capacity of cotton wool to absorb all forms of leucocytes,. By this method 98–100% of intact white cells are removed, but only part of the platelets. Slow centrifugation of the filtered product is necessary for removing these further. In our opinion it was of great importance to investigate whether filtered blood, in which no morphologically intact leucocytes are present, contains leucocyte fragments and/or soluble leucocyte antigens, as these might immunize the recipient.

We have studied this problem by heteroimmunization in rabbits, as this seemed the most sensitive method. Details of this study will be published elsewhere, but in brief the following results were obtained.

Preliminary experiments showed that when rabbits are immunized with 500,000, 1 million or 5 million leucocytes, leucocyte antibodies are formed in all animals. However, in the serum of rabbits injected with only 500,000 cells, leuco-agglutinins, but no lymphocytotoxins were demonstrable for quite some time during the course of the immunization and in some animals leuco-agglutinins were the only antibodies demonstrable, even after 8 injections. From absorption experiments with lymphocytes and granulocytes it became clear that in the serum of such animals granulocyte-specific antibodies are present, which do not react in the cytotoxic test with lymphocytes. Apparently, when relatively few cells or little antigen are injected, only granulocyte-specific antibodies are formed, indicating that granulocyte-specific antigens are the more immunogenic in the rabbit. For further experiments, the following component of filtered blood, in which no intact leucocytes were present, was prepared:

Red cells from filtered blood were resuspended in saline to a haematocrit of approximately 50% v/v and centrifuged at 625 *g* for 15 min. The supernatant was removed and centrifuged at 30,000 *g* for 120 min. The bottom 10 ml were removed and thoroughly mixed. This concentrated supernatant was used for further immunization studies.

The following results were obtained.

Rabbits injected with the concentrated supernatant prepared from blood filtered immediately after donation, formed only leuco-agglutinins, even after 8 injections, which were shown to be granulocyte-specific by absorption. All rabbits injected with the concentrated supernatant from blood stored 3 days before filtration, produced lymphocytotoxins also. These results indicate that, although leucocyte antigens are present in freshly filtered blood in which there are no intact leucocytes, they are in small quantity, as only granulocyte-specific antibodies are produced. In blood filtered after 3 days' storage, a larger quantity of antigen is present. As frozen/thawed glycerolized red cells are frequently used in patients in whom immunization against leucocytes must be avoided, because no or few morphologically intact leucocytes are present among such cells, rabbits were also immunized with the concentrated supernatant prepared from frozen/thawed glycerolized erythrocytes.

All animals produced both leuco-agglutinins and cytotoxins. The smallest quantity of leucocyte antigen seems to be present in blood which is filtered immediately after donation and it is therefore this blood that should be given to patients in whom immunization must be avoided. Whether such immunization does indeed not occur, can of course only be learned from the follow-up of patients treated in this way and in whose blood no leucocyte antibodies could be detected before the beginning of therapy. Investigations of this kind have been undertaken in our Institute. So far no immunization has occurred in the patients who only received blood filtered immediately after withdrawal.

II. Detection of non-agglutinating, non-cytotoxic antibodies against leucocytes or platelets

As we know from previous serological studies [4, 5, 10], both leucocyte and platelet antibodies may be of three serological categories, i.e. leuco-agglutinins, cytotoxic or complement-binding antibodies and antibodies that are neither agglutinins nor capable of activating the complement system: non-agglutinating, non-cytotoxic antibodies. The last category of antibodies may be demonstrated by means of the antiglobulin consumption test. This technique, however, is pretty well impossible with leucocytes, as nearly all antiglobulin sera are totally neutralized after incubation with leucocytes washed after incubation with normal serum. The test can be done on platelets, but it is an insensitive method, time-consuming and much blood is needed for one determination. Yet more and more need is felt for a convenient detection of non-agglutinating, non-cytotoxic antibodies. Platelet, and to a lesser extent, granulocyte therapy is increasing in clinical medicine. Since many recipients are immunized against platelet antigens, such as HL-A antigens, a convenient cross-match to select compatible donors would be of great importance. It is clear that such a cross-match, apart from the leuco-agglutination test and a cytotoxic or complement-fixation technique, must comprise a method for

the detection of non-agglutinating, non-cytotoxic antibodies. In many laboratories, methods for the preservation of platelets, also in the frozen state, are being evaluated. In order to test the efficacy of such platelets *in vivo*, the influence of immunological factors must be excluded. This can only be done if all possible kinds of platelet antibodies can be determined. Also, apart from these practical aspects, there are indications in the literature that non-agglutinating, non-cytotoxic antibodies are capable of blocking the activity of lymphocytes in the mixed lymphocyte culture. It will be most interesting to be able to detect these antibodies serologically, as among them there may be such as detect MLC determinants on the lymphocyte membrane.

Further, such antibodies may be of great importance in connection with the phenomenon of enhancement. The techniques that are being studied for this purpose in our laboratory by Dr. Francine Décary* are the mixed antiglobulin test, the immunofluorescent antibody technique and the immunofluorescent microphotometry technique, developed by van Boxtel [1]. Of these techniques, the mixed antiglobulin test seems at present the least promising. On lymphocytes this technique is difficult, as both the Ig receptor on B lymphocytes and the Fc receptor on part of the lymphocyte population interfere in the negative control and our results so far indicate that on platelets the technique is rather insensitive. The double sandwich fluorescence technique on lymphocytes, however, seems very promising. Although the Ig receptor on B lymphocytes gives a fluorescence of about 15% of the cells in this technique, the positive reactions are such that reading is not too difficult. Moreover, it was found that fixation of the lymphocytes with paraformaldehyde stops capping and shedding and gives an even fluorescence of the cell membrane.

It is already clear that with this technique strong positive reactions are found between serum/cell combinations which do not react in either the cytotoxic or the agglutination test.

Finally, the fluorescence microphotometry on platelets has been found to be a most sensitive method for the demonstration of platelet antibodies [1]. Its value for the above purpose has still to be evaluated.

III. The presence of HL-A antigens on the red cells of a normal donor

In connection with what is discussed under I, it is interesting to draw attention to the fact that not only leucocytes or platelets but, in rare cases, also red cells may be responsible for the formation of HL-A antibodies in a recipient.

Although HL-A antigens are thought to be lacking on human red cells, Harris and Zervas [7] demonstrated HL-A antigens on reticulocytes, but not on adult red cells. At first it was thought that while on tissue cells the HL-A antigens

* Medical Research Council Fellow (Canada).

are constantly renewed, this is not so on adult red cells, as the responsible messenger RNA is lacking in these cells. As certain HL-A antigens have now been detected on adult red cells, this theory is not tenable. A number of HL-A antigens have indeed been detected on adult red cells, either by a very sensitive method (autoanalyser) or by very special antisera. It has become clear that the red cell antigens Bg^a, Bg^b and Bg^c that are difficult to detect, are closely related to, or identical with, HL-A antigens. Recently, it has been found in our laboratory [8] that in exceptional cases all the HL-A antigens that are detectable on the lymphocytes, may be demonstrable on the red cells of a normal donor by means of common manual methods. The serum of a patient (vdK) gave a positive reaction in the indirect antiglobulin test with the red cells of donor O. The serum of this patient proved to contain leucocyte antibodies induced by previous transfusions, and reacted with 70% of the leucocyte samples tested. The patient's serum reacted with the red cells of one other donor out of 380. The cells of donor O. were then tested for the presence of rare red cell antigens and the serum vdK was tested with erythrocytes positive for rare blood group antigens. All reactions being negative and the cells of donor O. being positive for the antigens Bg^a, Bg^b and Bg^c, his cells were tested in the indirect antiglobulin test with the anti-HL-A sera of our cell panel. Positive results were obtained with the anti-HL-A2, anti-HL-A7 and anti-W10 sera and on HL-A typing of donor O.'s lymphocytes, his cells appeared to be positive exactly for these antigens.

Cross-absorption experiments showed his red cells to be capable of removing all anti-HL-A2, anti-HL-A7 and anti-W10 activity from the respective antisera, not only against his own red cells but also against his own as well as lymphocytes from other individuals positive for the corresponding antigens.

Finally, it was shown that the survival time of donor O.'s red cells in an individual whose serum contains anti-W10, anti-HL-A13 and probably also anti-HL-A7, was severely shortened. A biphasic survival curve was found. No immediate explanation for this phenomenon was evident, as IgG red cell antibodies capable of activating the complement system do not cause such curves. However, immunofluorescent studies with Mr. O.'s red cells showed that only part of his red cell population reacted with the anti-HL-A sera used, indicating an unequal distribution of HL-A antigens. This finding may explain the biphasic survival curve. It seems therefore that in this apparently healthy donor, there is an abnormal expression of the HL-A antigens on the adult red cells. Family studies gave no indication that this abnormal expression was genetically determined, although homozygosity for a recessive gene cannot of course be excluded. Immunization of rabbits with red cells from donor O. induced the formation of lymphocytotoxins, in contrast to immunization with red cells from an individual on whose erythrocytes the HL-A antigens were not so abnormally expressed. It is therefore clear that this phenomenon is of importance in connection with the immunization of recipients against HL-A antigens.

It even seems possible that a haemolytic transfusion reaction could occur when blood of a donor like Mr. O. is given to a patient whose blood contains

HL-A antibodies of a corresponding specificity, although a correctly performed cross-match should prevent such an unwanted reaction.

*

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Cross-Reactions of Mouse Anti-H-2 Sera with Human HL-A Antigens*

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Alloimmune pure anti-H-2 (anti-H-2.8, 37, 9) serum has been found to exert a strong cytotoxic reaction on human lymphocytes bearing the HL-A haplotypes in which the HL-A2 gene is involved.

Alloimmune mouse anti-H-2 sera were shown to exert a strong cytotoxic activity against human lymphocytes [4, 5]. In a previous experiment a battery of 22 alloimmune sera with anti-H-2 antibodies developed between H-2 congenic strains were tested against human lymphocytes from 115 unrelated Caucasian (French) individuals. The data were analyzed by the correlation coefficient (r) and a significant association was found between the reaction pattern of some mouse sera and the distribution of HL-A2 (MAC) antigen ($r = 0.30 - 0.40$). Absorption experiments with human thrombocytes indicated the specificity of the reaction. However, family studies failed to clarify whether all the specificities detected by the mouse sera segregated with the HL-A system.

The present paper reports on an analogous experiment performed on a lymphocyte panel from 258 unrelated Czech individuals. The confirmation of our previous finding complemented with preliminary family studies points to a possible cross-reaction of anti-H-2.8 with antigen HL-A2 (MAC).

Material and Methods

Anti-H-2 sera

The anti-H-2 serum tested (ASP-257) was prepared by intraperitoneal immunization with spleen, lymph node and thymus cells and collected 7 — 14 days after the last injection of a series of 10 injections (approximately one mouse donor per ten recipient) given at weekly intervals. The cell donor strain was B10.M ($H\text{-}2^f$); the recipients were $(B10 \times ASW)F_1$ hybrids ($H\text{-}2^b/H\text{-}2^s$). As strains B10.M and B10 are congenic strains differing at the H-2 locus only, the antiserum could contain only antigen-H-2 antibodies. According to the recently published H-2 chart

* This work is dedicated to Professor S. R. Hollán as a friendly remembrance of her successful effort to promote further cooperation in histocompatibility testing.

[1], the presence of anti-H-2.8, 37, 9 might be expected in this serum. Testing of a panel of inbred mouse strains actually showed the presence of anti-H-2 antibodies in accordance with the theoretical expectation.

Serology

The NIH recommended routine HL-A microlymphotoxicity technique was used; the tests were performed as blinds in the course of a routine large-scale screening programme for the detection of anti-HL-A specificities. The workers involved in the reading of the tests were not aware of the fact that mouse alloimmune serum was included between the human antisera. The mouse serum was tested at dilutions of 1 : 2, 4, 8, 16, 32. Those cells were considered H-2-positive where the reaction was strongly positive at two or more serum dilutions. Pooled normal mouse serum served as a negative control; these tests were regularly negative except for a few weak reactions. Absorptions of anti-H-2 sera were performed *in vivo*. Mice of the absorbing strain were injected intraperitoneally with 0.2 ml antiserum and bled 4 hrs later.

The lymphocyte cell panel

258 unrelated Czech individuals were tested; altogether 223 adults, of whom 25 were parents of tested children (one per family) and not included in the total number of unrelated adults and 75 children or adolescents younger than 20 years. In this panel of cells the distribution of 21 HL-A antigens (HL-A 1, 2, 3, 9, 10, 11, W28; 5, 7, 12, W5, W10, W14, W15, W16, W17, W18, W21, W22, W27) was known. The mutual relationship of the distribution of the HL-A antigens and the "H-2" antigen was tested by the correlation coefficient $\left(r = \sqrt{\frac{\chi^2}{N}}\right)$ with the help of a computer [2, 3]; $p < 0.01$ was considered significant.

Results

The anti-H-2 serum reacted positively with human cells in 64%. The reaction frequency was lower when the panel of cells from the 75 children was examined separately. The correlation coefficient was non-significant with all HL-A (and W) antigens tested except HL-A2 ($r = 0.49$; $p < 0.001$). The 2×2 table is given in Table 1.

Addition of cells from the subjects less than 20 years old was accidental due to the HL-A laboratory having tested a number of healthy children for other purposes. In these tests the reaction frequency of the anti-H-2 serum was significantly lower (45%) and the correlation with HL-A2 substantially higher than in the adults ($r = 0.75$, $p < 0.001$) (see Table 1).

Table 1

Cytotoxic reaction of anti-H-2 sera with human lymphocytes. Association with HL-A 2

H-2 antiserum	Donor mice strain	Recipients	Anti-H-2 specificity	Cytotoxic reactions on human lymphocytes								Statistical significance
				N	Reaction frequency (per cent)	2×2 associations****				χ	r	
						+/+	+/−	−/+	−/−			
AS-257	B10.M	(B10×A. SW) F_1	8, 37, 9	258*	64	111	53	15	79	63.958	0.498	yes
				223**	66	94	53	12	64	46.573	0.457	yes
				75***	45	30	4	6	35	42.526	0.753	yes
AS-285	B10.M	(B10×A) F_1	9	250*	34	52	32	70	96	8.649	0.186	yes

* Total number of unrelated individuals tested (adults and unrelated children).
** Total number of unrelated adult individuals tested
*** Total number of unrelated children tested (one per family).
**** "H-2 antigen"HL-A2 (MAC); individuals strongly positive in at least two dilutions of AS-257 were considered H-2-antigen plus.

Two informative families were also tested (Table 2). They were selected from a number of HL-A typed families and showed that the H-2 antigen segregates together with HL-A2.

Table 2

Family studies on the association of H-2 antigen with HL-A 2 (MAC) antigen on human lymphocytes

Family		HL-A genotype	HL-A haplotypes	H-2 antigen
A	Mother	3,7/1,8	a/b	−
	Father	$2,X_2/10,X_2$	c/d	+
	Child 1	$3,7/10,X_2$	a/d	−
	2	$3,7/2,X_2$	a/c	+
	3	$3,7/10,X_2$	a/d	−
	4	$1,8/10,X_2$	b/d	−
B	Mother	2,7/11,W15	a/b	+
	Father	$9,13/X_1,5$	c/d	−
	Child 1	2,7/9,13	a/c	+
	2	$2,7/X_1,5$	a/d	+
	3	$11,W15/X_1,5$	b/d	−
	4	$11,W15/X_1,5$	b/d	−
	5	$2,7/X_1,5$	a/d	+
	6	2,7/9,13	a/c	+
	7	$11,W15/X_1,5$	b/d	−
	8	2,7/9,13	a/c	+
	9	$11,W15/X_1,5$	b/d	−

Earlier absorption experiments of H-2 sera with human thrombocytes [5] indicated that human platelets are capable of a specific absorption of antihuman specificity from alloimmune mouse sera. In preliminary tests, absorption of the H-2 serum with donor mouse (immunizing) cells removed both the anti-H-2 and the antihuman specificities (Table 3).

Preliminary studies along the same line were performed with some other alloimmune mouse sera. The results were analogous to those obtained in our previous study [5], i.e. none of the further sera gave a significant correlation with HL-A2. Some correlation of a lower order appeared with other HL-A antigens; these results will be reported separately.

The reaction pattern of one other serum (ASP-285) seems noteworthy. This serum was in its composition analogous to the serum reported in detail in the present work, but its H-2 specificity was more narrow. The donor mouse strain was again B10.M, but the recipients were $(B10 \times A)F_1$ hybrids ($H\text{-}2^b/H\text{-}2^a$). Thus, only anti-H-2.9 could be and actually appeared to be present. This serum (with a reaction frequency of 34%) too showed a significant correlation with HL-A2, but the number of discrepancies was much higher ($r = 0.18$). The correlation between

Table 3

Absorption on anti-H-2.8, 37, 9 (ASP-257) serum

Human lymphocytes	Anti-H-2 serum No. 257 (anti-H-2.8, 37, 9)																	
	Unabsorbed						absorbed on mce^s train											
							B10						B10.M					
	2	4	8	16	32	64	2	4	8	16	32	64	2	4	8	16	32	64
HL-A2 −	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
HL-A2 −	++++	+++	++	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
HL-A2 +	+ ++	++++	++++	+++	+++	++	++	+	−	−	−	−	−	−	−	−	−	−
HL-A2 +	+±++	++++	++++	++++	+++	++	+++	++	+	−	−	−	−	−	−	−	−	−

the two anti-H-2 sera, i.e. anti-H-2.8, 26, 9 and anti-H-2.9, was, however, significantly positive ($r = 0.30$). The second (shorter) serum was basically but not conclusively included in the first (longer) serum.

Discussion

Typing of a panel of 258 unrelated Czech individuals with an alloimmune pure anti-H-2 (anti-H-2.8, 37, 9) serum has confirmed our previous finding of a significant correlation with HL-A2 observed in a French population [5]. Absorption experiments and family studies allowed the conclusion that antibodies present in this serum cross-reacted with human cells bearing antigen HL-A2.

The nature of this cross-reaction (1) may be due to biochemical similarities of HL-A2 and H-2.8 (37, 9), i.e. of the products of $H\text{-}2^f$ haplotype and HL-A2 antigen; (2) or this similarity concerns gene products closely linked with HL-A2.

The absence of a closer correlation in the population study may have been due to a number of mutually not exclusive situations, such as

1. differences in the composition of HL-A2 on different haplotypes;
2. or the cross-reaction was due to a gene closely linked with HL-A2 displaying a strong but not absolute linkage disequilibrium with HL-A2;
3. differences in the expressivity of HL-A and/or of the unknown closely linked gene product;
4. cross-reactions with some non-HL-A antigen(s). The expressivity of this non-HL-A antigen(s) might strongly be influenced by the haplotype marked by the gene for HL-A2. A model of this situation has recently been described in mice [6].

The third of the listed possibilities is of a special interest in the light of the finding that the correlation of H-2 with HL-A2 was closer in children than in adults. As a working hypothesis it is assumed that physiological factors such as the hormone level or its cycles may influence the expressivity of the HL-A2 associated human "H-2" antigen in lymphocytes. We also incline to believe that the cytotoxic reaction is not due to the HL-A2 gene product itself but is directed against or influenced by other closely linked genetic components of the human major histocompatibility system (MHS). The role and significance of this extraordinary human polymorphism detected by anti-H-2 serum must be tested in a number of different situations in which histocompatibility is known to play a major role. The understanding of the described phenomenon might lead to the elucidation of the evolutionary aspects of the MHS.

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Effectiveness of Rh Prophylaxis

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It gives me very great pleasure to know that in this celebration volume to Dr. Susan R. Hollán there is a paper from the Department of Medicine in Liverpool University, for it was here that the original work on the prevention of Rh haemolytic disease was carried out.

Much water has flowed down the Mersey since Finn (1960)[1] first put forward the suggestion of how prophylaxis might be effected, and it has given me very great satisfaction to see how in most parts of the world where Rh haemolytic disease is a problem the treatment is being carried out, not least by Dr. Susan R. Hollán and her colleagues in Hungary.

The paper by Dr. J. C. Woodrow assesses the present situation of anti-D therapy in many parts of the world.

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[1] Finn, R., *Lancet*, *1*, 526. (1960)

When experimental studies had shown that it was possible to prevent Rh immunization by administering IgG anti-D and clinical trials were started, it was not anticipated that complete success would be achieved. The degree of optimism about the likely results of giving anti-D postpartum depended on how important one thought primary immunization to be during the months of pregnancy, for it seemed likely that primary immunization occurring as the result of a stimulus during labour should be entirely preventable but priming during pregnancy might not be. The use in recent years of Rh prophylaxis on a wide clinical scale makes possible an assessment of the degree of success which can be achieved. When one looks at the results reported from several centres throughout the world two main questions can be asked. Firstly, what is the general effectiveness of the prophylactic treatment? Secondly, are there any important differences from one centre to another and, if so, what is their significance?

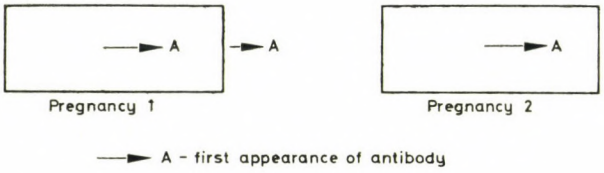

Fig. 1. Sequence of two Rh-positive pregnancies, e.g. the first and second, with the times at which antibody commonly appears for the first time

Before looking at the actual data it is profitable to review briefly the picture of Rh immunization as it occurs in the absence of treatment and to relate tests for antibody before and after treatment to this. Figure 1 shows a sequence of two Rh-positive pregnancies which might, for example, be the first and the second. Antibodies may appear in the first pregnancy and in a proportion of cases there may have been a previous Rh-positive transfusion or an abortion. Hindemann [1] has put forward evidence to suggest that in some instances there has been priming of the Rh-negative mother when she herself was in utero, resulting from materno-foetal transfusion from her own Rh-positive mother. However, in some instances primary immunization during the first pregnancy may be responsible. We might also expect that some mothers may have become primed before delivery but without antibodies being detectable. The actual figures for the incidence of antibodies during this first pregnancy vary from centre to centre and it is likely that the methods of antibody testing used could have an appreciable effect on the inclusion of cases in a clinical trial. A very weak antibody might be detected at one centre and the mother excluded from a trial but missed at another centre, the mother being treated, with the likelihood of failure resulting.

Antibodies may of course appear in the months subsequent to delivery, being about ten times more frequent where this first pregnancy is ABO-compatible than where it is incompatible. One common problem in treated women is that of

distinguishing active from passive antibody at this time. Although the T½ of IgG anti-D is of the order of 26 days, there is some evidence of considerable variation from individual to individual and what is later decided to have been passive antibody may be found by sensitive methods six months after treatment with 100–200 μg antibody. Clearly, differences in the way in which these antibodies are tested for, assessed and recorded, may make an appreciable difference when comparing one centre with another. When weak enzyme active antibodies are found it is only by a long follow up that a firm decision as to their significance can be made.

Antibodies may appear for the first time during the second pregnancy. Comparison of the incidence in untreated mothers with that for the first pregnancy strongly indicates that in most instances we are dealing with a secondary response, priming having occurred as a result of the first pregnancy. For example, our own studies on untreated mothers in Liverpool showed that 0.95 per cent of mothers developed antibodies during their first Rh-positive ABO-compatible pregnancy and that approximately 10 per cent did so during the second Rh-positive ABO-compatible pregnancy. Assuming that the incidence of primary antibody formation in the two pregnancies were the same, the marked excess of antibody development during the second pregnancy must have been due to mothers being primed by the first pregnancy but showing no detectable antibody during the first part of the second pregnancy. A small foeto-maternal transfusion during this second pregnancy would then lead to a secondary response with antibody becoming detectable. However, some instances of antibody development during the second pregnancy are likely to be due to a primary response and it is not to be expected that these would be preventable by anti-D given after the first pregnancy. Differences in the interpretation put on the appearance of antibody during the pregnancy subsequent to treatment may occur between different centres.

A detailed account of the appearance of antibodies at this time in untreated mothers was given by Bishop and Kreiger [2] who described two main patterns of response. In one there was the late development of an antibody active only by enzyme techniques with a negative direct Coombs test on the baby. At the other end of the scale there was the earlier development of an antibody, positive by antiglobulin methods, and often associated with a positive direct Coombs test on cord blood. It seems likely that the first pattern is typical of a primary response and the second of a secondary response. However, there is some doubt that one can always be dogmatic in separating these two patterns because the responsiveness of Rh-negative individuals is very variable.

Two other points are worth noting. Apart from differences in the routine dose of anti-D used in the trials, it is often the case that where an unusually large foetomaternal transfusion is detected, a dose of anti-D much larger than the standard one for the trial is given. The extent to which such cases are ascertained and the technical methods used to detect them vary from centre to centre. This may have an influence on the results of a clinical trial. Secondly, there is the possibility of differences in the natural history of Rh immunization from one area of the

world to another. For example, the incidence of antibody after ABO-compatible pregnancies appears to be lower for West Germany (4.9%) [3] and Finland (4.3%) [4] than that found in other Western countries where the incidence is of the order of 8%.

The possible variables to be considered in assessing the results of clinical trials are listed in Table 1.

Table 1

Variables associated with Rh trials

a) *Selection of cases*
   1) ABO-compatibility
   2) Parity
   3) Antibody tests before treatment

b) *Tests for immunization after treatment*
   1) Passive—active
   2) In subsequent pregnancy

c) *Dosage*

d) *Variations in natural history (?)*

Results

Antibodies in the post-delivery period

Table 2 gives the results for controlled clinical trials, based on tests for antibody in the months subsequent to treatment. The overall picture is of a 95 per cent reduction in the incidence of antibodies in treated mothers as compared to controls, i.e. a 5 per cent failure rate. Notable is the absence of failures in the Canadian trial. It is this group [5] which reports a relatively high incidence of antibodies at the end of the first pregnancy, often detectable only by sensitive enzyme methods and it is reasonable to speculate that instances of weak primary immunization are being detected and excluded from the trial. There is a suggestion in the Finnish data that treatment after ABO-incompatible pregnancies is relatively less successful but this has not been found by other groups.

Table 3 gives the corresponding results for uncontrolled trials. The overall incidence of antibodies six months after treatment is approximately 0.4 per cent.

Antibodies in the subsequent pregnancy

In Table 4 are shown the results of testing for antibody in the Rh-positive pregnancy subsequent to treatment in those trials where controls have been used. The overall incidence of antibodies in treated mothers is approximately 9 per cent of that in controls.

The corresponding results for the uncontrolled trials are shown in Table 5. The average incidence of antibodies is 1.6 per cent. Taking into account the

Table 2

Rh
Controlled clinical trials
Results of tests in months after delivery

Centre	Controls			Treated		
	No.	Anti-D present	Per cent with anti-D	No.	Anti-D present	Per cent with anti-D
Liverpool Group						
1000 μg trial	176	38	21.6	173	1	0.58
200 μg trial	362	13	3.6	353	0	0.0
United States	1476	102	6.9	3389	6	0.18
West Canada	500	36	7.2	2247	0	0.0
Holland	329	17	5.2	1563	7	0.45
West Germany	2458	96	3.9	3091	15	0.49
Finland						
ABO compatible	792	34	4.3	9569	12	0.13
ABO incompatible	220	1	0.5	3151	5	0.16
Sweden	595	25	4.2	2214	8	0.36

Table 3

Rh
Uncontrolled clinical trials
Results of tests in months after delivery

Centre	Total tested	No. with anti-D	Per cent with anti-D
Liverpool*	980	4	0.4
Ministry of Health (England and Wales)	5693	26	0.46
Canada (low protein)	677	2	0.3
Australia	3706	16	0.42
Edmonton	574	3	0.52
Belgium	1548	5	0.32

* Total experience with treated mothers (includes results of controlled trial)

Table 4

Rh
Controlled clinical trials
Results of tests in subsequent pregnancy

Centre	Controls			Treated		
	No.	Anti-D present	Per cent with anti-D	No.	Anti-D present	Per cent with anti-D
Liverpool						
1000 μg	65	20	30.7	88	2	2.4
200 μg	127	13	10.2	128	3	2.3
United States	179	24	13.4	438	5	1.1
West Germany	373	29	7.8	138	0	0.0

Table 5

Rh
Uncontrolled clinical trials
Results at end of subsequent pregnancies

Centre	Total tested	No. with anti-D	Per cent with anti-D
Liverpool*	439	7	1.6
Holland	98	2	2.0
Australia	1218	13	1.1
Canada	483	8	1.7
Finland	1027	10	0.97
Sweden	75	1	1.3
S. Africa	587	15	2.5
Leeds	437	7	1.6
St. Louis	240	3	1.3

*Total experience with treated mothers (figures include those for controlled trial)

variation in the selection of mothers for the trials, this represents a little over 10 per cent of the expected incidence.

Bowman [6] has suggested that it is likely that all the antibodies appearing in pregnancies subsequent to treatment in the Canadian trial are examples of primary responses but there is perhaps room for some doubt about this. In their analysis of the Finnish data, Eklund and Nevanlinna [4] estimated that of the 1 per cent of mothers developing antibody during the subsequent pregnancy, 0.35 per cent might be a primary and 0.65 per cent a secondary response (this assumes that the incidence of primary responses was the same in this subsequent second as in the first pregnancy, i.e. 0.35 per cent). In Liverpool 1.2 per cent of

mothers developed antibody during the pregnancy subsequent to treatment, and of these it is estimated that something like 0.5 per cent were primary and 0.7 per cent were secondary responses.

It is not easy to determine with certainty the causes of the apparent differences between centres. It is to be doubted whether differences in dosage have had a very marked effect on the results. In most centres 200 – 300 μg anti-D was being given. Sometimes an increased dose was given where unusually large foetomaternal transfusions were detected and as mentioned above this might have some relevance in the context. In some instances of apparent failure a comment was made that there was delay in administering the anti-D. It is possible that where a substantial stimulus has occurred and the mother is very sensitive, the time factor may be important.

In the series from West Germany there is included a proportion of mothers given anti-D immunoglobulin by the intravenous instead of the usual intramuscular route. Schneider [3] reported that 4 of 1032 (0.39 per cent) mothers treated by intravenous anti-D developed antibody compared to 10 of 1444 (0.69 per cent) given anti-D intramuscularly. Of a series of 3697 mothers given anti-D intravenously in doses varying according to the foetal cell count after delivery, two were reported as immunized during the subsequent months [7]. There is thus a suggestion of a somewhat lower failure rate when anti-D is given intravenously but because of the difficulties mentioned above in relating results from different centres, this question can only be answered with a degree of certainty by a well controlled trial carried out at one centre with the aim of comparing the two methods of administration. If intravenous treatment is somewhat more effective in some instances it might be in mothers who have been weakly primed just before delivery and who then received a further stimulus during labour. The very rapid build up of antibody in the tissues might be crucial in such a situation.

In those centres where foetal cell counts after delivery are done routinely, it has been observed that in most instances of failure of prophylaxis the volume of foetomaternal transfusion has been quite small or not detectable. In some of these mothers there might have been a delayed appearance of foetal cells in the maternal circulation consequent on absorption from the peritoneal cavity but it is likely that this only applies to a proportion. Thus inadequate dosage of anti-D is unlikely as the explanation in most instances of failure. More likely is that a primary immune response has been initiated during the weeks prior to delivery.

Because of this, trials of antepartum therapy with anti-D are being conducted in some countries. Table 6 shows some recent results from Canadian and Australian trials.* Although in the Canadian trial three control mothers had developed antibody by delivery, presumably there was passive antibody at this time in some of the treated mothers, and this would make it very difficult to know whether any of these mothers had been actively immunized. These trials are inconclusive and it is going to take a very large series with follow-up through subsequent pregnan-

* I am indebted to Dr. J. Bowman and Dr. M. G. Davey respectively for these data.

cies before a final answer is possible. Should a reduction in the failure rate be shown to result from this form of therapy it is still rather doubtful whether the practical problems involved will justify it as a routine measure.

Table 6

Antepartum anti-D

Canadian trial

300 μg anti-D at 28 and/or 34 weeks and postpartum
Primiparae — 340 tested 6—9 months after delivery
(Rh + infants)

— None immunized
324 mothers given postpartum anti-D only
— 3 had antibody at delivery
— no others immunized

Australian trial

250 μg anti-D (x1 or x2) in last 12 weeks and postpartum
— 364 tested 6 months after delivery
(Rh + infants)
— 2 had antibody — ? passive

Effect of Rh prophylaxis on disease incidence and mortality

The results of the clinical trials suggest that with the present wide application of Rh prophylaxis it should be possible to observe a progressive decline in the incidence of and the mortality from Rh haemolytic disease. The speed with which this decline occurs will depend on the efficiency of the prophylactic programme but also on the average family size and interval between successive births. Certain criteria may be affected by factors other than Rh prophylaxis. The crude incidence of Rh antibodies in a population is affected by the tendency in recent years to employ increasingly sensitive methods of detection. The use of phototherapy may lessen the frequency of exchange transfusion. It will probably therefore take several years before the full effect of the prophylactic programme can be assessed.

Hawes and Mordaunt [8] have produced some interesting data from California. Figure 2 charts the infant death rate from Rh haemolytic disease in California, between 1947 and 1971. It is seen that there was a gradual decline in mortality over this period. There were thought to be two main reasons for this, one being limitation of family size with an increasing percentage of primaparous deliveries, and the other improved methods of diagnosis and management of the condition. In 1968 Rh prophylaxis was introduced on an increasingly wide scale. In 1970 it became a legal requirement in the State that all pregnant mothers be Rh typed and that all cases of Rh haemolytic disease be notified. It is therefore very likely that the data on incidence and mortality from the disease are now accurate. The incidence of the disease fell from 4.1 cases per 1000 live births in the first quarter of 1970, to 2.9 in the corresponding quarter of 1972. The actual number of deaths

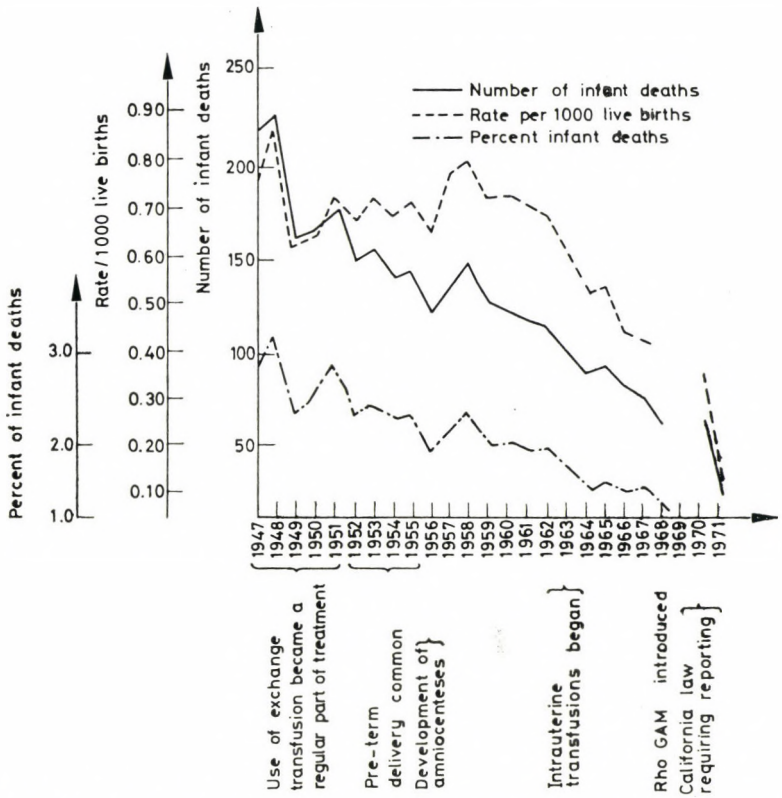

Fig. 2. Trends of infant death due to Rh haemolytic disease between 1947 and 1971. From Hawes and Mordaunt [8] by kind permission of the authors

attributed to the disease were 38 and 12 respectively during these two periods. The evidence strongly suggests that this marked fall was largely due to the widespread use of anti-D gammaglobulin.

*

I am grateful to many workers in the Rh field who have published data on clinical trials which I have quoted. I wish to thank the Californian Medical Association for permission to reproduce Fig. 2.

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Immunization of Rh_0(D)-negative Secundigravidae Whose First Pregnancy Was Terminated by Induced Abortion

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The occurrence of rhesus immunization was investigated in secundigravidae whose first pregnancy had been medically terminated during the first trimester.

a) Out of 301 *non-protected* women whose pregnancy had been terminated medically within the first trimester, the second pregnancy was terminated in 121 again by induced abortion within the first trimester. One of these was found to be immunized. She had received i.m. blood injection in childhood. Twenty-four women had spontaneous abortion. One of these became immunized. Immunization was detected in the fourth month of the second gravidity. The frequency of immunization was found about 1.0%. To evaluate the exact rate of immunization was not possible due to the low number of cases. Among the 156 secundigravidae who had delivered Rh_0(D)-positive newborns at term, antibodies could be detected in 6, about 4.0% (= 3.84%).

b) Among 96 women who *were protected* by 50 μg anti-D IgG when their first pregnancy was interrupted, immunization occurred in one case.

c) An attempt was made to determine secondary response in women whose first pregnancy was determined by induced abortion. Patients whose sera contained antibodies detectable with papain-treated RBC were considered immunized.

As close associates of Professor Susan R. Hollán, it gives us great pleasure to have the privilege of reporting in this dedicatory issue on our recent results in the field of anti-Rh_0(D) IgG prevention.

We are happy to offer our presentation so much the more because Professor Hollán, as a member of the WHO Expert Committee, has made valuable contributions to the improvement of the health and life of Rh_0(D)-positive children of Rh_0(D)-negative mothers. It gives us much satisfaction to witness the rapid progress of organized blood service in Hungary, and to see how Hungarian haematology is flourishing since Professor Hollán has become head of the Institute: modern haematology and transfusiology in Hungary are linked with her name.

The hazards to Rh_0(D)-positive foetuses and neonates of Rh_0D-negative mothers have often been discussed. It is also known that the 17 per cent risk of primary and secondary response observed in Caucasians can be reduced below 2.5 per cent by anti-D-IgG prophylaxis: There is an incidence of detectable immunization of about 0.5 per cent at 6 months and a further 1—2 per cent at the end of the next Rh_0(D)-positive pregnancy [1, 2].

Literary data are relatively scarce on the degree of risk in induced abortion and on possibilities of prevention. A survey of the literature makes difficult to establish the proportion of women at risk as the patients and methods reported vary considerably. Queenan et al. [10] combined their results with those of Jørgensen [5], Matthews [6] and Murray et al. [9]. Out of the total 145 $Rh_0(D)$-negative pregnant women who underwent induced abortion, 8 (5.5%) became immunized. Gestation time varied between 9 and 18 weeks, most of the patients were primigravidae. In the series of Goldman and Eckerling [4], 5 out of 122 untreated controls became immunized after induced abortion (4.1%). Murray and Barron [8] studied 177 $Rh_0(D)$-negative patients after spontaneous or therapeutic abortion: of these 48 were primigravidae. Rh antibodies were found in 3 (6.3%) primigravidae detected by the Coombs test and with enzyme-treated cells. Murray [7] found that the weak positivity with papain-treated cells became definitely positive at a subsequent pregnancy. In five follow-up studies among 252 women, in whom foetal red cell counts were carried out after abortion, Rh antibodies were found in 12 cases (4.8%). The risk of this occurring was found to be greater in the second than in the first trimester [2]. Freda et al. [3] estimate the risk of immunization following abortion at 3–4 per cent. They observed that the frequency of immunization increased with the age of the aborted foetus. The risk was negligible at 1 month, definitely appreciable at 2 months (2%) and considerable at 3 months and beyond (9%). Data published in this paper are in agreement with the publication of Freda et al. [3], who stated a 2 per cent primary immunization in primigravidae whose pregnancy was terminated by induced abortion in the second month. In Hungary induced abortion is legally permitted in the first trimester, however, in practice it is generally performed in the second month.

One of the present authors has recently reported on the failure rate of anti-D prophylaxis [11]. In 1971 and 1972 1588 $Rh_0(D)$-negative primigravidae were examined who had had induced abortion in the first trimester of pregnancy. Within 72 hours following the intervention they were given 50 μg anti-D IgG intramuscularly and only 4 became immunized (antibody detected with papain-treated cells), a failure rate of 0.25% [11]. The 1973 serological follow-up of the 887 primigravidae who had been protected after abortion showed 2 cases of immunization, amounting to a failure rate of 0.24% [12].

To our best knowledge no report has been published on the frequency of a secondary response manifesting itself during the second pregnancy of women whose first pregnancy had been terminated by induced abortion.

The present investigations were aimed at clarifying the frequency of antibody formation during the second pregnancy in women whose first pregnancy was interrupted in the first trimester.

Material and Method

The method of pregnancy interruption in primigravidae, the serological examinations, the preparation and control of the applied anti-D IgG have been reported previously [13].

The present paper presents data of secundigravidae (a) who had been protected after the first abortion, and (b) who had not been protected after the first abortion and whose second pregnancy had terminated in induced or spontaneous abortion, or in delivery in the period 1971 to 1973.

The serological tests in every case were made immediately after the obstetrical event and three, resp. six months later.

This conjoint study was carried out by the National Institute of Haematology and Blood Transfusion and five regional centres of the National Blood Service.

Results

Table 1 shows data of $Rh_0(D)$-negative secundigravidae whose first pregnancy had been terminated by induced abortion followed by the administration of 50 μg anti-D IgG.

It can be seen that in 4 counties of Hungary all the 96 $Rh_0(D)$-negative secundigravidae *who had been protected* by 50 μg anti-D IgG after the termination of their first pregnancy were subjected to serological control. None of them were found to be immunized. In the course of the second pregnancy one parturient woman was found to be immunized. Detailed data are given in Table 2.

Table 1

Data of secundigravidae whose first pregnancy had been terminated by induced abortion followed by the *administration of 50 μg anti-D IgG* (1971 to 1973)

County	No. of controlled secundigravidae	Serological findings							
		Termination of second pregnancy							
		Before first abortion		Induced abortion		Spontaneous abortion		Delivery	
		neg.	pos.	neg.	pos.	neg.	pos.	neg.	pos.
Bács	20	20	—	12	—	1	—	7	—
Baranya	39	39	—	22	—	2	—	15	—
Békés	25	25	—	15	—	—	—	9	1
Zala	12	12	—	4	—	—	—	8	—

n = 96. Immunized: 1.

The serological findings of the 301 pregnant women *who had not been protected* following induced abortion of their first pregnancy are shown in Table 3.

As indicated in Table 3, in 121 of the 301 non-protected women, the second pregnancy was terminated again by induced abortion. Among these antibodies were found in 1 case (she received i.m. blood injection in childhood).

Anti-D-IgG was detected in the sera of one woman out of the 24 who had spontaneous abortion and in 6 (3.84%) out of the 156 who had term delivery. Detailed serological findings of the 6 immunized women are given in Table 4 and 5. Table 4 shows the detailed serological data of three $Rh_0(D)$-negative secun-

Table 2

Serological data of the

Patient	Date of induced abortion	Administration of 50 μg anti-D IgG	Serological control		
			July 4, 1972		
			Blood group	Papain-treated RBC	ICT[1]
F. L. (1953)	Jan. 25, 1972 9^h 40	Jan. 25, 1972 10^h 20	A $Rh_0(D)$ neg	neg	neg

[1] Indirect Coomb's test

Table 3

Data of secundigravidae whose first pregnancy had been terminated by induced abortion and who were *not protected* after the first induced abortion (1971 to 1973)

County	No. of controlled secundigravidae	Serological findings							
		Termination of second pregnancy							
		Before first abortion		Induced abortion		Spontaneous abortion		Delivery	
		neg.	pos.	neg.	pos.	neg.	pos.	neg.	pos.
Bács	90	90	—	44	—	12	—	31	3
Baranya	81	81	—	39	—	4	—	38	—
Békés	99	99	—	26	—	4	1	67	1
Zala	31	31	—	11	1*	3	—	14	2
Total	301	301	—	120	1*	23	1	150	6

* Received i.m. blood injection in childhood.

digravidae by whom no antibodies were detected in the mother's sera between the two pregnancies. Table 5 illustrates the detailed serological data of 3 $Rh_0(D)$-negative secundigravidae who were not subjected to serological control after the first abortion.

Discussion

Of 156 $Rh_0(D)$-negative primiparae, whose second pregnancy was terminated by term delivery of $Rh_0(D)$-positive newborns 6 (3.8%) were found to be immunized. This is a high percentage, but taking into account that these 156 primiparae

immunized patient

Second pregnancy							
Last menses	Serological control			Partus Nov. 24, 1973			
				Mother		Newborn	
		Papain-treated RBC	ICT[1)]	Blood group	Papain-treated RBC	Blood group	Papain-treated RBC
Febr. 5, 1973	May 8	neg	neg	A $Rh_0(D)$ neg	1 : 256	O $Rh_0(D)$ pos	1 : 4

were in reality secundigravidae, whose first pregnancy was terminated in the first trimester by induced abortion further questions arise:

In the non-protected group, data of the 156 parturients and 24 women who had spontaneous abortion can be classified into two groups.

Table 4 illustrates the detailed serological findings of 3 $Rh_0(D)$-negative secundigravidae who were left unprotected after the termination of their first pregnancy. *In this group after the first abortion there was a 6-month period when no antibodies were detected in the mothers' sera.*

Immunization became manifest at the end of the second pregnancy. Most probably the 3 (i.e. slightly less than 2%) parturients gave a secondary response, although the possibility of early primary immunization during the second pregnancy cannot be excluded.

Table 5 shows the findings of 3 immunized parturients (near 2%) who were not subjected to serological control examinations after the first abortion. Therefore, it is now impossible to establish whether in these cases immunization had occurred as a primary or a secondary response.

A frequency of 1 per cent immunization in protected women (Table 1) does not seem realistic. Primary response after abortion amounted to 0.25 per cent, while that after delivery to 0.5 per cent. This high rate of failure may be due to the small number of cases under investigation.

As indicated in Table 3, in the 4 counties 30 $Rh_0(D)$-negative secundigravidae did not receive anti-D IgG following interruption of the first pregnancy, despite the fact that anti-D IgG protection is free of charge in Hungary. Comparing this figure with that of the protected women (see Table 1), it can be seen that 75% of the women had remained unprotected, owing mainly to the failure of convincing obstetricians about the necessity of prophylaxis. To achieve an effective and universal protection, the administration of anti-$Rh_0(D)$ IgG to all previously non-immunized $Rh_0(D)$-negative pregnant women will be compulsory in Hungary.

Table 4

Serological data of immunized secundigravidae whose first pregnancy had been after the first

Patients	First induced abortion	Serological control between the two pregnancies				Second		
		Time	Blood group	Anti-D		Last menses	Serological	
				Papain-treated RBC	ICT		Time	Blood group
B. G.[1]	Sept. 1968	Jan. 1970	AB Rh_0 neg	neg	neg	Nov. 1972	April 11, 1973	AB $Rh_0(D)$ neg
N. J.[2]	1970	—	—	—		Jan. 1973	April 12, 1973	AB $Rh_0(D)$ neg
B. B.[2]	1970	—	—	—		Sept. 1970	Dec. 10, 1970	O $Rh_0(D)$ neg

[1] Negative by blood donation.
[2] Negative at the first trimester of second pregnancy.

Table 5

Serological data of immunized secundigravidae whose first pregnancy had been terminated had been subjected to serological

Patient	Year of birth	First induced abortion	Before first abortion		Second				Serological
			Papain-treated RBC	ICT	Last menses	First serological control			
						Time	Blood group	Papain-treated RBC	ICT
J. J.	1945	?	neg	neg	?	Dec. 21, 1971	AB $Rh_0(D)$ neg	1 : 2	neg
Sz. G.	1945	?	neg	neg	?	Jan. 25, 1973	B $Rh_0(D)$ neg	1 : 2	neg
D. J.	1955	1970	neg	neg	Febr. 14, 1971	May 10, 1971	A $Rh_0(D)$ neg	1 : 8	1 : 4
Z. M.	1944	1968	neg	neg	Oct. 6, 1970	Febr. 2, 1971	A $Rh_0(D)$ neg	1 : 256	1 : 128

terminated by induced abortion and who were not protected nor had detectable antibodies induced abortion

pregnancy		Partus				
control		Mother				Blood group of the neonate
Papain-treated RBC	ICT	Time	Blood group	Papain-treated RBC	ICT	A $Rh_0(D)$ pos
1 : 32	?	July 5, 1973	AB $Rh_0(D)$ neg	1 : 128	1 : 8	A $Rh_0(D)$ pos
neg	neg	Sept. 3, 1973	AB $Rh_0(D)$ neg	1 : 64	1 : 4	A $Rh_0(D)$ pos
	neg	June 12, 1971	O $Rh_0(D)$ neg	1 : 4	—	—

by induced abortion and who were not protected after the first induced abortion nor control after the first abortion

findings							
pregnancy			Partus				
Second serological control			Time of delivery	Mother			Blood group of neonates
Time	papain	ICT		Blood group	Papain-treated RBC	ICT	
April 2, 1972	1 : 2	neg	May 9, 1972	AB $Rh_0(D)$ neg	1 : 2	neg	A $Rh_0(D)$ pos
May 8, 1973	1 : 4	neg	May 28, 1973	B $Rh_0(D)$ neg	1 : 8	1 : 1	B $Rh_0(D)$ pos
—	—	—	Nov. 9, 1971	A $Rh_0(D)$ neg	1 : 256	1 : 64	O $Rh_0(D)$ pos
spontaneous abortion Febr. 24, 1971				—	spontaneous abortion		—

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The Role of the Antibody in Immunological Cell Triggering Processes

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The idea is developed that the role of the antibody in immunological cell triggering processes is that of a transducer of the stimulus afforded by specific antigen. In another sense it can be considered to be a "pro-hormone", which is capable of recognizing and "priming" (i.e. sensitizing) the appropriate target cell so that subsequent interaction with antigen brings about the mobilization of specific effector groups which trigger the cell into action. Although the emphasis has been placed on the mode of action of anaphylactic antibodies in the initiation of vasoactive amine release, it is suggested that other types of cytophilic antibodies such as those engaged in the mediation of phagocytosis and lymphocyte stimulation could be fulfilling an essentially similar role. Taking this analogy further, it is tentatively suggested that IgG mediator antibody molecules possess cell-binding sites with differing specificity located within a similar region of their C_H3 domains and that the effector sites responsible for triggering different target cells are probably located within a different part of the antibody Fc region.

Introduction

Of the wide range of cellular processes implicated in normal and abnormal immunological responses, those mediated by surface-bound antibody are of particular interest mechanistically. These include: complement-dependent lysis of erythrocytes and bacteria, phagocytosis by macrophages and monocytes, lymphocyte activation and release of vasoactive amines from mast cells and basophils. A fascinating question, awaiting answer by the experimentalist, is how combination of antibody molecules with specific antigen at the cell surface initiates the ensuing lysis or activation culminating in secretion of specific mediators. In other words, what precisely is the nature of the primary immunological trigger process (the "first message" in the terminology of the endocrinologist)?

In this article the idea will be developed that immunological triggering processes, leading to the active secretion of mediators of subsequent biological events, possess certain important characteristics in common irrespective of the nature of the target cell and the class of antibody involved.

Emphasis will be placed, however, on the mode of release of histamine in immediate-type hypersensitivity reactions, elucidation of which has been greatly facilitated in recent years by increased knowledge about the characteristics of

anaphylactic-type antibodies and by the ready manner in which such antibodies (and certain of their cleavage products) can be attached to their target cells *in vitro* "at will". Indeed, it will be suggested that the mechanism of triggering of mast cells which will be presented (as a stimulus for further discussion) could well serve as a useful model in furthering our understanding of other immunological cell-triggering processes, which have very different biological outcomes.

Role of the antibody in the initiation of histamine release from mast-cell and basophils

Anaphylactic type antibodies are involved in three crucial aspects of immediate-type hypersensitivity reactions (as illustrated schematically in Figure 1) culminating in the release of vasoactive amines:

1) Identification and sensitization of the appropriate target cells (i.e. mast-cells or basophils).

2) Subsequent reaction with specific antigen (allergen).

3) Translation of the effect of this reaction to the target cell (presumably, initially, to "activation" sites on the plasma membrane).

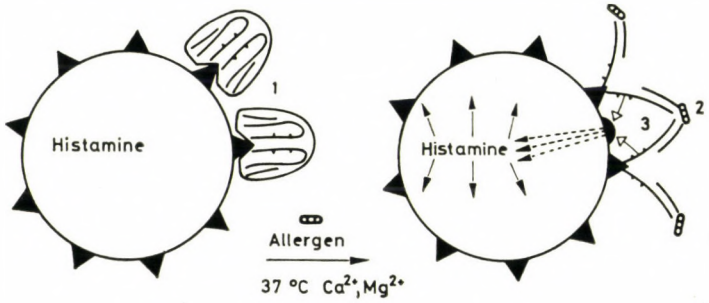

Fig. 1. Schematic representation of 3 crucial steps in immediate-hypersensitivity reactions: (1) target cell sensitization; (2) stimulation by specific antigen; (3) triggering of histamine release

In a sense, therefore, the antibody molecule can be considered to be a "transducer" of information from antigen to target cell, furnishing it with the appropriate "message" to stimulate it into histamine secretion. A fundamental question to be answered is how precisely does this triggering occur? Observations on the role of the antibody in each of the above steps will be considered in turn in the development of a hypothesis which, it is suggested, offers a plausible explanation of the mechanism of this particular immunological trigger process; and which, as already pointed out, might stimulate new ways of looking at other forms of immunological cell-triggering. But first, it is worth making some general comments on the nature of the antibodies involved in this type of reaction.

Anaphylactic, tissue sensitizing, antibodies are not restricted to the IgE class: certain IgG antibodies in human and other mammalian species are capable of

similar activity; which has never been shown, however, by antibodies of the other three major immunoglobulin classes (IgM, IgA or IgD). Two types of IgG anaphylactic antibody have been recognized in many species. These have been conveniently referred to as $\gamma 1$ and $\gamma 2$ types [39], corresponding to the electrophoretic "fast" and "slow" forms of IgG first studied in the guinea pig; but they are now known to be representative of different IgG sub-classes. Unlike antibodies of the IgE

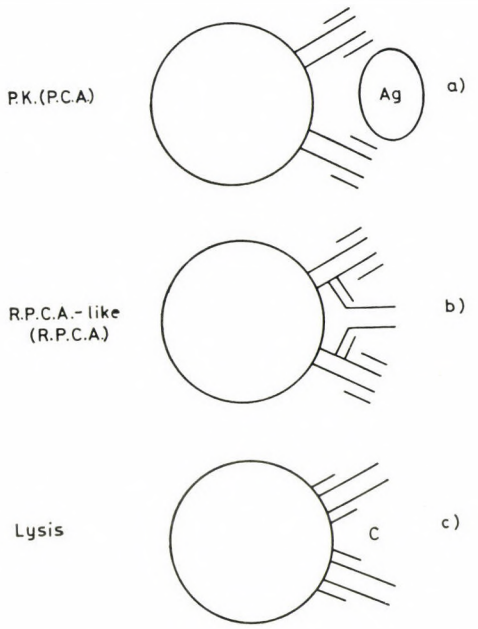

Fig. 2. Diagram illustrating basically different roles of IgG antibody in: a) and b) histamine secretion from mast cells; c) haemolysis

class, they are heat-stable and bind much more weakly to target mast cells. Furthermore, sensitizing antibodies of the "$\gamma 2$ type" bind to tissues (e.g. skin) of heterologous species and should therefore be regarded as "pseudo-anaphylactic" in the sense that they probably are not involved in immediate-sensitivity reactions in the parent animal.

It seems highly significant that none of these three major types of anaphylactic antibody (IgE, isologous and heterologous tissue-sensitizing IgG) appear to rely on complement for their activity; activation of neither the classical nor alternative pathways [12] seeming to be involved. As suggested in an earlier article in this journal [38], complement-mediated cell lytic processes would seem to be distinguished by the manner in which the transducer/mediating antibody (IgG or IgM) is bound to erythrocyte surface antigens through sites within its Fab regions, thus leaving Fc sites available for interaction with the Clq subcomponent of the

complement system. In contrast (see Fig. 2), as will be discussed further later, anaphylactic antibodies (IgE or IgG) bind to their target mast cells and basophils the "opposite way round" and are therefore in an appropriate configuration for subsequent interaction with challenging antigen (allergen). Furthermore, as no other serum protein constituents appear to be required for histamine release, the implication is that "effector" sites mobilized within the cell-bound antibody molecules themselves following interaction with antigen are capable of triggering the target cell without any help from external agents (other than essential divalent cations such as Ca^{++} and Mg^{++} ions).

If this is the case, the lack of anaphylactic activity shown by antibodies of the IgM, IgA and IgD classes might be due to an absence of the appropriate effector structures; or, alternatively, it could equally well be ascribed to an incapacity to bind to (i.e. sensitize) target mast cells. The latter possibility is suggested by the failure of human isoagglutinins of the IgM [32] and IgA [11] classes to induce direct and reverse passive cutaneous anaphylactic (RPCA) reactions in guinea pigs.

Obviously detailed structural comparisons of anaphylactic and non-anaphylactic immunoglobulin classes will eventually provide important clues about the nature of mast cell binding and effector sites. In the meantime alternative, less direct, approaches are beginning to prove rewarding as will now be discussed.

1) *Nature of the binding of anaphylactic antibodies to mast cells*

A certain degree of specificity in the binding of anaphylactic antibodies to target cells is indicated both from cross-species sensitization studies and from attempts to sensitize cells other than mast cells and basophils. Thus, human IgE antibodies are only capable of sensitizing isologous human or closely related non-human primate skin, and isologous tissue sensitizing antibodies of the IgG class are likewise incapable of binding to the tissues of other than closely related species. Furthermore, anaphylactic antibodies appear to be selective with regard to their binding to mast cells and basophils, suggesting that they are capable of identifying these target cells.

It is conceivable, of course, that one class of anaphylactic antibody binds to different target cell receptors to those to which another binds. But evidence has been obtained from application of a rosetting technique [31] that mouse IgE and IgG-1 antibodies fix to the same receptors on mouse peritoneal mast cells; and, similarly, we have indirect evidence [42] that human IgE and IgG-4 (the sub-class in which, quite possibly, human non-reaginic anaphylactic antibodies are found) bind to the same or closely located receptors on baboon skin mast cells.

The strength of binding of the two classes of anaphylactic antibody to the target cell would seem, however, to be markedly different. IgE antibodies appear to be much more avidly bound; it having been recently estimated [17] that they possess an association coefficient of $10^8 - 10^9$ lit. per mole and to be linked to mast cells through non-convalent bonds. Consequently IgE anaphylactic antibodies

are found to persist for appreciably longer periods than IgG ones on transfer to isologous skin sites.

At the chemical level, some evidence has been obtained from studies involving PCA testing with chemically substituted rabbit IgG anaphylactic antibodies [22] that one or two charged ε-ammonium groups may play a critical role in antibody binding to mast cell receptors, which could contain complementary anionic carboxylic or phosphate groups (which would be expected to be found, respectively, in protein or lipid regions of the target cell membrane).

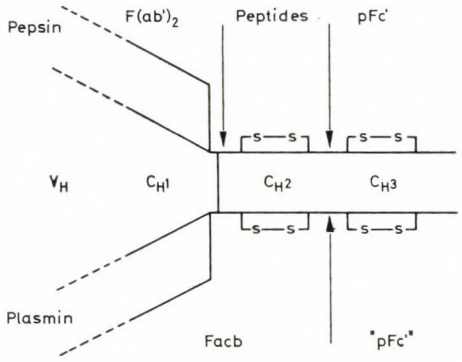

Fig. 3. IgG (rabbit) structure showing point of plasmin cleavage to give Facb fragment

Attempts to define the location of the cell-binding sites within the anaphylactic antibody molecule have relied so far on the testing of the PK and PCA-inhibition activity of proteolytic cleavage fragments of human myeloma IgE. Of the various IgE fragments examined in this manner, none smaller than the whole Fc fragment proved inhibitory [37, 39, 41]. This was disappointing because it had been hoped by the testing of Fc sub-fragments, to pin-point the location of the target cell binding sites more precisely. The finding was, however, consistent with the observations [47], that relatively limited proteolytic cleavage at the N-terminal end of the Fc fragment of rabbit IgG led to a loss in RPCA activity.

It seemed, therefore, that amino acid residues close to the hinge region were implicated in both IgE and IgG cell-binding activity. In this event other types of immunoglobulin cleavage fragment which retained these residues might be expected to show PCA and RPCA activity. The more recently described Facb fragment [6] was particularly interesting in this connection because (as is shown in Fig. 3) it retains the whole of the C_H2 structural domain following plasmin cleavage of acid-treated rabbit IgG, which produces also a C_H3 domain fragment of similar characteristics to the pFc′ fragment obtained by peptic cleavage of IgG. But neither the rabbit IgG Facb fragment, nor the residual pFc′-like fragment were found to bind to guinea pig skin when tested for inhibition-RPCA activity by the con-

ventional procedure employing intravenously injected Evans blue dye to indicate the extent of extravasation occurring [43].

This failure to find activity in either fragment could be due to the destruction of a binding site located between the C_H2 and C_H3 structural domains of the rabbit IgG molecule, as a result of the plasmin cleavage treatment (as the acid pre-treatment *per se* was shown not to influence inhibition-D RPCA activity). But recent observations by Minta and Painter [26] suggest that a technical inadequacy was probably responsible: because these workers found that the pFc′ fragments of rabbit IgG antibody, which appeared to lack inhibition-PCA activity using the conventional blueing technique, were in fact inhibitory as indicated by radioassay of the accumulation of intravenously injected ^{125}I-serum albumin in the test sites. Moreover, in recent collaborative studies with Drs J. Morley and T. Greenland, we have shown the pFc′-like fragment obtained from plasmin cleavage of rabbit IgG likewise inhibits RPCA reactions as measured by the more sensitive radio-labelled albumin accumulation test.

Thus, it is beginning to look as if mast cell-binding sites might be located within the terminal C_H3 structural domain of IgG antibodies, a conclusion which has several important implications. Of particular interest are the observations that macrophage [30_a, 44, 49] and cytotoxic lymphocyte [23] binding sites are likewise located within the C-terminal (C_H3 domain) of human and rabbit IgG molecules (as is indicated in Table 3). Furthermore, as will now be discussed, there is reason to suppose, at least as far as histamine release is concerned, that *separate* "effector sites", supposedly responsible for applying the trigger to the target cell (following antigen combination), are located within a *different* part of the antibody Fc region to that comprising the cell-binding sites.

It is tempting to suppose that the mast cell binding sites of IgE antibody molecules will prove to be similarly located within the C-terminal (C_H4) structural domain; a fact which could possibly be demonstrated by repeating our earlier baboon PCA-inhibition tests on myeloma IgE Fc sub-fragments using the improved technique mentioned earlier. In this connection, it is interesting to note that Bennich and his associates [1] have recently reported a greater degree of structural homology between the C-terminal domains of the heavy polypeptide chains of human IgE and IgG than between the C-terminal domains of IgE and the corresponding heavy chain regions of human IgM and IgA.

2) *Nature of histamine-releasing stimulus*

Substantial evidence has been obtained to suggest that the cross-linking of adjacent cell-bound anaphylactic antibodies (IgE and IgG) by specific allergen is the crucial event which initiates the release of histamine. This has come from two types of approach, involving PCA testing with Fab-deficient IgG anaphylactic antibody preparations or with hapten (e.g. benzyl penicilloyl)-conjugated polylysine "allergens" of varying valency, as has been discussed fully elsewhere [39]. Thus, it has been shown that "univalent" sensitizing antibodies which possess only one

viable antigen (allergen)-binding Fab sub-unit are just as capable of mediating anaphylactic reactions as divalent antibody molecules (presumably because they can still be bridged by allergen, when situated on adjacent target cell receptor sites). It is interesting, therefore, that recent quaternary structural studies on human myeloma IgE [3] have indicated that the molecule is not conformationally flexible in the same way as the IgG molecule, its Fab regions not seeming to possess that same freedom of independent movement as IgG Fab regions. Could this, therefore, lead to an impairment in one of the IgE antibody molecules' antigen binding sites, so that it behaves (like the artificially altered IgG antibody) as an "univalent antibody"?

Conversely, findings from studies on hapten-polylysine conjugates of varying valency have indicated that a minimum allergen valency of two is required to form the induction of histamine release in previously sensitized skin sites of humans and experimental animals [7]. Furthermore, it could be that a similar "valency" requirement needs to be met if native antigen molecules are to prove allergenic [39].

How is the effect of the cross-bridging of adjacent cell-bound antibody molecules by allergen translated to the surface of the target mast cell, to initiate the release of histamine and other vasoactive amines? The hypothesis of cell-triggering which I have evolved over the last six or seven years (illustrated schematically in Fig. 1) postulates that interaction with allergen induces a conformation change within the cell-bound antibody molecules resulting in the mobilization of effector sites within the Fc regions, which activate complementary sites on the target cell surface (quite possibly comprising a membrane enzyme system). This suggestion is consistent with the idea first mentioned in the Introduction, that target cell triggering (i.e. effector) site(s) could be located within a different part of the sensitizing antibody Fc region to that comprising the cell-binding site(s); and, by implication, that the activation sites triggered on the cell membrane by the antibody effector sites are probably distinct from the antibody-binding receptors.

What evidence is available to support this hypothesis? Admittedly, it is mainly based on indirect findings at the moment, but many such observations of this type – particularly those from studies of alternative ("artificial") modes of inducing histamine release from mast cells and basophils – fit into the basic release mechanism which is proposed. For instance, as might have been anticipated, the cross-linking of adjacent cell-bound anaphylactic antibody molecules by anti-antibody (anti-IgE or anti-IgG) as occurs in RPCA reactions also induces histamine release; as does reaction with protein A, the *Staphylococcus aureus* cell wall constituent which precipitates IgG of certain species *in vitro* and initiates "pseudo-anaphylactic" reactions *in vivo*. It would seem reasonable to suppose that, like cross-bridging by specific allergen, these artificial bridging agents induce the appropriate conformational change necessary for the mobilization of the target cell effector groups within the Fc regions of the cell-bound antibody molecules. On the other hand, pre-aggregated IgE (or IgE Fc fragment) or pre-formed antigen-IgE antibody complexes (of certain proportions) are capable of inducing

immediate histamine release from *non-sensitized* mast cells. Presumably the appropriate effector groups have been already mobilized in these structures *before* presentation to the target cell, so that a prior cell-sensitizing step is not required. This conclusion is supported by our failure to inhibit such immediate histamine release reactions in baboon's skin by monomer IgE, which has been shown to block IgE antibody receptors.

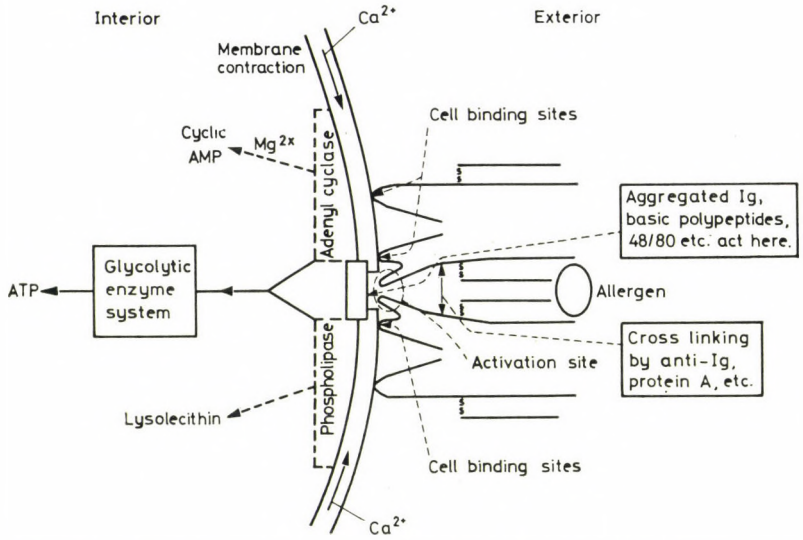

Fig. 4. Summary of various direct and indirect methods of triggering mast cells into histamine release, suggesting a common activation site on the plasma membrane (reproduced from [39]).

Hence, it would seem that irrespective of the antibody cross-linking agent, or even if this occurs *off the target cell* (as in pre-aggregate or antigen-antibody complex formation), the same subsequent cell-triggering process is initiated involving activation of the same site on the target cell membrane. Furthermore, it is conceivable that other artificial (non-immunological) histamine releasing agents such as compound 48/80 and various basic polypeptides could also trigger the same site on the mast cell surface (as is illustrated schematically in Fig. 4). For such substances, in suitably low doses, behave like immunological triggers in initiating the active secretion of histamine; so that they too, like pre-formed aggregates of anaphylactic antibody, would appear to be capable of "short-circuiting" the regular two stage antibody-mediated release process (depicted in Fig. 1). Consequently, the identification of that part of the structure of artificial liberators directly involved in the selective histamine release process could provide important clues about the value of the cell-triggering effector sites mobilized in the Fc regions of cell-bound antibody molecules following bridging by specific allergen.

In adopting this approach we have chosen to undertake structure-activity studies on certain well-defined basic polypeptides and their derivatives; because, if the hypothesis which I have outlined is correct, they might be expected to possess histamine releasing amino-acid sequences similar to those which supposedly comprise the effector sites mobilized within the Fc regions of anaphylactic (IgG and IgE) antibody molecules. In other words, we ought to be able *to predict* the nature of the antibody effector sites from such indirect studies.

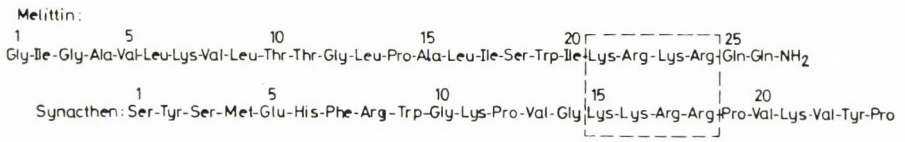

Fig. 5. Primary amino-acid sequences of melittin and Synacthen (ACTH β^{1-24} polypeptide)

Although we have investigated the structural basis of the histamine releasing activity of a wide variety of non-immunological polypeptide histamine liberators [18] we have paid particular attention to ACTH polypeptide and melittin (derived from bee venom). A range of synthetic ACTH polypeptides of varying chain length were obtained through the courtesy of the CIBA Company, and well defined melittin proteolytic cleavage products were provided by Dr. G. Kreil (University of Vienna). The capacity of varying doses of each compound to release histamine in a standardised *in vitro* rat peritoneal mast-cell system was determined, by use of a spectrofluorometric histamine assay. Comparison of the activities of the various compounds on a molar basis in relation to their known primary amino-acid sequences permits one to begin to formulate the polypeptide structural requirements for histamine release. Thus it appears that a cluster of basic amino-acids (a "hot-spot") is of critical importance (see Fig. 5); but the activity of such a sequence is influenced by neighbouring amino-acids, being enhanced by a relatively long chain of N-terminally situated essentially non-basic residues and depressed by the presence of acidic residues on the C-terminal side of the cluster. It is also noticeable that similar types of structure with greater rigidity, such as mast cell degranulating substance (which possesses two intra-molecular disulphide bridges) tend to show even greater histamine releasing activity. Likewise, histamine releasing activity is enhanced by the introduction of residues which facilitate membrane penetration, such as the high proportion of hydrophobic amino-acids in the $1-20$ region of the melittin sequence and the N-terminal 6-methyl octanoic acid residue in polymyxin B (another potent artificial histamine liberator). Indeed, in these cases, a non-selective "lipid reactive" element has been added to the selective releasing region of the non-immunological liberator structure; the outcome of such a chemical combination being reflected in a drastic effect on mast cells similar to that elicited by detergents (such as Triton X 100), whose interaction with the lipid of the plasma membrane results in cell lysis (as is indicated in Table 1). A similar effect can be achieved by interaction with anti-mast cell antibody in the

Table 1

Classification of agents which elicit histamine release from mast cells

NON-SELECTIVE (CYTOTOXIC) AGENTS		SELECTIVE (PROTEIN) REACTANTS	
GENERAL SURFACTANTS	PHOSPHOLIPID REACTANTS	DIRECT v MEMBRANE ENZYME	INDIRECT v MEMBRANE BOUND-Ig
Detergents	Lysolecithin	Sens. Ab-Ag Aggreg. Ig	Anti-Ig, Protein A
Ab v surface antigens			
		Basic Polypeptides	
Polycations			
	Melittin ⟨– – – – – –⟩		Melittin, 48/80
Hypotonicity			

presence of complement, which induces a cytolytic effect similar to that effected by haemolytic antibodies; which are likewise directed against target cell antigenic determinants and activate the complement system through sites in their Fc regions (as was illustrated in Fig. 2). In contrast, as already emphasized, anaphylactic antibodies appear to be capable of triggering their target cells directly into active secretion; and it would appear that the basic polypeptide analogues, such as the ACTH peptides investigated in our laboratory are acting in a similar selective manner, quite possibly on the same activation sites on the mast cell membrane.

If this contention is valid, it is tempting to look for similar clusters of histamine-liberating basic amino-acids in the Fc region primary sequences of those IgG and IgE immunoglobulins whose structures have already been elucidated. Although no consecutive "runs" of basic amino-acid residues are observed in the primary sequence of the Fc region of human IgG-1 [9], there are five regions in which two basic amino-acid residues are separated by a neutral residue. Of those, one comprises a separating cysteine residue and is therefore probably not relevant to the present discussion; whilst, of the others, three (Lys^{246} Pro Lys^{248}; Thr^{289} Lys Pro Arg^{292}; Lys^{338} Ala Lys^{340}) are located in or near the C_H2 domain and one (Lys^{414} Ser Arg^{416}) within the C_H3 domain. It is interesting to note that the 289–292 tetra-peptide has been synthesized under the name "Tuftsin" and shown to possess phagocytosis-stimulating activity [29]; but we found it to be relatively inactive on testing its ability to release histamine from rat mast cells. "Tailor-made" synthetic peptides, incorporating the other three sequences mentioned above, have proved on the other hand [50] to have substantial *in vitro* histamine releasing activities, as has a synthetic tetrapeptide Arg-Lys-Asp-Arg which corresponds to a structure found in the primary sequence of the C_H4 domain of human myeloma IgE recently reported [2]. Admittedly, the histamine releasing activities of these

synthetic structures are around 100 – 100 times less than those of a standard ACTH 1 – 24 peptide; but this does not necessarily exclude the involvement of one (or more) of these sequences in the cell-triggering sites of whole anaphylactic antibodies, where (on the basis of the data discussed earlier) more remote parts of the Fc polypeptide structure might be expected also to contribute to effector activity.

In contrast to the several potential mast-cell triggering sequences observed in the Fc region of the human myeloma IgG-1 protein, only one such region (namely Arg^{443} Pro Lys^{445}) is present in the Fc region of the recently reported [34] sequence of a human IgM; an immunoglobulin class, which as mentioned earlier, has never been implicated in anaphylactic activity.

3) *Consequences of the immunological triggering of mast cells*

Returning to the question of immunological mast cell triggering, the proposed mechanism which has been outlined envisages that the target cell is "primed" by a sensitizing antibody capable of identifying it and binding to its receptors: so that when the antibody interacts with specific antigen (allergen) the resulting conformational change brings the effector site (presumed to contain basic amino-acid residues) into precisely the correct juxtaposition relative to complementary activation sites on the plasma membrane. Such a triggering process has certain features in common with that supposedly responsible for the action of the polypeptide hormones like ACTH on their target (i.e. adrenal) cells (as defined recently for example by Seelig and Sayers [36] in studies on isolated adrenal cells). As is illustrated in Fig. 6, one part of the single chain polypeptide hormone sequence, namely 10 – 24, is thought to carry the structure which "addresses" (i.e. recognizes) the target adrenal cells; whilst another part, namely the 5 – 10 amino-acid sequence, supposedly contains the "message". It is suggested that one can liken the initial sensitization of target mast cells and basophils by anaphylactic antibody as an address step in immediate hypersensitivity reactions, the subsequent message (i.e. trigger of histamine release) being conveyed by the effector site(s) mobilized as a result of antibody combination with specific antigen. In other words, as mentioned in the Introduction, the antibody can be regarded as a kind of transducer of information from the antigen to the target cell.

Moreover, the findings from our studies on peptide analogues already described have suggested that the message conveyed by the antibody in this manner might merely comprise a sufficient concentration (i.e. a pulse) of negative charges (as indicated in Fig. 7), which might be sufficient to initiate the complex series of events which culminate in the release of mediators of anaphylaxis.

But, in this connection, it should be mentioned that membrane potential changes measured in individual mast cells (rat) during stimulation by compound 48/80 were found not to correlate precisely with degranulation [46]; and electrical stimulation of individual mast cells did not induce degranulation [8]. Hence, on the basis of these and other observations on the lack of a firm correlation between

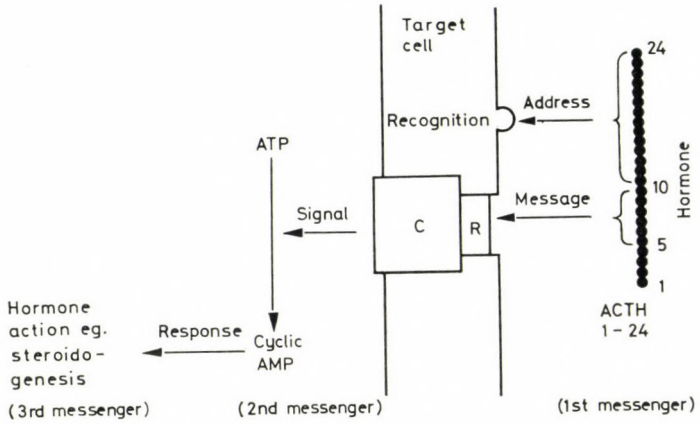

Fig. 6. Supposed mode of triggering of target adrenal cell by ACTH β^{1-24} polypeptide hormone

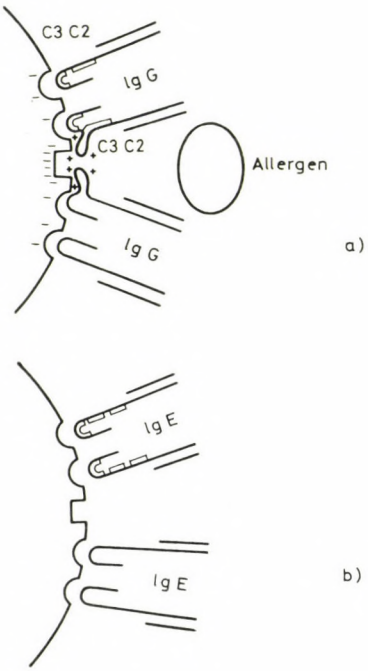

Fig. 7. a) Possible mode of triggering of target mast cell by IgG antibody effector sites comprising pulses of negative charge. b) The extra structural domain within the Fc region of IgE antibody molecules could have a critical influence on such a mechanism

histamine release and ion flux across stimulated mast cell membranes [19] it seems that the triggering process probably involves more than a mere charge re-distribution on the target cell plasma membrane. It is conceivable, therefore, that a more selective membrane enzyme-activation process along the lines already discussed cannot be ruled out.

If one were to take further my suggested analogy with the supposed mode of action of polypeptide hormones (outlined earlier), one might suspect that adenyl cyclase would fulfil such a role. But, there is growing evidence that cyclic AMP exerts a negative modulatory control on histamine release processes; implying that adenyl cyclase is acting in a secondary capacity as might also be membrane-located phospholipases (as is illustrated in Fig. 4). It should be recognized, however, that one of the many other types of regulatory enzymes known to be orientated across cell membranes might accept the primary signal from the cross-linked antibody molecules' effector sites (as has been discussed in more detail elsewhere [39, 40]. Of the various candidates for such a role, a Ca – Mg ATPase is an attractive possibility, as this could control the passage of Ca^{++} ions across the membrane; which, as some investigations have recently suggested [30b], might cause a contraction of microfilaments sited close to the mast cell plasma membrane resulting in the extrusion of the mast granules. Another possibility is that liberated Ca^{++} ions act on contractile protein within the membrane itself. In this connection, it is interesting that mast cells can be induced to secrete histamine when Ca^{++} ions are carried into them by an artificial ionophore which, because of its lipophilic nature, is able to penetrate the plasma membrane [10]. This observation has prompted the suggestion that antibody-antigen interaction on the mast cell surface might initiate histamine secretion in a similar manner; but, if this is the case, it would be necessary to postulate that a predominantly hydrophobic part of the antibody Fc structure facilitates the membrane transmission of the divalent cations, possibly after being cleaved off by action of a surface protease.

Hence, it still has to be established whether the first response of target cell mast cells to anaphylactic antibody-antigen interaction at their surface is of a specific or non-specific nature. Indeed, is the primary trigger itself a selective one, as has been suggested throughout this article; or does the cross-linking of cell bound antibody molecules by antigen merely stimulate the cell by increasing the binding energy of the aggregated antibody molecules' Fc regions, as has been argued by Metzger [25]. This is a fundamental question which applies also to other immunological triggering mechanisms.

Admittedly, the evidence that a conformational change occurs in the Fc regions of antibody molecules on combination with specific antigen is of an indirect kind. Nevertheless, as we [14] and other investigators, e.g. Callahan et al. [5] have shown, changes in the optical characteristics of the antibody molecule occur when such interactions are carried out in moderate antigen excess. Furthermore, if (as has been demonstrated) new antigenic determinants appear within the Fc regions of the antibody molecules, why should not target cell effector groups of the type discussed earlier be also formed in a similar manner following interaction

of cell-bound antibody molecules with antigen (allergen)? The fact that such changes appear to be similarly induced in immunoglobulin G and E molecules as a result of non-specific aggregation induced by heating or some other denaturing process is not necessarily contrary to this idea as Metzger claims [25]. Because, as has been discussed elsewhere [24], there are reasons for supposing that immunoglobulins (such as IgG) undergo similar conformational changes when induced to aggregate *non-specifically*, as when they interact with antigen specifically. In both cases, it would seem that an initial cross-linking of the antibody molecules' Fab regions either directly following denaturation treatment or indirectly (through specific antigen) leads to an unfolding and subsequent inter-molecular association of the Fc regions. It would seem, therefore, that the onus is on the "non-conformation change" school to provide evidence that the new groupings which are known to appear in the antibody Fc region following antigen combination are not providing activation signals to target cell surfaces. For this would seem to be a much more effective way of achieving selective stimulation than by a process which merely involved an increase in the binding affinity of the cytophilic antibody molecules for the target cell.

Moreover, in this latter connection, it is worth noting that it now seems [21] that there is no correlation between the triggering of lymphocytes into blast formation and the "capping" effect (i.e. accumulation of surface aggregates) resulting from the latticing of the complexes formed on reaction of cell surface immunoglobulin with anti-immunoglobulin. It should also be mentioned that a temperature dependent distribution of endogenous IgE molecules on human basophils, not unlike the capping effect revealed on lymphocytes by immunofluorescence analysis, has been observed by electron microscopic examination [45].

4) *Other immunological trigger processes*

It is interesting to consider whether the immunological triggering process which I have proposed applies only to the release of vasoactive amines from mast cells and basophils, or whether an essentially similar mechanism is responsible for the immunological triggering of other cells such as macrophages and lymphocytes.

Irrespective of the type of target cell mentioned, it would seem that the processes involved possess certain basic features in common (as will be seen from Table 2). For instance, like mast cell exocytosis, lymphocyte activation and phagocytosis by macrophages (via the cytophilic antibody pathway) is mediated by antibody molecules with their Fc regions directed towards the target cell; the trigger occurring from the subsequent combination with specific antigen. Furthermore, in these cases too the ultimate outcome is the release of mediators (e.g. lymphokines and lysosomal enzymes) which, like histamine release, is dependent upon Ca^{++} ions but which does not necessarily require complement, at least as far as IgG mediated phagocytosis is concerned [16]. Moreover, it would seem to be more than of little significance that (as will be seen from Table 2) lymphocyte activation

and phagocytosis by macrophages (and monocytes) can also be "short-circuited" by presentation of pre-formed antigen-antibody complexes to the target cell (e.g. as in phagocytosis mediated through the opsonizing antibody pathway) as well as by cross-bridging of the cell-bound antibody by anti-immunoglobulin (as in the artificial triggering of lymphocytes referred to earlier). Just how far, therefore, can the analogy with the histamine release system be taken?

Table 2

Similarities between different immunological cell triggering processes

Target cell	Anti-body	Direct triggers	Indirect triggers	Artificial triggers	End results
Mast cell Basophil	IgE, IgG	Specific antigen (allergen)	Ag-Ab (IgG) complex, (IgG)n	Anti-Ig, protein A, basic poly-peptides	Exocytosis: Vasoactive amine secretion; degranulation,
Lymphocyte	IgG	Specific antigen	Ag-Ab (IgG) complex	Anti-Ig, phytomito-gens, etc.	Blast formation, protein synthesis, lymphokine secretion
Macrophage	IgG	Specific antigen	Ag-Ab (IgG) complex, (IgG)n		Phagocytosis, lysosomal dis-ruption, enzyme release

More than one group has observed the stimulation of normal cultured lymphocytes (from non-sensitized donors) by pre-formed antigen-antibody complexes, but there is differing opinion about the mechanism of the effect and there is doubt about the role of complement. Thus, for example, Bloch-Shtacher and his associates [4] feel that antigen (BSA)-antibody complexes probably stimulate normal lymphocytes by non-specific attachment to the plasma membrane thereby causing injury with the aid of complement. Other investigators [20], on the other hand, have suggested that stimulation of lymphocytes by antigen-antibody complexes can be attributed to an increased immunogenicity of the antigen when complexed with antibody. In other words, the effect of the antibody-antigen association process is manifested in the antigen rather than in the antibody (as is suspected is the basis of the stimulatory effect of aggregated IgG antibody molecules on mast cells and macrophages); which is not too surprising if one looks on this as a latticing of antigen in preparation for its reactivity with receptor immunoglobulin already present on the lymphocyte surface. (Particularly, if as Leiken and Oppenheim [20] suggest, the supposedly normal lymphocytes which they tested were from adults who may have unknowingly become already sensitized to the test BSA antigen employed.) As might have been expected, therefore, non-specifically

Table 3

Evidence for Fc-located target cell binding site(s)

Target cell (For Ig)	Species	Reactive Ig class	Assay	Binding site location	Reference
Monocyte	Human	Human IgG1, IgG3	Inhib. phagocytosis (C)	C_H3	Okafor et al. [30a]
Macrophage	Guinea pig	Human IgG1	Inhib. rosetting	C_H3	Yasmeen et al. [49]
Macrophage	Guinea pig	Rabbit IgG	Inhib. rosetting	C_H3	Stewart & Stanworth [44]
Macrophage	Guinea pig	Rabbit IgG Ab-Ag	Inhib. phagocytosis by neutrophils (O)	C_H3	Maclennan et al. [23]
Cytotoxic lymphocyte	Human	Rabbit IgG	Sens. of Chang cells for lysis	C_H3	Maclennan et al. [23]
(Mast cell)	Guinea pig	Human IgG	Inhib. PCA	C_H3	Minta & Painter [26]
(Mast cell)	Guinea pig	Rabbit IgG	RPCA, inhib. PCA	C_H3	Stewart et al. [43]
(Mast cell)	Human Monkey	Human IgE	Inhib. PK, PCA	?	Stanworth et al. [41, 42]

C = cytophilic pathway; O = opsonic pathway

aggregated human IgG, i.e. (IgG)n, was found to be incapable of stimulating *normal* human lymphocytes [15, 27]. But, in view of the difficulty of stimulating human lymphocytes with antihuman IgG, it will be wise to confirm that artificially aggregated rabbit IgG likewise failed to stimulate normal rabbit lymphocytes (which are readily transformed by treatment with anti-rabbit IgG antibody). As is now well known, lymphocytes can also be stimulated non-specifically by the action of lectins such as phytohemagglutinin and conconavlin A, which would appear to possess multiple (i.e. more than one) combining site for lymphocyte surface groups and, therefore, to be capable of fulfilling a similar bridging function to anti-immunoglobulin and protein A in the artificially-induced histamine release from mast cells (discussed earlier).

The analogy between mast cell exocytosis and phagocytosis by macrophages is more clear-cut inasmuch as the latter process is mediated by cytophilic antibody

either pre-bound to the target cell or in combination with antigen in a pre-formed complex; and it has been suggested that the part of the Fc structure of IgG molecules responsible for cytophilic binding may be identical with the phagocyte binding site of opsonizing IgG antibodies [13]; a view which is supported by the observation that the attachment of human IgG1 and IgG3 to human monocytes is inhibited by soluble antigen-antibody complexes (in a similar manner to inhibition, by free IgG1 and IgG3, of phagocytosis of opsonized red cells). But although Hay and his associates [13] suggest that the multivalent linkage of antigen is more likely to occur by the opsonization than the cytophilic antibody pathway, do their observations necessarily exclude the possibility that phagocytic cells can be directly triggered by effector sites localized within the Fc regions of antibody molecules as a result of combination with specific antigen? For there is evidence [35] of discrete attachment and ingestion steps in phagocytosis by macrophages; and the observed phagocytosis of glutaraldehyde-treated normal (i.e. non-opsonized) erythrocytes could possibly be attributable to the formation of a phagocytosis effector group within the normal IgG on the erythrocytes' surfaces as a result of aggregation by the chemical treatment.

The crucial question is whether separate target cell binding and effector sites within the Fc regions of IgG molecules are involved in lymphocyte stimulation and phagocytosis by macrophages and monocytes, as is suggested is the case as far as histamine release from mast cells is concerned. There is growing evidence that macrophage and monocyte binding sites, like mast cell binding sites, are located within the C_3H terminal domain of IgG molecules (as is indicated by the findings from many laboratories referred to in Table 3). Could it be, therefore, that – as appears to be the case with regard to histamine release – a phagocytosis effector site (or sites) is located within a different part of the mediatory IgG antibody Fc region; possibly within the C_H2 domain, where at least one other effector site (i.e. that involved in Clq activation) is known to be located?

Although, as already indicated (and as is apparent from Table 3), there is now substantial evidence on the manner in which cytophilic antibody molecules become attached to phagocytes, there is surprisingly little reliable information available about the mechanism of subsequent events including the fate of soluble antigen-antibody complexes. It has been suggested that although "free" IgG antibody molecules have an exposed binding site for attachment to phagocytes before reaction with antigen, more stable binding results from the formation of antigen-antibody complexes with multiple sites of attachment and that it is this which initiates subsequent phagocytosis [33]. Yet as these investigators admit, their data have not excluded the possibility that antigen-mediated aggregation may be necessary to bring about an allosteric change within the antibody molecule; particularly as it was observed that combination of IgG antibody with simple haptens failed to produce any enhancement of binding to phagocytes.

Hence, as has been suggested with regard to the immunological stimulation of histamine release from mast cells, it is still possible that a phagocytosis effector site is mobilized in cell bound cytophilic IgG antibody molecules on their combi-

nation with specific antigen and that such a site could be pre-activated in opsonizing antigen-antibody complexes. In this connection, it is worth referring again to the identification of the phagocytosis-stimulating tetrapeptide ("Tuftsin")* sequence Thr^{289}-Lys-Pro-Arg^{292} [29] located within the C_H2 domain of human IgG, because this was predicted quite independently by me [40] to be a potential histamine liberating sequence (on consideration of the results of our studies mentioned earlier on the structural basis of the direct histamine releasing activity of various well-defined polypeptides). It might also not be entirely fortuitous that an anaphylatoxic fragment (3a) of the third component of complement is a basic polypeptide capable of inducing histamine release in human skin [48]. Could it be, therefore, that the two human monocyte receptors for the third component of complement and for IgG, which have been shown to exert a co-operative effect on ingestion of erythrocytes by monocytes [16], are complementary to IgG effector sites comprising basic amino-acid residues possibly located within the C_H2 cytophilic antibody molecules?

Concluding Comments

It is obvious that, before the mechanisms of the various immunological triggering processes discussed here are fully elucidated, many more detailed investigations will be necessary. Moreover, if these are to be really fruitful, it will be important to develop new methods of measuring *directly* "in situ" the effect of antibody-antigen combination on the surface of target cells.

In the meantime, as has been suggested, it might prove profitable to look upon the mode of activation of lymphocytes and phagocytes as a process not too unlike the proposed mode of triggering of mast cells and basophils. Admittedly the "second messengers" involved in the various processes are probably different, as are the ultimate responses, but the mechanisms of the primary immunological trigger could yet prove to be remarkably similar in each case. Indeed, I would go further and suggest that it is not impossible that mediator IgG antibody molecules will be found to possess cell-binding sites located *within the same part* of their C_H3 domains, irrespective of the type of target cell involved (e.g. mast cell, macrophage, etc.), with fine structural differences within that part of their Fc region being responsible for recognition of the different cell types. Likewise, it is not entirely improbable that different effector sites responsible for different triggering processes will prove to be located within an essentially similar part of the Fc region (but different to that involved in cell binding) and they, too, could prove to possess some structural characteristics in common (at least as far as histamine release and phagocytosis are concerned).

* It should be pointed out that Najjar and his associates [28] have claimed (somewhat surprisingly) that "Tuftsin" is a peptide hormone covalently linked to the Fd region of a leucophilic IgG as *a branch off the main γ chain.*

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Changes in Lymphocyte Circulation after Administration of Antigen

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The recirculation of lymphocytes has been studied using the model of Bede Morris whereby vessels afferent to and efferent from single lymph nodes are cannulated (in sheep), enabling the collection of *all* efferent lymph over long periods of time in conscious animals when antigen is introduced *via* a cannulated afferent lymph vessel. Antigen and specific antigen-reactive cells are confined to the lymph node or diverted from the animal by the chronic efferent lymphatic fistula. The sequential changes in cell traffic, the appearance of blast cells and specific antibody forming cells, and the appearance of biologically active materials such as MIF and a mitogenic factor are described. Evidence is presented that when allogeneic lymphocytes are confined to a single lymph node, that node is responsible for removing all lymphocytes specifically reactive to those allogeneic lymphocytes from the recirculating pool of lymphocytes of the whole animal. The potential of this finding and of the model is discussed.

The migratory habits of lymphocytes

It is fully recognized that the cellular basis of the specific immune response is a property of lymphocytes and their interactions with each other and with other cells of the reticulo-endothelial system. It is perhaps less widely recognized that the movement of lymphocytes between various tissue compartments is an essential prerequisite for the normal immune response in the intact animal. This movement or traffic of lymphocytes has been examined in various ways in different experimental models. Gowans [1] demonstrated a large scale recirculation of lymphocytes from blood to lymph using a model based on the chronic collection of thoracic duct lymph from rats. This model has since been exploited to great advantage in rats and mice, and much has been learned about the migratory habits of thymus-derived (T) lymphocytes and marrow-derived (B) lymphocytes which has been comprehensively reviewed by Sprent [2]. One disadvantage with such models, however, is that thoracic duct lymph is a conglomerate of lymph from many different organs and tissues. Although it reflects, it does not directly measure, the finer details and kinetics of lymphocyte passage through individual organs and tissues or clearly defined lymphatic drainage areas. Ford [3] attempted to overcome this problem by using a different model, i.e. isolated perfused rat spleen, and he was

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able to provide the first direct measurements of lymphocyte traffic through the spleen.

It has become increasingly important to examine lymphocyte traffic in closer detail in the light of the rapid accumulation of new data on cell interactions in immune reactions. One may cite in this respect the cooperation of T and B cells and their interaction with macrophages and antigen [4], the surface receptors on lymphocytes and their relationship to lymphocyte activation [5], allogeneic inter-

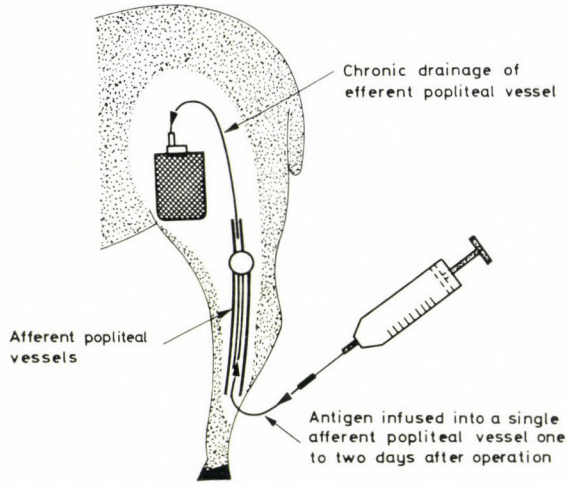


Fig. 1. Diagram showing the chronic lymphatic drainage model of a single lymph node originally developed by Bede Morris and used with various modifications in our laboratory

actions between various populations of lymphocytes [6, 7]. All of these areas are of major concern to immunologists today but there are stringent limits to the amount of new information that can be obtained about such interactions from purely *in vitro* methods. We must recognize that in order for cells to cooperate and interact in immune responses they must first be brought together. That is to say, immune reactions are the result of interacting systems and not just interacting cells.

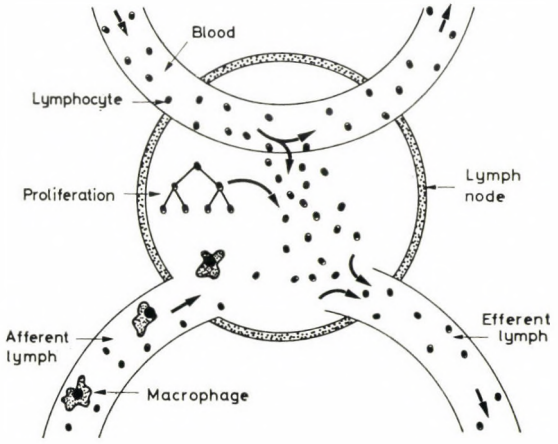
Another model exists for studying lymphocyte traffic, which is not simply a modification of chronic thoracic duct drainage but which allows one to follow an immune response isolated to a single lymph node, and subsequently to study the interaction of that "isolated" lymph node with the rest of the immune system of the body; at the same time enabling the passage of lymphocytes from blood to lymph to be studied in a precise and quantitative fashion. It is the model devised by Bede Morris [8, 9] (Fig. 1) whereby lymphatic vessels afferent to and efferent from single lymph nodes in sheep are cannulated and the lymph collected in conscious animals over long periods of time, and in amounts which do not prejudice the physiological integrity of the animal. This model permits a close examination of the interaction of the blood and lymphatic vascular systems and offers added

perspective to the physiological and pathological aspects of immune reactions as they occur in the intact animal.

It is our intention in this paper to summarize some of the work in our laboratory using this model. In particular to summarize the evidence that when antigen is confined to a single lymph node it is responsible for the specific removal of antigen-reactive cells from the recirculating pool of lymphocytes. This is a notion of potentially great practical importance since it raises the possibility that animals (including humans) may be rendered for several months specifically unresponsive to an antigen by removing one single lymph node into which the decisive majority of the appropriate specific antigen-reactive cells have been recruited.

Chronic lymphatic drainage from single lymph nodes

The preparation involves the continuous collection of efferent lymph from single lymph nodes (a resting lymph node of approximately 1 g weight produces with only minor daily fluctuations around 5 ml of lymph per hour which contains between 30 and 50 million small lymphocytes) enabling the simultaneous analysis of several plasma and cellular components. Macromolecules issuing from the node,



Resting lymph node of 1g (e.g. popliteal)
Output: $30x10^6$ cells/hr.
Input: $1x10^6$ cells/hr.; proliferation within the node
$3x10^6$ cells/hr. from afferent lymph
$26x10^6$ cells/hr. from blood via postcapillary venules

Fig. 2. Scheme of the blood and lymph compartments in a lymph node with special emphasis on the origin of efferent lymph. Note the uni-directional flow of lymphocytes. Quantitative aspects of lymphocyte migration of a resting lymph node of 1 g weight are given

for example antibody, can be obtained from the lymph in high concentration. During a vigorous immune response the cell output is increased to such an extent that an amount of cells equal to the wet weight of the entire node (1 g) can be collected in 24 hours. By cannulating an afferent vessel, antigen can be introduced directly into, and thereby confined to, a single lymph node. This is evident from the experimental fact that if all the efferent lymph is continuously collected *via* an indwelling cannula the development of systemic memory and the presence of antibody in the blood circulation are completely prevented [10]. There is evidence that proteins of a molecular weight greater than that of albumin, when injected *via* an afferent lymphatic directly into a lymph node, cannot enter the blood stream directly from the lymph compartment of the lymph node, but depend rather on an intact system of lymphatic vessels for their return to the blood *via* the thoracic duct [11]. The movement of plasma proteins is exclusively from blood to lymph within the lymph node and furthermore it is unlikely that cells can re-enter the blood directly from within the lymph node [12]. The lymph node is in fact "washed" in its own volume of lymph 3 times every hour and these "medium changes" are quantitatively collected so that soluble substances produced within the lymph node during any type of immunological reaction might be expected to appear in this efferent lymph. This is illustrated, for example, by the high titres of antibody which can be obtained in the lymph. Such an isolated lymph node preparation then possesses many of the advantages of a closed *in vitro* system but does not preempt the physiological interactions of the blood and lymphatic vascular systems (Fig. 2).

The events in efferent lymph during an immune response

When a single lymph node is stimulated with antigen, a characteristic sequence of events occurs in its efferent lymph. Essentially similar regardless of dose, type, and primary or secondary injection of antigen, this sequence of events has been systematically studied and documented for over twenty different antigens (Fig. 3). Thus, in a series of publications over the last 10 years the responses of single lymph nodes to numerous antigens have been examined, including bacteria [13], viruses [14], proteins [15], skin grafts [16], renal grafts [17] and during the development of adjuvant-induced granulomata [18] and delayed hypersensitivity reactions [19]. The changes which occur in efferent lymph after stimulation with antigen can be summarized as follows.

1. Shutdown

The output of lymphocytes, as already mentioned, from an unstimulated node is normally constant with only minor daily fluctuations. This resting output is approximately 30×10^6 cells/hr/g node weight and typically in a popliteal lymph node is between 30 and 50×10^6 cells/hr [20]. The immediate effect after introduction of antigen is to cause a drop in the cell output (but not in lymph plasma flow) which varies in severity and duration from antigen to antigen but which

may be profound, as for example with influenza virus where the efferent lymph is practically devoid of lymphocytes for the 24 hrs immediately following stimulation [14].

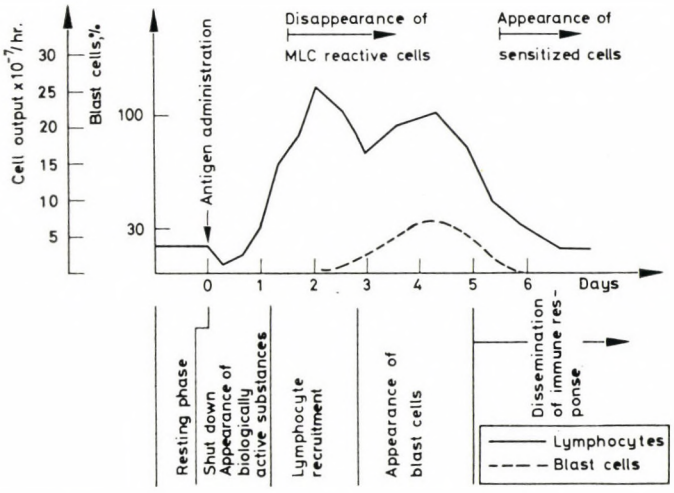


Fig. 3. Summary diagram of the events in the efferent lymph during an immune response based on the experimental results described in detail in the text. The diagram is a general one based on the results obtained from many individual experiments

2. *Appearance of biologically active factors in lymph plasma*

The first 24 hours after antigen are associated with many of the classical changes of an acute inflammatory response. There are for example permeability changes in the vascular endothelium of the lymph node and, depending on the antigen, there may be a transient release of neutrophils, or eosinophils in the efferent lymph [11, 21]. This is also a period characterized by the release of biologically active materials such as macrophage inhibition factor (MIF) and mitogenic factor (MF) which have been intensively studied in our laboratory. In fact, the discovery of a highly potent MIF by Hay, Lachmann and Trnka [18, 19] in the efferent lymph draining a tubercular reaction or following stimulation with mitogen concanavalin A by Hay [21] was the first clear demonstration that MIF was actually produced as an immediate consequence of antigenic stimulation *in vivo*.

3. *Lymphocyte recruitment*

The second 24 hours after antigen are marked by a sustained increase in the output of cells from the node. This increase, the first of 2 peaks in cell output

during a response, is due almost entirely to small lymphocytes which have migrated from the blood stream *via* the postcapillary venules into the lymph compartment and subsequently into the efferent lymph [22] and has been called lymphocyte recruitment by Morris [20]. More recently we have shown that this period of lymphocyte recruitment is a phase marked by the disappearance of specific antigen-reactive cells [23].

4. Antibody synthesis and the dissemination of immunological memory

The recruitment period is succeeded over the fourth and fifth days after antigen by a second increase in cell output which, unlike the initial recruitment peak which consists mostly of cells in transit, contains a significant proportion of blast cells which have emerged from the node in response to the antigen. Many of these newly formed basophilic cells have been shown to incorporate ^{3}H-thymidine *in vitro* [12] and, after certain antigens, to be secreting specific antibody [10]. These antibody forming cells are highly motile and differ from the residential plasma cells which remain in the node, in that they do not contain significant amounts of rough endoplasmic reticulum [24]. As mentioned earlier it has been shown that if the animal is deprived of the cells originating from the node in response to antigen by a chronic fistula of the efferent lymphatic duct, the systemic amplification of the immune response and the establishment of immunological memory are completely prevented [10, 24]. Furthermore, these effects can be reversed by the intravenous infusion of cells free of antigen collected at this stage of the response [24]. This is an important observation because it provides direct evidence that antigen-stimulated cells in the lymph node are precluded from direct access to the blood and that they depend on an intact lymphatic system for their dispersal and the consequent dissemination of the immune response.

Production of biologically active factors in lymph during delayed hypersensitivity reactions

Over recent years it has been clearly demonstrated that when sensitized T lymphocytes are cultured *in vitro* together with specific antigen they release soluble products into the culture medium [25]. Depending on the test situation used, such soluble lymphocyte products have been shown to exhibit a wide range of biological activities [26]. The driving force behind much of this work has been the attractive notion that soluble lymphocyte products might play an important role as "mediators" *in vivo* of the delayed hypersensitivity (DH) reaction.

It follows that a basic requirement of any plausible mediator of DH is that it should be produced locally at the site of a DH reaction in a high concentration and preferably that it should be extractable from the tissues at the time of its proposed action. Recent work in our laboratory has provided evidence that, with respect to one potential mediator of DH, namely macrophage inhibition factor

(MIF), this important requirement is indeed fulfilled [18, 19, 21]. It has been shown that during a tuberculin reaction [18, 19] and following the subcutaneous injection of 50 μg of concanavalin A [21] the draining lymph plasma (of the regional lymph node) contains two biologically active materials in high concentrations which were able to a) inhibit the normal migration of guinea-pig macrophages *in vitro* and b) stimulate normal sheep lymphocytes to synthesize DNA *in vitro*. The MIF produced in this way shared many of the properties of MIF produced by guinea-pig or human lymphocytes *in vitro*. It was first detectable about 6 hours after the injection of PPD into the sensitized animal and was present in maximum concentration after 24 hours and it had a MW in the albumin range on Sephadex G-200 columns. Unlike guinea-pig or human MIF, however, it was destroyed by heating at 56°C for 1 hour. MIF produced in response to concanavalin A had the same properties and was detectable as early as 3 hours after stimulation.

Although the demonstration that MIF is produced *in vivo* does not prove that it functions as a mediator, it does provide a firm basis for further investigation. The delayed hypersensitivity reaction is an inflammatory reaction and the attendant cell migration changes in vascular permeability and tissue destruction are unique to the intact animal. It seems hardly coincidental that a suspected mediator of the DH reaction is present in efferent lymph, not only when the changes in vascular permeability occur, but also immediately preceding the changes which occur in cell migration. In any event the possibility of collecting MIF in such large amounts should facilitate the difficult task of its purification which is essential if MIF is to be unequivocally shown to participate as a mediator of delayed hypersensitivity reactions.

Selective removal of antigen-reactive cells during lymphocyte recruitment

Lymphocyte recruitment, the increased traffic of lymphocytes from blood to lymph, which occurs within antigen-stimulated lymph nodes, was first described by Hall and Morris [22]. By continuous infusion of ^{3}H-thymidine they proved that the increased cell output was not due to proliferation within the node and by chronic drainage of afferent vessels they clearly demonstrated that it was not due to increased numbers of cells entering the node from the drainage area *via* the afferent lymphatics. They suggested that lymphocyte recruitment was non-specific and that it provided a mechanism whereby a large number of cells was brought into the lymph node so that cells of the appropriate specificity for the particular antigen could be selected for within that node.

If their interpretation was correct then it would imply that the efferent lymph of an antigen-stimulated node would be depleted of specific antigen-reactive cells at some time during an immune response. This hypothesis was tested in our laboratory [23] by exploiting the ability of certain antigen-reactive cells to proliferate

in vitro in the presence of the priming antigen. The experimental approach was to assay antigen-reactive cells in efferent lymph before and sequentially after introduction of the specific antigen into the lymph node. In one series of experiments involving tuberculin-sensitive animals, cells reactive to PPD were followed during the course of a tuberculin reaction. In a second group of experiments, one-way mixed lymphocyte reactions were used to assay for reactive cells directed against allogeneic lymphocytes. The reactive cells were followed after subcutaneous immunization with allogeneic lymphocytes or after the infusion of cells directly into a lymph node *via* an afferent lymphatic vessel. The results of these experiments can be summarized as follows.

1) Stimulation of single lymph nodes with allogeneic lymphocytes results in the sequential changes in the cellular composition of efferent lymph outlined in the introductory portion of this paper. In general the lymphocyte recruitment peak occurred between 1 to 3 days after challenge with antigen. Recruitment was followed by a second increase in traffic associated with a blast cell response and was succeeded by the appearance in the lymph plasma of specific antibody directed against the donor lymphocytes. Both agglutinating and cytotoxic antibodies were produced but they were never detected earlier than 96 hours after the node had been stimulated with antigen. It was therefore clear that, in terms of cell traffic changes, the appearance of blast cells and the production of specific antibody the response of single lymph nodes challenged with allogeneic lymphocytes was in principle not different from the responses to any other antigens tested in this system.

2) Stimulation of single lymph nodes with allogeneic lymphocytes produces a characteristic series of changes in one-way mixed lymphocyte cultures (MLC) obtained from efferent lymph cells draining the immunized node. Prior to immunization there is a certain level of MLC reactivity which depends on the genetic differences in histocompatibility between the recipient and the potential donor. In general, the incorporation of ^{3}H-thymidine into one-way MLC (where the responding cells were recipient in origin and the stimulator cells were donor lymphocytes prevented from reacting by irradiation with 4500 R immediately prior to culture) was 5 to 10 times the amount incorporated into either of the cell populations cultured alone. When $4-5\times10^8$ donor lymphocytes are infused *via* an afferent lymphatic into a single lymph node, there is a progressive fall in MLC reactivity so that by 30 to 40 hours after infusion no MLC reactive cells can be detected in the efferent lymph draining the node. This disappearance of MLC-reactive cells against the donor lymphocytes is maintained on the third and fourth days of the response. In the next phase of the response corresponding to the interval between 5 and 8 days after infusion of donor lymphocytes, there is a dramatic increase in MLC reactivity which usually peaked at 6 to 7 days before returning after the eighth day to preimmunization levels. During this period, ^{3}H-thymidine incorporation of stimulated cells in the MLC was as much as 100 times that in control cultures. These changes in the level of MLC-reactive cells in the efferent lymph draining the node were associated with changes in the *in vitro* kinetics of ^{3}H-thymidine incorporation in the MLC. Prior to immunization the peak of *in*

vitro ^{3}H-thymidine incorporation in MLC occurred on day 4 of culture. During the disappearance phase there was no difference in ^{3}H-thymidine incorporation between MLC and control cultures over the entire 6 days for which the *in vitro* cultures were maintained. However, during the period of greatly increased MLC reactivity, peak ^{3}H-thymidine incorporation occurred as early as the 1st day of *in vitro* culture and progressively declined over the next 5 days of culture. When the responses had ended and the MLC reactivity in the efferent lymph had returned to normal levels, the *in vitro* MLC kinetics reverted to the pattern observed before immunization.

3) The immunological specificity of the disappearance of MLC-reactive cells is difficult to determine in these experiments because of the lack of satisfactory assays for antigen reactive cells specific for other antigens. The obvious specificity control was to use a third party sheep (presumed to be genetically distinct from both the recipient and the donor sheep) and to look for any changes in recipient MLC-reactive cells to that third party control animal. MLC-reactive cells in such control combinations were depressed during the period when the MLC-reactive cells from the experimental recipient–donor combination had completely disappeared. Significant MLC reactivity in the control cultures was, however, maintained and the depression which occurred could be explained on the basis of cross-reactivity between antigenic similarities in the histocompatibility locus of the third party sheep and the donor sheep.

In order to test more critically whether the disappearance of antigen-reactive cells was specific for the immunizing antigen, two further systems were employed in which the recipient sheep had been immunized with BCG two to three months before the individual node was stimulated with antigen.

In the first series of experiments the level of PPD-reactive cells in the efferent lymph from a single node was established by culturing efferent lymph cells in the presence of 10 μg of PPD for 3 days *in vitro* and determining the amount of ^{3}H-thymidine incorporation which had occurred. The lymph node was then challenged with 100 μg of PPD and efferent lymph from the node during the subsequent response was sequentially examined for PPD-reactive cells using the assay outlined above. It was found that there was a progressive fall in PPD-reactive cells early in the response and that during the second day of the response they were undetectable and on the third day they emerged in greatly increased numbers. Thus the sequence of events was similar to the changes in the MLC-reactive cell during an allogeneic response. This disappearance of PPD-reactive cells was specific because there was at no stage during the response to PPD any decline in the level of MLC-reactive cells as assayed by one-way MLC with a second control animal.

The reverse of this experiment provided further evidence of the specificity of the depletion of antigen-reactive cells from the efferent lymph of antigen-stimulated nodes. When MLC-reactive cells were sequentially assayed during an allogeneic response in a BCG-primed animal, MLC-reactive cells disappeared but PPD-reactive cells remained unaffected.

4) The most provocative observation was noticed when the recirculating pool of lymphocytes was examined by studying the efferent lymph draining a contralateral lymph node remote from the site of antigen administration. Three days after allogeneic lymphocytes were introduced into the test node, specific MLC-reactive cells were undetectable in the recirculating pool of the whole animal as assayed by the above-mentioned MLC reactivity technique. Moreover, unlike the immunized lymph node where this period of undetectable MLC reactivity was succeeded by a period of greatly enhanced MLC reactivity, specific MLC-reactive cells remained undetectable in the recirculating pool. When the immunized node was surgically removed after the recruitment period, the recirculating pool was still specifically MLC-negative 2 months later when the last MLC was performed.

Problems in understanding the phenomenon of the disappearance of specific antigen-reactive cells from the recirculating pool of lymphocytes

The results of the experiments outlined above raise important questions about the influence of antigen administration on the pattern of lymphocyte recirculation. If one accepts a precommitment of immunological specificity by lymphocytes [27], then the most economical explanation of our findings is that there has been a selective removal of specific antigen-reactive cells during lymphocyte recruitment. Partial depletion of specific antigen-reactive cells in thoracic duct lymph following intravenous injection of the corresponding antigen has been described in mice by Sprent et al. [28, 29] and in rats by Ford et al. [30, 31] and Rowley et al. [32, 33]. Ford and Atkins [30] demonstrated this effect for cells directed against histocompatibility antigens following the intravenous injection of allogeneic lymphocytes. Recently with higher doses of antigen Sprent and Miller [34] have shown that after the intravenous injection of sheep red blood cells (SRBC) there is specific and complete disappearance of specific antigen-reactive cells from thoracic duct lymph. Furthermore they were able to recover the specific antigen-reactive cells from the spleen (a major site of SRBC localization after intravenous injection; the spleen, as it were, functioning as a type of lymph node of the blood vascular circulation) but were unable to recover them from mesenteric lymph nodes.

When the notion of selective removal of specific antigen-reactive cells is applied to our experiments, important differences between the results for mice and rats have to be considered. In the latter experiments the implication is that antigen-specific cells are continuously recirculating and that after administration of antigen such cells fail to recirculate when they encounter a site of antigen localisation. In the experiments of Sprent and Miller [34] antigen appears to be localized in the spleen rather than in lymph nodes but even if antigen was distributed throughout the animal, for example, in the spleen, liver and lymph nodes, specific selection of antigen-reactive cells would still cause depletion in thoracic duct

lymph; indeed it might be expected to occur more rapidly since there would be more sites available for specific "filtering" of the recirculating pool. However, since Ford and Gowans [35] have shown that the entire recirculating pool could pass through the spleen during a period of about 3 days, the interpretation of Sprent and Miller that specific antigen-reactive cells were removed from the recirculating pool during the physiological passage of lymphocytes through the spleen is reasonable even if antigen was entirely localized to the spleen, because the depletion observed by them occurred over a period of time sufficient to allow the spleen to screen the entire recirculating pool.

In our experiments if one considers first the situation with respect to the lymph node which received antigen *via* a cannulated afferent lymphatic vessel, then the concept of specific selection of antigen-reactive cells as they pass from blood to lymph through a lymph node which contains a high concentration of antigen provides a plausible and appealing explanation for the absence of specific antigen-reactive cells in the efferent lymph.

It is not, however, the only possible explanation. Specific antigen-reactive cells may not have disappeared but instead may be in a state of specific unresponsiveness and therefore "silent" in our *in vitro* assays. Specific unresponsiveness would have to be an antibody-mediated phenomenon and there are only two possible sources of such antibody. On the one hand recipient cells could produce specific alloantibody directed against the donor lymphocytes and thereby "block" the *in vitro* MLC. There are two reasons which make this possibility unlikely. First, recipient alloantibody has never been detected before 96 hours, and second, it is present in the lymph at the time of greatly enhanced MLC reactivity. Alternatively, alloantibody specifically directed against the recipient cells could be released by the donor cells which were infused into the lymph node and as a result render the recipient cells silent in the MLC. This possibility is unlikely because occasionally the MLC reactivity is absent as long as 8 days after immunization and secondly, because in preliminary experiments the disappearance of MLC-reactive cells has occurred after the infusion of irradiated donor cells where significant production and release of alloantibody by the donor cells is unlikely. Furthermore, in the case of the disappearance of PPD-reactive cells the possibility of specific blocking antibody is most unlikely. In summary then the selective removal of specific antigen-reactive cells by the lymph node acting as a biological filter is supported by our evidence obtained with single lymph nodes and by the evidence gathered in other systems notably rats and mice [28–30, 32, 33]. The important difference in our experiments is that there is strong evidence that antigen and/or antigen-stimulated cells are either confined to the single lymph node which received the infusion of antigen, or they are drained away from the animal via the chronic efferent lymphatic fistula. Yet the entire recirculating pool of the animal appeared to be specifically depleted of MLC-reactive cells, not only under conditions where clearly the whole recirculating pool did not pass from the blood stream *via* the lymph node into the lymph and into our collecting bottles, but such MLC-reactive cells disappeared under conditions where the immune response was con-

fined to the single lymph node which received the antigen. If the explanation of our results is the selective removal of specific antigen-reactive cells from the recirculating pool, then one is faced with the difficulty of explaining how one single lymph node could effectively screen the entire recirculating pool in a period of only 2 or 3 days and at the same time remove from it all MLC-reactive cells variously estimated at as much as 12% of the total recirculating pool [6, 36]. One prerequisite of any acceptable explanation based on the concept of selective removal is that the blood flow through the lymph node must be sufficient to enable the total recirculating pool of lymphocytes to reach the blood vascular network of the stimulated lymph node over the period of disappearance of specific antigen-reactive cells. This prerequisite is supported by the demonstration of the dramatic increase in blood supply to an antigen-stimulated node [12, 37].

Two possible mechanisms of selective removal might be considered. First, recirculating lymphocytes entering into the lymph compartment of the lymph node from the blood might not use the lymphatic system exclusively for their return to the blood. Instead there might be a large scale re-entry of irrelevant antigen-reactive cells directly from the lymph compartment of the node back into the blood circulation. There is no direct evidence of the existence of such a circulation and arguments for and against it must remain in the realm of pure speculation.

A second alternative is to invoke a selective mechanism for lymphocyte subpopulations at the level of the vascular endothelium of the postcapillary venules. If antigen or some product of antigen could confer specificity at the level of the endothelium, then the efficiency of specific selection could be greatly increased by this additional endothelial component. Specific MLC-reactive cells recognizing an "endothelial receptor" could preferentially leave the blood stream and superimpose a specific element on lymphocyte recruitment. This second alternative in the absence of direct evidence must also remain entirely speculative. Nevertheless when there is strong evidence that the concept of selective removal of specific antigen-reactive cells accounts for our failure to detect such cells in the efferent lymph draining antigen-stimulated nodes, a unifying concept that would apply the same mechanism to explain our failure to detect specific antigen-reactive cells in the entire recirculating pool is tempting despite all its inherent difficulties.

The possibility that a better understanding of the pathophysiology of lymphocyte traffic through single lymph nodes may lead to the development of a simple and practical method to obtain a state of long-lasting and specific unresponsiveness in a clinical situation more than amply justifies further studies in this complex field. Indeed, regardless of the explanation for the phenomena we have described the contingency our experiments have raised that antigen-induced changes in the circulation of lymphocytes through a single lymph node induce remarkable changes in the circulation of subpopulations of lymphocytes in the animal as a whole, emphasizes the poverty of our insight into the homeostasis of lymphocyte circulation.

The detailed documentation of this work from our laboratory is either in press or in preparation for publication in an additional series of individual papers.

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The Usefulness of Scatchard Plots in the Analysis of Antigen-Antibody Interactions at the Surface of the Red Cell Membrane*

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The Scatchard conversion of the law of mass action has been used extensively for quantitating the reactions of antibodies with red cells. We have found that it is not completely satisfactory for these measurements and suggest that use of the red cell concentration at which 50% of the absorbable antibody is bound is more suitable. The required value is obtained by plotting the percentage of antibody absorbed using a probit scale on the ordinate against the concentrations of red cells on a logarithmic scale on the abscissa.

Introduction

In 1949 Scatchard [1] published a method based on the law of mass action for estimating how many and how tightly small molecules and ions reacted with proteins. He pointed out "the great importance of the greatest possible precision over the widest possible range in order that these curves may be extrapolated to the intercepts which tell us 'how many' and 'how tightly bound' ". The Scatchard plot has been used extensively for estimating the number of receptors on red cell membranes and for quantitating blood group antibody activity [2]. Our interest in the quantitative immunobiology of the red cell surface and its use as a model for studying the general phenomena of the interaction of biological macromolecules required us to re-evaluate the applicability to and the accuracy of the resulting estimates of protein-membrane reactions which can be measured with precision only over a relatively narrow range of reactant concentrations. The purpose of this paper is to describe and evaluate preliminary observations made by us in quantitating the reactions between antibodies and their receptors on intact human red cells.

Materials and Methods

Blood samples of laboratory staff members (T. G., N. B., S. L. and J. F.) were collected in acid-citrate-dextrose NIH formula B in a proportion of 1 part ACD: 6 parts blood (Vacutainer S3204X, Becton-Dickinson, Rutherford, N. J.).

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The anti-D concentrates used for radioiodination were prepared by precipitating the gamma globulin fractions of the antibody-containing plasma with an equal volume of saturated $(NH_4)_2SO_4$. After removal of the $(NH_4)_2SO_4$ by exhaustive dialysis against saline, an eluate was prepared by using an insoluble immunoadsorbent developed in this laboratory [3] and concentrated by $(NH_4)_2SO_4$ precipitation.

Radioiodination using chloramine-T was performed by a slight modification of the method of McConahey and Dixon [4] employing 2 mCi of carrier-free $Na^{125}I$ (New England Nuclear, Boston, Mass.) per 5 mg of gamma globulin. Remaining free $Na^{125}I$ was removed by dialysis and the specific activity of the labeled eluate was determined by conventional methods using Folin-Ciocalteu reagent for measuring the protein content [5]. In all subsequent calculations it was assumed, as usual, that almost all the protein in the labeled eluates was gamma globulin and that non-antibody and antibody IgG molecules were uniformly labeled.

The ^{125}I eluate used for the quantitative studies was prepared by absorption and elution from red cell ghosts using the digitonin-pH 3 glycine method of Kochwa and Rosenfield [6].

The assays with the ^{125}I-labeled eluates of anti-D were performed essentially as described by Hughes-Jones and his associates [7, 8].

The calculations for the Scatchard plots were obtained by substituting observed values in the following equation derived by him from the law of mass action

$$\frac{r}{[A]} = Kn - Kr$$

in which r is the amount of antibody in moles taken up by 1 ml (10^{10}) of red cells at equilibrium; [A] is the moles of free antibody per ml of red cells at equilibrium; K is the equilibrium or binding constant; and n theoretically represents the moles of antibody which can be bound by all the specific antigen sites available on 1 ml of red cells; theoretically when the extrapolated curve crosses the abscissa $\frac{r}{[A]} = 0$ and $r = n$. It must be assumed that the antibodies behave as if they were univalently attached to the red cells by only one of their receptors [9].

The log-probit conversion [10] makes it possible to obtain a straight line when the probit of the percentage of antibody taken up by the red cells at each red cell concentration used is plotted on the ordinate [11] and the logarithm of the concentration of red cells expressed as ml of red cells/ml of reaction mixture is plotted on the abscissa.

Results

Typical log-probit plots of ^{125}I anti-D uptake by different D positive red cells are shown in Fig. 1. The straight line is calculated by the method of least squares [11]. These data are then used to draw the Scatchard curves illustrated in Figs 2 and 3.

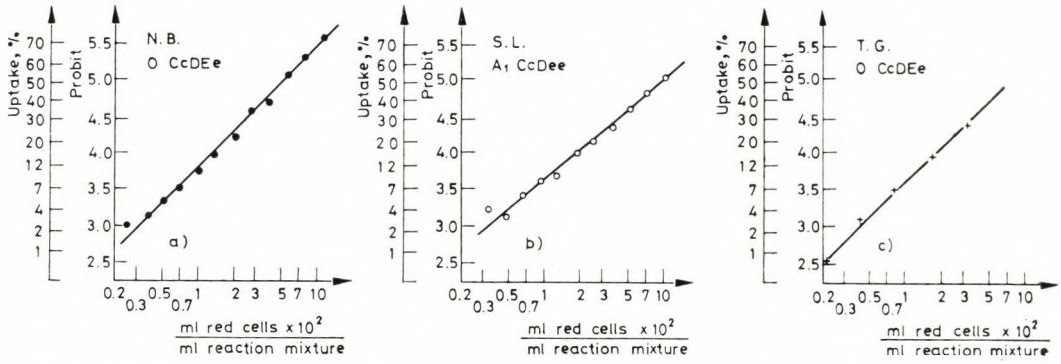

Fig. 1. Absorption of ^{125}I anti-D antibodies by increasing numbers of red cells reacted to equilibrium at 37°C. The regression line was calculated by the method of least squares [11]

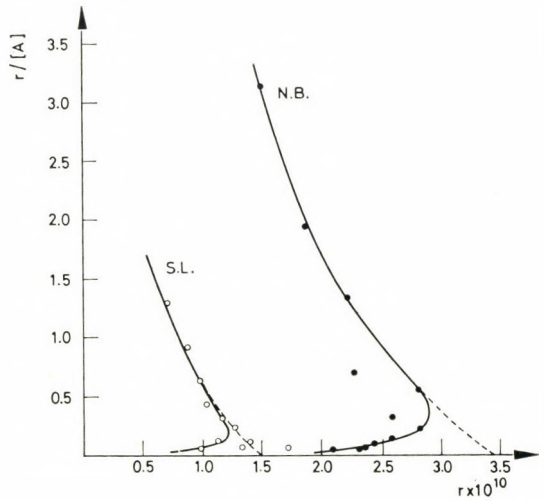

Fig. 2. Scatchard plots constructed from the least squares regression lines given in Fig. 1. The points shown are the actual experimental data which were used in plotting the log-probit regression lines. r = moles of antibody bound per ml (10^{10}) red cells; [A] = moles of free antibody per ml red cells

Table 1

Comparison of the number of D sites per red cell calculated by the three methods described in the text

Blood sample	Number of D sites determined by		
	conventional Scatchard extrapolation	r value at "turn" of Scatchard plot	2×50% uptake
N. B. O CcDEe	20 659	17 226	26 966
S. L. A_1 CcDEe	8 914	7 378	9 374
T. G. O CcDEe	*	14 455	29 700
J. F. A_1 CcDee	12 648	11 323	14 156

* extrapolation not possible, see Fig. 3.

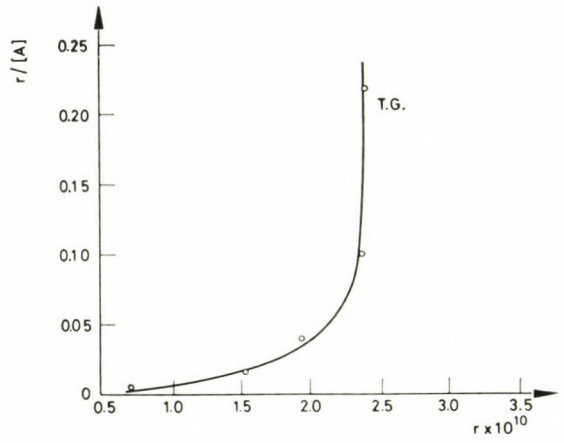

Fig. 3. Scatchard curve plotted as Fig. 2, but these data were collected to emphasize the shape of the curve at extremely high relative antibody excess. Note the difference in the scale of the ordinate

The number of antigenic sites per cell calculated from conventional Scatchard extrapolation, the point at which reversal of the curve occurs, and the point at which 50% of the antibody is bound are compared in Table 1.

Discussion

Fong and Masouredis [12] found that with a fixed number of red cells measurements in excess amounts of radioiodinated anti-D could sometimes be fitted to a straight line on the Scatchard plot but some ^{125}I eluates showed inhibition and reversal of the curves. Hughes-Jones's observations at equilibrium with varying

concentrations of red cells in the presence of a fixed concentration of antibody always result in curvilinear plots. The non-linear nature of Scatchard-type plots is recognized as characteristic of hapten-antibody systems [13] and therefore our observations should not be surprising. The preparation of antibody concentrates of high potency and specific activity made it possible for us to carry out measurements at high and low relative red cell concentrations which brought to our attention the fact that $\frac{r}{[A]}/r$ plots appear asymptotic at both ends and extrapolation at best gives a relatively crude approximation of n. We have found reversal of the binding curve when the experiments were carried out to sufficiently low red cell concentrations. The least squares log-probit plot enables us to pick theoretical points for our Scatchard curves based on the same data which demonstrate that theoretically the Scatchard curves should always be expected to turn around when the ratio of antibody molecules to red cell numbers exceeds a certain maximum. We chose to use the log-probit method of analysis because it has been found so useful by pharmacologists to study drug dose-response reactions in biological systems and has the advantage of being linear in the greatest range of usefulness in our systems except when there is marked antigen excess. Certainly the reaction of 7S antibodies with red cells is dose-related and is at least the first stage of a biological response.

In Table 1 we have compared the number of D-sites on the red cell using three different methods of calculation: (1) the value of n using conventional Scatchard extrapolation; (2) the value of n assuming that the turning point of the Scatchard plots approaches complete saturation or $r = n$; and (3) the value of n assuming that 50% of the antigen sites are occupied at the cell concentration at which 50% of the absorbable antibody is bound.

In view of the subjectivity of the values obtained by Scatchard extrapolation, we propose that a nonarbitrary point (i.e., 50% antibody absorption) provides a more objective basis for comparing the number of available antigenic sites on red cells, and for calculating antibody concentrations and thermodynamic constants.

*

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Die Funktion der neutrophilen Granulozyten und der Monozyten im Rahmen der zellulären Abwehr

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Unter den Abwehrmechanismen des menschlichen Körpers hat das durch die Funktion der neutrophilen Granulozyten und der Monozyten vermittelte phagozytäre System eine besondere Bedeutung. Die durch den Blutstrom bedingte praktisch ubiquitäre Verteilung und große Reserven an verfügbaren Zellen kennzeichnen diese myelogenen Systeme unter normalen Bedingungen. Phagozytose, Bakterizidie, Abbau und Beseitigung bzw. Abkapselung organischer und anorganischer Materialien sowie verschiedene Aufgaben im Rahmen der humoralen und zellgebundenen Abwehr stellen die wesentlichsten Funktionen dieser Zellsysteme dar. Neutrophile Granulozyten und Monozyten-Makrophagen weisen zahlreiche funktionelle Unterschiede auf, ergänzen sich jedoch in ihren Aufgaben. Die wechselnde Zusammensetzung der zellulären Exsudate spiegelt die unterschiedliche funktionelle Bedeutung der Entzündungszellen wider.

Reife neutrophile Granulozyten sind hoch differenzierte und spezialisierte, nicht proliferationsfähige Zellen. Sie weisen eine hohe Phagozytoseleistung auf und sind durch ihren hohen Gehalt an zytoplasmatischen Granula gekennzeichnet, welche — abhängig vom Zeitpunkt ihrer Produktion im Rahmen der Ausreifung der neutrophilen Reihe — einen unterschiedlichen Enzymgehalt aufweisen. Unter diesen Enzymen finden sich eine Reihe kataboler lysosomaler Enzyme sowie einige bei neutralem pH besonders aktive Proteasen. Verschiedene bakterizide Enzyme und Proteine ergänzen die funktionelle Ausstattung der Granula. Aufgrund ihrer metabolischen und zytologischen Eigenschaften sind die neutrophilen Granulozyten nur zu einer kurzdauernden, wenn auch sehr spezialisierten Funktion befähigt.

Im Gegensatz zu den Neutrophilen weisen die Monozyten des peripheren Blutes eine vergleichsweise geringe Differenzierung auf, und sie erreichen ihre volle funktionelle Leistungsfähigkeit erst nach ihrer Umwandlung in Makrophagen, Histiozyten, Epitheloid- oder Riesenzellen. Diese Umwandlung erfolgt im extravasalen Milieu unter entsprechender Stimulation und durch Enzyminduktion können zytochemisch differierende Makrophagen entstehen in Abhängigkeit vom umgebenden Milieu und von den abzubauenden Substanzen. Die wesentlichsten Ereignisse bei der Umwandlung sind die Aktivierung des RNS-Stoffwechsels, Zunahme der Zellgröße und Vergrößerung und Aktivierung des GERL (Golgiapparat, endoplasmatisches Retikulum, Lysosomen) sowie Umstellungen im Bereich des oxydativen Stoffwechsels. Im Gegensatz zu den kurzlebigen neutrophilen Granulozyten sind Makrophagen und Epitheloidzellen in der Lage, Wochen und Monate zu überleben und — unter besonderen Bedingungen — zu proliferieren. Die bakteriziden Mechanismen sind in den Neutrophilen und Monozyten-Makrophagen grundsätzlich ähnlich, in den letzteren sind sie allerdings viel wirksamer und vielfältiger, wenn auch erschöpfbar.

Angeborene und erworbene Störungen der besprochenen intrazellulären Funktionen dieser Phagozyten führen zu zahlreichen Krankheiten. Einige der pathophysiologisch interessanten Formen werden kurz besprochen.

Die Abwehr körperfremder, belebter Organismen und die Beseitigung anorganischer Materialien und auch krankhafter körpereigener Zellen wird im menschlichen Körper wie auch in dem höher entwickelter Tierarten durch die Funktion von Zellen und von zellulären Stoffwechselprodukten bewirkt. Durch den unmittelbaren Kontakt der Effektorzellen mit dem zu beseitigenden Material ist die zelluläre Abwehr gekennzeichnet. Die humorale Abwehr wird durch Eiweißkörper des Plasmas und durch die Zellen, die diese produzieren, vermittelt. Diese begrifflich klare Unterscheidbarkeit darf nicht darüber hinwegtäuschen, daß erst der Synergismus dieser komplexen Funktionssysteme eine optimale Abwehrfunktion des Organismus gewährleistet. Die Phagozytose und der intrazelluläre Abbau des phagozytierten Materials sind zytologische Eigenschaften, die den meisten Zellen des Organismus gemeinsam sind. Für die zelluläre Abwehrleistung des Organismus sind im wesentlichen die Zellen des RES und die neutrophilen Granulozyten wie die Monozyten verantwortlich. Während die ersteren in verschiedenen Organen weitgehend fixiert und überwiegend für die Entfernung im Blut zirkulierender Partikel verantwortlich sind, können die polymorphkernigen Neutrophilen und Monozyten aufgrund ihres raschen Transportes durch das Blut und ihrer Motilität, die es ihnen gestattet aus den Gefäßen – gelenkt durch Chemotaxis – in die Entzündungsgebiete einzuwandern, als praktisch ubiquitär im Organismus betrachtet werden. Die Ubiquität wird noch weiter unterstrichen durch die Tatsache, daß durch die Verfügbarkeit der intravasalen und des Speicherpooles des Knochenmarkes fast beliebige Mengen Neutrophiler rasch am Ort des Bedarfes angehäuft werden können.

Die besondere Verfügbarkeit dieser Zellen ist jedoch nicht das einzige Kriterium, auf dem ihre Bedeutung für die zelluläre Abwehr beruht. Diese wird in viel größerem Ausmaß durch die speziellen zytologischen Eigenschaften der Neutrophilen und der Monozyten bedingt. Diese Besonderheiten sollen nun besprochen werden.

Reife, polymorphkernige neutrophile Granulozyten des peripheren Blutes weisen im Gegensatz zu ihren Vorstufen wie Promyelozyten, Myelozyten und Metamyelozyten eine sehr geringe Fähigkeit zur Proteinsynthese und einen geringen Gehalt an Ribonucleinsäuren auf [16]. Morphologisch äußert sich diese in einem geringen Gehalt an Ribosomen, Ergastoplasma und in einem kleinen Golgi-Apparat [9]. Kennzeichnend für diese Zellform ist der Gehalt an etwa 600 bis 1000 primären und sekundären Granula; erstere werden im Stadium des Promyelozyten gebildet und stellen ca. 20% der Granula der polymorphkernigen Neutrophilen dar [3, 9]. Sie enthalten typische lysosomale hydrolytische Enzyme mit einem pH-Optimum im Bereich von 4.0 bis 5.0 (z. B. saure Phosphatase, Desoxyribonucleasen, β-Glucuronidase u. v. a.) sowie etwas Lysozym, eine große Menge Peroxydase und eine hohe zytochemisch nachweisbare Aktivität von Naphthol-AS-D-Chloroacetat-Esterase [2, 15]. Diese Naphthol-AS-D-Chloroacetat-Esterase konnte als neutrale Proteaseaktivität der Granulozyten identifiziert werden. Durch Polyacrylamid-Gelelektrophorese waren insgesamt 12 distinkte Esterasebanden darstellbar, die keine Überlappung mit der Aktivität der Peroxydase, sauren

Phosphatase, β-Glucuronidase, Leucinaminopeptidase, Alaninaminopeptidase bzw. dem Lysozym zeigten. Die neutralen Proteasen konnten durch 0.15 M NaCl fraktioniert solubilisiert werden. Die Gelfiltration der in 0.15 M NaCl, pH 7.0 löslichen Granuloproteine über Sephadex G-75 ergab zwei Proteasefraktionen, von denen die erste Proteine mit einem Molekulargewicht von ungefähr 70 000, die zweite Proteine mit einem Molekulargewicht von ungefähr 33 000 enthielt, darunter die Elastase-ähnliche Aktivität der Granulozyten. Die Solubilisierung der kationischen Proteasen, die die Chymotrypsin-ähnliche Aktivität der Granulozyten darstellen, gelang mit 1 M NaCl pH 7.0 [15].

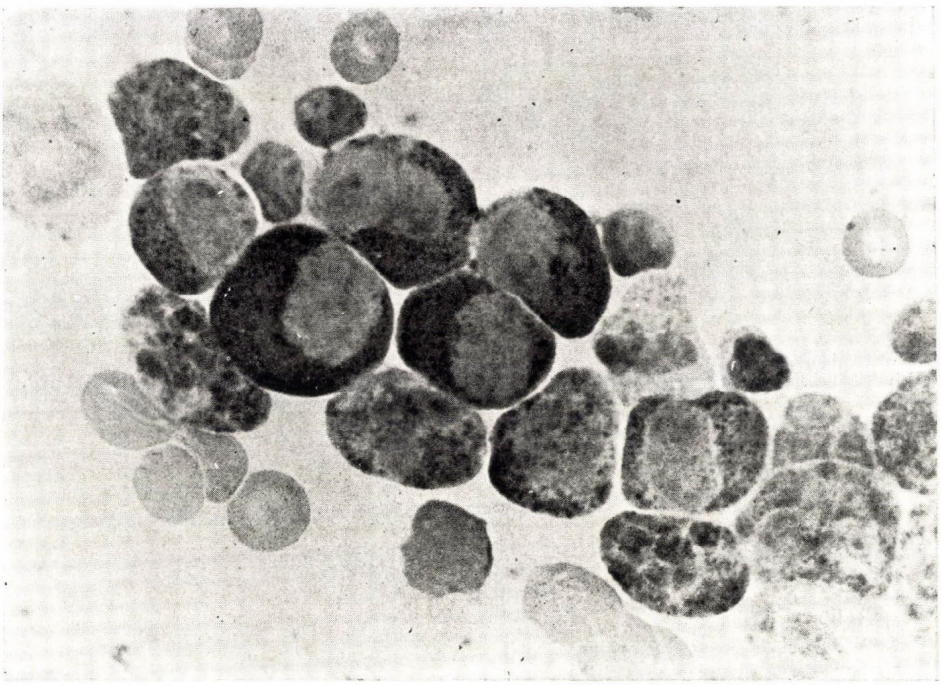

Abb. 1. Starke Naphthol-AS-D-Chloroacetat-Esterase-Aktivität (neutrale Proteasen, siehe Text) in den Zellen der neutrophilen Reihe, Monozyten (schwach) und Eosinophilen (negativ) 1250×

Die Bildung der sekundären Granula findet in den Stadien des Myelozyten und des Metamyelozyten statt [3] und liefert elektronenoptisch etwas weniger dichte Granula mit einem Gehalt an Lactoferrin und Lysozym (2/3 der Gesamtmenge), um die wichtigsten Granulakomponenten zu nennen [2]. Die Lokalisation der alkalischen Phosphatase erscheint neuerdings wieder unsicher. Microtubuli sind äußerst labile subzelluläre Strukturen, die maßgeblich für die Funktion der Granula und zusammen mit den Mikrofilamenten möglicher-

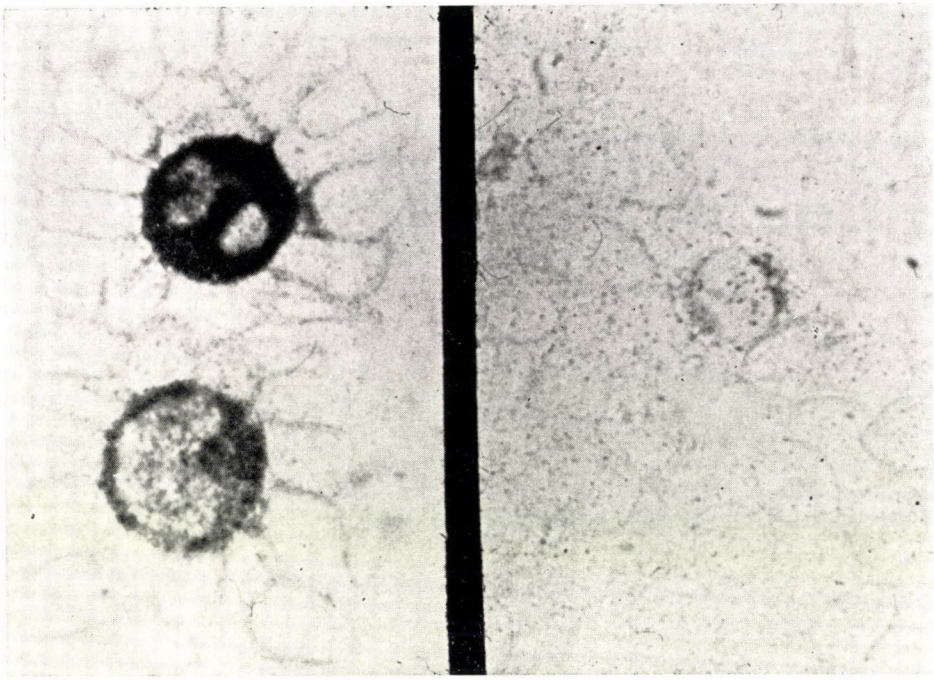

Abb. 2. Glukose-6-Phosphat-Dehydrogenase in einem neutrophilen Granulozyten, einem Monozyten und einem Lymphozyten. 1250×

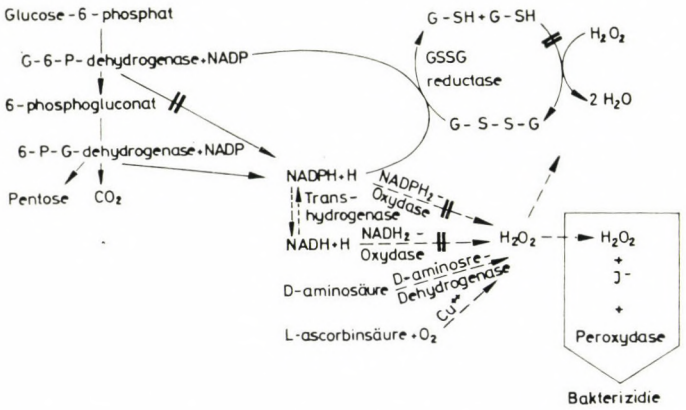

Abb. 3. Stoffwechselvorgänge in den Leukozyten, die zur Produktion von H_2O_2 führen können. || bezeichnet Blockierungen der Stoffwechselprozesse, die als Ursachen der chronisch granulomatösen Erkrankung des Kindesalters in Betracht gezogen werden

weise auch für die Motilität der Neutrophilen und Monozyten von Bedeutung sind [13]. Eine gegenüber den meisten anderen Körperzellen abweichende Stoffwechselsituation findet sich im Hinblick auf das Überwiegen der Glykolyse, über welche in Ruhe mehr als 95% der utilisierten Glukose abgebaut werden [16]. Durch diese Stoffwechselsituation werden die Neutrophilen in die Lage versetzt, energieliefernde Phosphate notfalls auch unter völlig anaeroben Bedingungen zu erzeugen und damit auch unter diesen Bedingungen die Zellfunktion und insbesondere die sehr hohe Phagozytoseaktivität aufrecht zu erhalten. Entsprechend der geringen oxydativen Phosphorylierung finden sich wenige Mitochondrien. Der sehr hohe Glykogengehalt der Neutrophilen stellt das Depot für den hohen Glukoseverbrauch dar. Die Phagozytose, d. h. die Aufnahme extrazellulären Materials in intrazytoplasmatisch gelegene Phagosomen, geht mit einer massiven Stoffwechselsteigerung einher [1, 4, 11, 16]. Eine Besonderheit der Neutrophilen stellt die relativ hohe Aktivität des Hexosemonophosphat-Shunts dar, die insbesondere bei Phagozytose um maximal das Zwanzigfache gesteigert werden kann [6, 11, 16]. Mit großer Wahrscheinlichkeit dient dieser Prozeß der Bereitstellung des für die Bakterizidie wichtigen Wasserstoffsuperoxyds [11] (siehe Abb. 3). Der Abbau des in den Phagosomen vorhandenen Materials erfolgt durch die Enzyme der Granula. Letztere entleeren ihren Gehalt an Enzymen (siehe Tabelle 1) in das Innere des Phagosoms, d. h. der Phagozytosevakuole unter Erzielung einer hohen Enzymkonzentration, ohne daß dabei das restliche Zytoplasma den hochaktiven Enzymen ausgesetzt wird [7]. Ein graduelles Absinken

Tabelle 1

Inhalt der Granula der neutrophilen Granulozyten

Mit sicherer oder wahrscheinlicher Lokalisierung in:	
Primären Granula	Sekundären Granula
Lysosomale Enzyme	
Saure Phosphatase	Naphthylamidase (= Protease?)
DNase	Lysozym
RNase	Lactoferrin
β-Glucuronidase	Bakterizide Proteine
Glycosidasen	
Arylsulphatase	
"neutrale" Proteasen:	
= Naphthol-AS-D-Chloroacetat-Esterasen	
Elastase	
Kollagenase	
Histonase	
Chymotrypsin-ähnliche Protease	
u. a.	
Peroxydase	
Lysozym	
Bakterizide Proteine	
Saure Mucopolysaccharide	

des pH im Phagosom ermöglicht den Enzymen eine Funktion in ihrem pH-Optimum [10]. Bakterien und Pilze können durch mehrere Systeme in den Phagosomen abgetötet werden. Peroxydase und Wasserstoffsuperoxyd stellen in Gegenwart von Halogeniden ein wirksames bakerizides System dar, das insbesondere Staphylokokken, Candida, Escherichien u. a. zerstört [1, 11, 12]. Weiters entfalten das in den Granula enthaltene Lysozym sowie indirekt auch das Lactoferrin eine bakteriostatische bzw. eine bakterizide Wirkung. Eine hohe Bakterizidie weisen auch stark basische Granulaproteine auf, an deren Charakterisierung derzeit noch gearbeitet wird [15]. Bei gesteigerter Phagozytoseaktivität, z. B. auch bei Phagozytose von Ag-AK-Komplexen kommt es zu einer Abgabe von Granulaenzymen in die Umgebung und dadurch zur Möglichkeit einer enzymatischen Schädigung der umliegenden Gewebe.

Im Gegensatz zu den sehr spezialisierten Neutrophilen weisen die Monozyten einen wesentlich einfacheren Bau auf. Die Untersuchung der Entstehung und der Funktion dieser an sich schwer identifizierbaren Zellen wurde besonders durch ihre Charakterisierbarkeit an Hand der Natriumfluorid-hemmbaren Naphthol-AS-Acetat-Esterase ermöglicht [16]. Diese gestattete den Nachweis der Monozytenentstehung im Knochenmark und die Verfolgung des Entwicklungsweges dieser Zellform über die Monozyten des Blutes zu den Funktionsformen – die Makrophagen – in den Geweben und in den Entzündungsgebieten. In der im Blut zirkulierenden Form vermögen die Monozyten zwar eine mäßige Phagozytoseleistung zu vollbringen und verfügen wie die Neutrophilen über katabole Enzyme, die ebenfalls in den Granula lokalisiert sind und in der gleichen Weise zur Funktion gelangen [11, 16]. Bakterizidie wird besonders durch das Lysozym, jedoch auch durch das Peroxydase-System bewirkt. Immunglobulinrezeptoren an der Oberfläche der Monozyten und ihrer Funktionsformen führen zu einer selektiv gesteigerten Phagozytose immunglobulinbeladener Partikel (z. B. Bakterien oder Erythrozyten) [8]. Neutrophile Granulozyten besitzen ähnliche, allerdings in ihrer Immunglobulinspezifität verschiedene und offenbar weniger empfindliche Rezeptoren [14]. Im Gegensatz zu den Neutrophilen ist der Ribonucleinsäurestoffwechsel und damit auch die Proteinsynthese der Monozyten deutlich aktiver [11, 16]. Ausgiebiges Ergastoplasma, deutlicher Golgi-Apparat und zahlreiche Ribosomen stellen das ultrastrukturelle Korrelat zur Proteinsynthesefähigkeit dar [9]. Hinsichtlich des energieliefernden Stoffwechsels stimmen Monozyten und Neutrophile weitgehend überein; neben der bei weitem überwiegenden Glykolyse zeigen allerdings der Krebszyklus und die oxydative Phosphorylierung eine höhere Aktivität als in den Neutrophilen, während der für die Bakterizidie wichtige Hexosemonophosphat-Shunt eine den Neutrophilen vergleichbare Aktivität aufweist [16]. Nach der Auswanderung aus den Gefäßen wandeln sich Monozyten in ihre Funktionsformen – die Makrophagen – um. Hautfensterversuche, insbesondere unter Anwendung zytochemischer Verfahren, gestatten die Beobachtung dieser Umwandlung *in vivo*, die nach 1 bis 3 Stunden bereits deutlich zu erkennen und nach 16 bis 24 Stunden in den Grundzügen abgeschlossen ist [16]. Die Umwandlung äußert sich in einer Größenzunahme des Zytoplasmas, in einer aus-

geprägten Steigerung der Phagozytose und einer starken Aktivierung der RNS- und Protein-Synthese im Rahmen einer stark vermehrten Produktion von Lysosomen bzw. Granula [4, 16]. Diese massive Steigerung des Gehaltes an katabolen Enzymen beruht jedoch nicht nur auf einer indiskriminierten Vermehrung vorhandener Enzymtypen, sondern durch Enzyminduktion können normalerweise kaum vorhandene Enzyme in reichlicher Aktivität für den Abbau besonderer Materialien bereitgestellt werden [16]. Durch diese Fähigkeit können sich die monozytären Funktionsformen den jeweiligen Bedingungen anpassen und stellen einen im Endeffekt ebenfalls sehr spezialisierten Zelltypus dar, dessen Bedeutung für die Abwehrfähigkeit des Organismus nicht wegen der geringeren Häufigkeit im Blut unterschätzt werden darf. Die Anpassungsvorgänge im Rahmen der Makrophagenbildung sind zusätzlich mit ausgeprägten Veränderungen des energieliefernden Stoffwechsels verbunden, bei denen es neben einer starken Zunahme des Glukoseverbrauches zu einer Vermehrung der Mitochondrien und der oxydativen Phosphorylierung und damit zu einer besseren energetischen Ausnützung der verbrauchten Glukose kommt [11, 16]. Parallel kommt es zu einer Einlagerung von Glykogen. Diese Vorgänge gestatten es den Makrophagen wochen- und ev. monatelang zu überleben und bei Bedarf am Ort der Funktion sich mitotisch zu teilen. Diese Eigenschaften bilden die Voraussetzung zur Bildung von Granulomen. Riesenzellen stellen wahrscheinlich den Versuch dar, durch Vermehrung der Zellmasse besondere Aufgaben zu bewältigen, so z. B. bei den Fremdkörper-Riesenzellen. Anzumerken ist, daß die Umwandlung leukämischer Monozyten sowohl *in vivo* wie auch *in vitro* in ähnlicher, wenn auch quantitativ reduzierter Weise abläuft.

Vergleicht man Neutrophile und Monozyten, so erscheinen die ersteren als Endzellen, die – ausgestattet mit einer hohen Konzentration bakterizider und kataboler Enzyme und einer auf ihre Funktion abgestimmten Stoffwechselsituation – für die kurzfristige Erfüllung einer sehr spezialisierten Aufgabe bestimmt sind. Ihre Lebensdauer beträgt wesentlich weniger als einen Tag. Durch die Vielzahl der in ihnen enthaltenen Funktionssysteme sind sie in der Lage, ein breites Spektrum von speziellen Aufgaben zu erledigen. Sie sind jedoch nicht mehr in der Lage,

Tabelle 2

Bakterizide Systeme der Neutrophilen Granulozyten und Monozyten

1. Peroxydase + H_2O_2 + Halogenid
   Produktion von H_2O_2 durch:
   a) $NADPH_2$-Oxydase
   b) $NADH_2$-Oxydase
   c) d-Aminosäure-Dehydrogenase
   d) l-Ascorbinsäure
2. ? Catalase; l-Ascorbinsäure; H_2O_2-Produktion wie unter 1.
3. Basische bakterizide Proteine
4. Lysozym
5. (Lactoferrin)

sobald sie das Knochemark verlassen haben, ihre funktionellen Fähigkeiten sekundär auf ein bestimmtes Problem hin auszurichten. Im Gegensatz dazu zeigen die monozytären Funktionsformen (Makrophagen, Epitheloid- und Riesenzellen) die Fähigkeit, sofern sie nicht vom phagozytierten Agens zerstört werden, Wochen bis Monate zu überleben. Dies ist ihnen durch die beschriebene Umstellung des energetischen Stoffwechsels möglich und durch die Möglichkeit, immer neue lysosomale Strukturen aus ihrem leistungsfähigen Ergastoplasma und Golgi-Apparat für die Phagozytose nachzubilden.

Tabelle 3

Enzyme in den Granula der Monozyten

Azurophile Granula	Spezifische Granula
Saure Phosphatase	Lysozym
Saure Desoxyribonuclease	NaF-sensitive Naphthol-AS-Acetat-Esterase
Saure Ribonuclease	Naphthylamidase (?Protease)
Saure Proteasen	
β-Glucuronidase	
Napthol-AS-D-Chloroacetat-Esterase (= neutrale Proteasen)	
Peroxydase	

Aus den beschriebenen pathophysiologischen Grundlagen ergibt sich zwangsweise, daß Störungen in der Funktion der Neutrophilen und Monozyten zu schweren Erkrankungen führen.

Krankheiten durch Versagen der Abwehrfunktion der Neutrophilen und Monozyten

Zusätzlich zu den überwiegend quantitativen Störungen der myeloischen Leukopoese – auf die hier jedoch nicht eingegangen werden soll – können qualitative oder besser funktionelle Störungen klinisch weitgehend analoge Krankheitsbilder bedingen. Zu erwähnen sind hier unter den angeborenen Störungen das sehr seltene, meist im frühen Kindesalter tödliche *Chediak-Higashi-Syndrom* mit rezidivierenden Infektionen, Granulombildungen, Lymphadenopathien und okulokutanem Albinismus. Als pathophysiologische Grundlage hierfür wird eine Störung der Lysosomen-Bildung bzw. der Lysosomenfunktion und damit ein behinderter Abbau phagozytierter Substanzen und eine verminderte Bakterizidie angenommen [1, 12]. Beeinträchtigungen der Bakterizidie sind auch für die *chronische granulomatöse Erkrankung* des Kindesalters und ähnliche, allerdings seltene Krankheitsbilder verantwortlich. Hierbei scheint es sich um ein Versagen im Bereich der Wasserstoffsuperoxyd-Produktion zu handeln, die an mehreren Stellen durch Enzymstörungen eingeschränkt sein kann (siehe Abb. 3). Folge des Wasserstoffsuperoxydmangels ist eine Störung der Peroxydase-gekoppelten Bakterizidie und damit eine Neigung zu Infektionen durch Staphylokokken, Pseu-

domonas, Escherichien, Candida u. ä. Dies wiederum führt zu therapierefraktären Pneumonien, Neigung zu granulomatösen Prozessen und meist Tod im frühen Kindesalter [1, 12]. Ist bei derartigen Störungen der zugrundeliegende biochemische Defekt wenig ausgeprägt oder läßt sich der metabolische Block umgehen, sind die Folgen entsprechend geringer oder fehlen. Bemerkenswert ist die Tatsache, daß beim kongenitalen familiären Peroxydasedefekt der Neutrophilen und Monozyten *(Alius-Grigniaschi-Undritz)* zwar eine eingeschränkte Bakterizidie beobachtet werden kann, daß es jedoch nur in Ausnahmefällen zu schweren Infektionen kommt. Im Gegensatz dazu wurde berichtet, daß schwere erworbene Peroxydasedefekte bei Leukämien zu einer deutlichen Abwehrschwäche führen können [1]. Abgesehen von diesen Störungen der Bakterizidie kann die Chemotaxis kongenital oder erworben, z. B. beim Diabetes mellitus, gestört sein [14a]. Beim Mongolismus wurde neben den bekannten Syndromen auch eine Phagozytose-Störung der Neutrophilen beobachtet [1, 12]. Bei Leukämien, insbesondere bei unreifzelligen myelogenen Formen, kommen neben Peroxydasedefekten auch solche neutraler Proteasen und anderer wesentlicher Enzyme der Granula vor, und bedingen eine eingeschränkte funktionelle Leistungsfähigkeit der leukämischen Granulozyten [1, 12]. Beim *Morbus Kostmann* konnten wir neben der obligaten Agranulozytose qualitative Anomalien der Monozyten und der Monozytenfunktion feststellen [17]; so wiesen die Monozyten einen etwas gesteigerten Gehalt an Enzymen der azurophilen Granulation auf, ließen jedoch bei der Umwandlung in Makrophagen eine verzögerte und verminderte Steigerung der sauren Phosphatase erkennen. Eine exakte Kenntnis der pathophysiologischen Grundlagen der Leukozytenfunktion bildet die Basis des Verständnisses der beschriebenen Erkrankungen und auch die Grundlage der Pharmakologie mancher antiphlogistischer Substanzen. Obwohl diesbezügliche Untersuchungen erst am Anfang stehen, lassen sich doch schon sehr distinkte Angriffspunkte antiphlogistischer Substanzen erkennen. Neben der Beeinträchtigung zytologischer Funktionen – wie Motilität-Chemotaxis (Colchicin, Glucocorticoide), Phagozytose (Colchicin, Glucocorticosteroide, Phenylbutazon), Verwertung der Granula (Colchicin, Vincristin), Umwandlung der Monozyten in Makrophagen (Glucocorticosteroide) – lassen sich auch Enzymhemmungen als Ursache des antiphlogistischen Effektes (z. B. Phenylbutazon, Goldsalze) nachweisen [12, 13].

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The Function of Neutrophil Granulocytes and Monocytes in the Frame of Cellular Defence

Among the mechanisms protecting the integrity of the human body the neutrophil granulocytes and the monocytes form a peculiar phagocytic system whose most important features include ubiquitous distribution guaranteed by the blood circulation and, at least under normal conditions, large pools of the disposable cells. The functions include phagocytosis, bactericide property, degradation or segregation of organic and inorganic materials as well as various functions within the humoral and cell mediated immunologic mechanism. As the performance of these tasks is concerned, neutrophile granulocytes and monocyte-macrophages distinctly differ complementing each other in their functions. The variations in the composition of the cellular exudates and the granulomatous process reflect the different functional peculiarities of the cells involved.

Mature granulocytes are highly differentiated and specialized non-proliferating cells. They are highly phagocytic and are characterized by conspicuous cytoplasmic granules which depending on the time of production during the neutrophile development, differ in their content of very active enzymes. These include a large set of catabolic lysosomal enzymes as well as some enzymes, especially proteases, acting preferentially at a neutral pH; various

bactericidal enzymes and proteins complete the equipment. The metabolic and cytological features predispose the neutrophile PMN to a highly specialized but only short-termed function.

In contrast, the monocytes in peripheral blood display poor differentiation and reach their full functional abilities as they transform into macrophages, or histiocytes or epitheloid or giant cells. In an extravasal environment under adequate stimulation and enzyme induction a transformation occurs into cytochemically different forms of macrophages, depending upon the environment and the substances to be degraded.

The transformation into macrophages includes activation of the RNA metabolism with increase in cell size and enlargement and activation of the Golgi apparatus, endoplasmic reticulum and lysosomes, as well as marked changes of the oxidative metabolism. In contrast to the short-lived neutrophile granulocytes, the macrophages and epitheloid cells may survive for weeks and months and under special conditions they are capable of proliferating at the site of their action. The bactericidal mechanisms are essentially similar in monocytes or macrophages and in neutrophile granulocytes; in the latter they are, however, more potent and manifold but rapidly exhausted.

Congenital or acquired disorders of the intracellular functions among these phagocytes are the causes of various diseases; some of the most interesting syndromes are briefly reported.

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Technical Problems of Spontaneous Rosette Formation

A suggestion for standardization of the method

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A high percentage of human lymphocytes forms rosettes with sheep red blood cells. The percentage of rosette forming cells (E-RFC) varies considerably according to the technique of the test. In the present work some technical conditions influencing the number of E-RFC have been investigated. The criteria of an optimum rosette technique capable of detecting a maximum number of E-RFC is described. It is important to keep the rosettes at 0°C, because resuspension at higher temperatures destroys them and a smaller percentage is counted.

Rosette formation of human lymphocytes with sheep erythrocytes (SRBC) has been demonstrated by many workers to represent a specific marker for human T cells [16—18, 22—27].

All the techniques applied were, however, found to show differences and fluctuations in the number of rosette forming cells (E-RFC). Slight modifications of the methods may cause unexpected changes in the percentage of E-RFC. This suggested that the E-RFC number was unreliable and incomparable unless the method is carefully standardized.

The basic steps of rosette techniques are: preparation of SRBC suspension, separation of human lymphocytes, mixing of lymphocytes with SRBC, resuspension and counting. The most important details influencing the resulting percentage of E-RFC are: pretreatment of lymphocytes before mixing them with SRBC, circumstances of enhancing the contact between lymphocytes and SRBC (centrifugation time, r.p.m., temperature, lymphocyte : SRBC ratio), incubation time and temperature, pH and composition of the medium, mode of resuspension and criteria of the rosette.

Since the binding between lymphocytes and SRBC is rather loose, to avoid losses the demonstration has to be careful. According to the technical conditions applied by the different authors the E-RFC% approaches the real number of T cells.

In the present report some technical aspects have been investigated to produce the maximum number of E-RFC without artefacts.

Materials and Methods

Lymphocytes. Fresh human peripheral blood lymphocytes were prepared from heparinized venous blood by separation on a Ficoll gradient [5]. After washing the cells twice in medium 199, the cell count was adjusted to 5×10^6 cells/ml. The viability of lymphocytes was checked before each test with trypan blue. Samples were discarded if viability was less than 90%.

Preparation of SRBC. SRBC were washed 3 times with phosphate buffered saline (PBS, pH 7.4) and the final concentration was adjusted to $1{,}5 \times 10^8$ cells/ml.

Test procedure for rosettes. 0.2 ml lymphocyte suspension was mixed with 0.2 ml SRBC suspension and spun at 200 *g* for 5 min. The pellet was resuspended by gentle shaking, and in one drop spread on a glass slide and covered by a coverslip 200 lymphocytes were counted. All lymphocytes binding more than two SRBC were counted as E-RFC.

Labelling of Ig-bearing lymphocytes. $2{,}5 \times 10^5$ lymphocytes in 0.05 ml were incubated with an equal volume of monospecific FITC-labelled anti-human immunoglobulin (anti IgG, IgA and IgM, Hyland) at 4°C and then washed with PBS. 200 cells were counted in each sample.

Details which influence the binding of SRBC and the proportion of E-RFC

1. *Diluting media.* Lymphocytes, before mixing them with SRBC, were pretreated by PBS, medium 199 and medium 199 containing 20% calf serum, human AB serum and autologous serum at 37°C for 60 min. (The sera were heat-inactivated and absorbed with SRBC before use.)

2. *Neuraminidase pretreatment.* 25 U of neuraminidase (Koch-Light Laboratories Ltd.) were added to 5×10^6 cells/ml lymphocyte suspension. After incubation at 37°C for 30 min the lymphocytes were washed in PBS, recounted and distributed for fluorescent staining and rosette formation.

3. *Pretreatment with PHA.* The lymphocyte suspension was pretreated with 25 μg/ml PHA-P (Difco) for 30 min at 37°C [14]. After the incubation the cells were carefully washed, and resuspended in medium 199. The rosette test was carried out according to the conditions described with incubation at 0°C and 37°C for 60 min.

4. *Incubation of the pellet.* The test tubes were incubated at 0°C, 7°C, 23°C and 37°C for 60 min after rosettes had been formed on centrifugation.

Results

The percentages of E-RFC from peripheral blood of healthy human donors varied from 3% to 76% depending upon the procedures used.

Effect of temperature. Maximum adherence occurred when contact between the cells was first established at room temperature and followed by incubation

at 0°C. On mixing the cells at 0°C no rosette was formed. With incubation at higher temperatures (7°C, 23°C and 37°C) after centrifugation a variable but rather high percentage of the rosettes already formed had disintegrated.

Effect of diluting media and pretreatment of the lymphocytes. A significant pecrease in the number of rosettes was observed if the test was performed in PBS

Table 1

Effect of pre-incubation and post-incubation on the percentage of E-RFC

Post-incubation temperature (60 min)	Pre-incubation at 37°C for 60 min						Without pre-incubation (in medium 199)
	PBS	medium 199	medium 199 +29% CS	CS	human serum		
					ABS	autologous	
37°C	3 (0–7)	14 (12–16)	13 (11–15)	14 (12–16)	15 (8–19)	11 (4–16)	10 (9–12)
23°C	5 (2–9)	62 (53–69)	61 (54–70)	64 (53–71)	63 (45–68)	48 (40–52)	58 (51–63)
7°C	11 (5–15)	65 (56–71)	64 (57–72)	65 (60–69)	66 (48–73)	54 (45–62)	61 (56–66)
0°C	27 (23–32)	74 (72–78)	75 (70–79)	73 (69–78)	73 (63–80)	67 (58–74)	75 (70–80)
Without post-incubation	4 (1–11)	60 (51–66)	63 (60–68)	62 (58–67)	68 (55–75)	56 (52–60)	55 (45–63)

The figures represent mean and range in per cent (n: 7)
ABS = AB serum, CS = calf serum

alone. Pretreatment of lymphocytes with medium 199 and, medium 199 containing 20% calf serum, or calf serum and human AB serum alone had no significant effect on the number of E-RFC as compared to the rosette test performed without pretreatment. On the other hand human autologous serum as diluting medium caused a slight decrease in the number of E-RFC. The rosette test performed in the presence of medium 199 and of medium 199 containing calf serum, and in the presence of calf serum or human AB serum gave similar results if after centrifugation the incubation was carried out at 0°C for 60 min. This seemed the best for answering the maximum of E-RFC (Table 1).

With the test standardized for maximum performance we have obtained an E-RFC proportion of 74.3% ± 6.4 with healthy human peripheral lymphocytes. The percentage of cells positive for surface immunoglobulin from the same blood was 26.8 ± 6.1. The sum of the percentages of the B cells and E-RFC for each person was approximately 100%; this suggested that the percentage of E-RFC might represent the total number of T cells (Table 2).

Pretreatment of the lymphocytes with neuraminidase increased the E-RFC number and decreased the percentage of B cells (Table 3).

Table 2

Percentage of E-RFC and SIg bearing cells from peripheral blood

No.	E-RFC	SIg	No.	E-RFC	SIg
1.	88	12	29.	74	20
2.	80	19	30.	78	28
3.	76	28	31.	82	21
4.	65	36	32.	72	34
5.	76	23	33.	79	23
6.	70	30	34.	81	19
7.	79	20	35.	66	32
8.	74	21	36.	67	35
9.	78	27	37.	75	27
10.	70	24	38.	74	28
11.	70	30	39.	85	18
12.	69	33	40.	60	38
13.	82	27	41.	74	35
14.	67	32	42.	79	31
15.	64	38	43.	73	24
16.	74	35	44.	80	18
17.	73	25	45.	85	19
18.	76	33	46.	78	23
19.	77	26	47.	72	27
20.	87	22	48.	72	31
21.	70	30	49.	78	29
22.	70	38	50.	64	21
23.	68	29			
24.	66	26			
25.	75	18	Mean:	74.3	26.8
26.	75	21	SD:	6.4	6.1
27.	74	21	Range:	60–88	12–38
28.	80	29	E-RFC+SIg = 101.1%		

SIg = presumptive B cells bearing surface immunoglobulins

Pretreatment with PHA increased the number of E-RFC after incubation at 37°C, but only a slight increase was obtained by centrifugation followed by incubation at 0°C (Table 4).

Discussion

The nature of spontaneous rosette formation in man is not clear. On the basis of several lines of evidence it has been suggested that E-RFC of peripheral human blood are thymus derived (T) cells. It is obvious that E-RFC percentages can be compared only if a standardized technique is used for maximum performance. To show the variability of results due to the different details of the E-RFC techniques we have compiled in Table 5 the most important items of the E-RFC test.

Table 3

Effect of neuraminidase on the E-FRC and SIg cells

No.	E-RFC %	$E\text{-}RFC_{(N)}$ %	SIg %	$SIg_{(N)}$ %
1.	74	89	35	18
2.	79	85	31	20
3.	73	91	24	12
4.	67	87	35	16
5.	75	94	27	18
6.	76	83	33	24
7.	74	85	38	25
8.	73	92	25	16
9.	64	76	38	31
10.	70	88	24	15
11.	66	89	32	22
Mean:	72	87	31	19
Range:	64–69	76–94	24–38	12–33
Δx:	+15		−12	

Table 4

Effect of PHA on the proportion of E-RFC

	After incubation for 60 min			
	at 37°C		at 0°C	
	without PHA	with PHA	without PHA	with PHA
E-RFC % (Mean)	13.5	66.2	72.7	76.3
Range	11–16	20–98	66–88	60–97

The number of E-RFC varies according to the technical conditions of the rosette test. The techniques of Jondal et al. [16], Ross et al. [20], Brouet et al. [7] and Bentwich et al. [3] detect practically all E-RFC, while the techniques of Bach et al. [2], Coombs et al. [10], Lay et al. [17], Papamichail et al. [18] and Brain et al. [6] detect only an E-RFC subpopulation. The method of Wybran and Fudenberg [27] identifies only a subpopulation which has high affinity receptors for SRBC (active rosette test).

On the basis of the literature and our earlier findings [13] we have studied the role of different factors influencing the rosette formation and we determined the criteria of an optimum technique for counting all E-RFC. These are:

1. Lymphocyte separation from defibrinated or heparinized venous blood by Ficoll gradient,

2. use of medium 199 or Hanks' balanced salt solution (pH 7.4) at room temperature,

Table 5

Different rosette techniques

Authors	Pretreatment	Centrifugation	Incubation	Medium	Result %
Bach et al. [2]	—	200 *g* 5 min	—	PBS	0.4—2.6
Coombs et al. [10]	—	N. D.	N. D.	Hanks	5—43
Brain et al. [6]	—	200 *g* 5 min	variable	Hanks, ABS	10—30
Lay et al. [17]	—	200 *g* 5 min	0°C 60 min	Hanks	20—40
Papamichail et al. [18]	—	200 *g* 5 min	4°C 30 min	Hanks, FCS	29
Jondal et al. [16]	37°C 5 min	200 *g* 5 min	0°C 60—120 min	PBS	52—81
Wybran and Fudenberg [27]	37° 60 min	200 *g* 5 min	—	FCS, CS, BS	28.4
Wybran et al. [28]	—	200 *g* 5 min	20°C 60 min	FCS	62—73
Farid et al. [11]	37°C 15 min	200 *g* 5 min 4°C	0°C 60 min	TC-199	66—68
Ross et al. [20]	37°C 90 min	200 *g* 10 min	0°C 120 min	FCS	70—75
Aiuti et al. [1]	37°C 15 min	200 *g* 5 min	4°C 18 h	Hanks	40—69
Brouet et al. [7]	37°C 5 min	200 *g* 5 min 4°C	4°C 2—4 h	Hanks, ABS	55—70
Bentwich et al. [3]	37°C 5 min	50 *g* 5 min 4°C	4°C 60 min	Hanks, ABS	61—84

Abbreviations: N. D. = not determined, ABS = AB serum, BS = bovine serum, CS = calf serum, FCS = foetal calf serum, PBS = phosphate buffered saline.

3. washing the lymphocytes twice,
4. lymphocyte : SRBC ratio 1 : 30,
5. centrifugation at 200 *g* at room temperature for 5 min,
6. incubation at 0°C for 60 min before resuspension,
7. resuspension by gentle shaking immediately before counting.

Maximum adherence was not influenced by pretreatment of the lymphocytes with medium 199, calf serum or human AB serum, but all other changes in the test conditions reduced the rosette-forming capacity. It was surprising that autologous serum inhibited the rosette formation despite optimum technical conditions. This suggested the presence in normal serum of a substance which masks the SRBC receptors [8, 9]. We obtained similar results with Wybran's active rosette test [27] when lymphocytes had been pretreated with autologous serum.

The fact that the maximum number of rosettes depends upon the incubation at low temperature suggests that the microvilli detectable by scanning electron microscopy [19] form a so delicate contact between lymphocytes and SRBC that even slight turbulences are capable of loosening the binding. It is not likely that the SRBC receptor itself should be involved in a change at low temperature making it more accessible for the SRBC, because no rosettes are formed at 0°C

[15]. Low temperature may be essential only for preventing the dissociation of rosettes formed at temperatures above 0°C. Using higher temperatures before resuspension of the centrifugate, SRBC are freed from the E-RFC and only the strongest (most active) cells retain the SRBC during resuspension.

Neuraminidase caused a population of presumed B cells to acquire the rosette forming property. The most likely explanation is perhaps that there are two B cell populations, or alternatively that there are two subpopulations of T cells, one of them absorbing immunoglobulins from the serum. The SRBC receptors as ancient characteristics of lymphocytes may be masked on normal B cells which appear later as T cells. After removing this mask by neuraminidase [3, 21] or papain [8] the microvilli for SRBC [19] are exposed on the surface of a subpopulation of B cells. On the other hand, pretreatment of SRBC with neuraminidase enhances rosette formation [4]. The explanation of this phenomenon is not clear.

The number of E-RFC is altered by short incubation with PHA under special conditions [14]. Using conditions other than optimum for rosette formation, PHA increases the number of E-RFC, but under optimum circumstances it does not increase significantly the rosetting capacity of lymphocytes. This suggests that the effect of PHA may be due to a configurational change making the receptors more accessible to SRBC.

Though on the basis of available data the essential character of spontaneous rosette formation by human lymphocytes with SRBC remains uncertain, a standardized sensitive technique is absolutely necessary for obtaining comparable data for E-RFC.

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Functional Properties of T and B Lymphocytes in Cell-mediated Immunity

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Recent data on the characteristics and functional behaviour of T and B lymphocytes are summarized. The importance of surface markers (antigens, receptors, immunoglobulins) in the distinction of these two lymphocyte populations is stressed. Some evidence is offered to show that the equilibrium between T and B lymphocytes is one of the basic rules of normal immune reactivity. The functional characteristics of T and B lymphocytes, important in cell-mediated immunity, are discussed together with the different sensitivity to mitogens, the specific and aspecific cytotoxic activity and the "soluble factor" release of T and B lymphocytes. Adherence, mobility and motility, and the subpopulations of the lymphocytes and some results of studies on PHA sensitivity, spontaneous cytotoxicity and moving properties of human lymphocytes are reviewed.

The lymphocyte has long been considered one of the most mysterious cells of the organism. Its role as a regulator of the multipotential stem cell, tissue growth and metabolism has been assumed [30, 31, 184], and its significance in immunological processes recognized [113]. Then about two decades ago it has been shown that it plays a significant role in the maintenance of homeostasis [106]. Billingham et al. [17] and Gowans and McGregor [65] have determined its primary role in cell-mediated immune functions.

Similarly to the classification of cell-mediated and humoral immunity [95, 102], the lymphocytes have also been classified according to their participation in these processes. Roitt et al. [145] classified them according to origin: T lymphocytes (thymus-derived, thymus-processed) and B lymphocytes (thymus-independent, bursa equivalent-derived). Our knowledge of the immune function of these cells is extending daily, thus a detailed description of this role would be beyond the scope of this review. Therefore, we shall survey only the most important results elaborated in the last few years, including our own experience concerning cell-mediated immunity.

Origin and distribution in the organism of T and B lymphocytes

Both cells belonging to the T and B cell axis are formed as a result of clonal proliferation and differentiation induced by humoral factor(s) of a common multipotential stem cell, derived from the bone marrow [10, 107, 182]. The

multipotential stem cells migrating to the thymus, besides the already present histocompatibility antigens, form theta (θ), thymus leukaemia (TL) and other lymphocyte specific antigens (Ly) on the surface of T cells [126]. T cells form two subpopulations: 80 to 90% of the population sensitive to gamma rays and cortisone consist of small cells and occur in the cortex. The population consisting of larger cells occurring in the medulla is cortisone-resistant, its θ antigen content is lower and has no TL antigen [18, 98, 141]. The latter subpopulation, regarding its surface structure, is similar to the T lymphocytes circulating in peripheral blood [18, 35, 75]. This mature subpopulation migrates at an extremely fast rate to the peripheral lymph organ [32].

The origin of B lymphocytes has been defined in fowls. Their central organ being analogous to the thymus, the primary lymphoid organ is the bursa of Fabricius [39, 112]. In mammals the "bursa equivalent" central lymph organs (bone marrow, Peyer's patches, appendix, tonsils, sacculus rotundus) seem to be the sites where the B lymphocytes responsible for antibody formation are differentiated [5, 22, 54, 114]. Knowledge concerning the origin and development of B lymphocytes is rather poor.

A large number of lymphocytes migrating from the central organs form a recirculating lymphocyte pool between blood and interstices. A smaller number is found in the peripheral lymph organs. According to indirect evidence, migration and recirculation are more the properties of T lymphocytes [64, 94, 108]. According to some recent data, however, B lymphocytes also migrate, although their recirculation is slower and less systematic [85]. Specific (antigen) and aspecific (adjuvants, heparin) stimulators increase the recirculation of lymphocytes [58, 81, 158].

As T and B lymphocytes can be distinguished by the difference of their surface characteristics, their distribution pattern in peripheral and central lymph

Table 1

Percentage distribution of mouse T and B lymphocytes on the basis of surface properties [76]

	T cell properties	B cell properties			
		MBLA+	Surface Ig+	Receptor C3+	Receptor Fc+
Thymus	95–100	0	0– 1	0	0– 1
Thoracic duct	80– 90	10–15	5–15	10–20	10–20
Blood lymphocytes	60– 85	20–40	10–20	–	15–25
Lymph node	55– 80	30–35	10–20	10–20	20–25
Spleen	25– 45	50–65	30–50	25–50	40–45
Peyer's patches	20– 40	60–75	–	–	60–70
Peritoneal lymphocytes	25– 50	–	5–15	–	–
Bone marrow	0– 1	25–45	5–15	5–15	10–15

organs could be determined (Table 1). The two sorts of lymphocytes have sites of predilection: T lymphocytes are found in the paracortical region and diffuse cortex of lymph nodes, while B lymphocytes occur in the primary and secondary follicles [128, 144].

The ratio of T and B lymphocytes in peripheral blood is important from both theoretical and practical aspects. It is now generally accepted that 60 to 70% of the circulating lymphocytes are T cells and the rest belong to the B cell axis [1, 147, 160, 180]. Any alteration of this ratio means a clinical disorder of immune function, occurring generally in association with congenital immune deficiencies and malignant haematological diseases. T cell deficiency is characteristic of DiGeorge's syndrome [38], B lymphocyte deficiency occurs in Bruton-type agammaglobulinaemia [1]. In combined immune deficiencies the ratio of T and B cells differs according to the deficiency of which cell line dominates [1, 57, 155]. An absolute increase in B cell count and relative T cell deficiency occur in chronic lymphoid leukaemia [18, 88]. In this disease, 98% of lymphocytes are of the B type [18, 136]. In recent years, various changes in the T and B lymphocyte ratio have been reported in several autoimmune diseases [59].

The optimal ratio of T and B lymphocytes in blood and lymph organs is ensured by the primary efflux of lymphocytes from the lymph organs and by recirculation between lymph vessels, blood vessels, tissues and secondary lymph organs. Hereditary factors are presumably responsible for the maintenance of this significant dynamic equilibrium. It has been shown in inbred mice that part of the genes responsible for the characteristic T–B cell ratio is bound to the H-2 complex region [44].

Surface characteristics of T and B lymphocytes

T and B lymphocytes do not differ morphologically. Recently, differences in the functional and structural features of their surfaces have been recognized, and on this basis the two cell types can be differentiated. The differences in surface antigens, immunoglobulins and receptors are summarized in Table 2.

Lymphocyte surface antigens have first been described in mice. The most important are as follows.

1. *Histocompatibility (transplantation) antigens* are most explicit in lymphocytes. These antigens are determined by one of the basic genetic systems of the organism, denoted in mice as H-2 complex (for a review, see [42]), in humans as "major histocompatibility system" (for review, see [8, 41, 170]). Knowledge of these surface antigens is surveyed in another paper of this volume (G. Kaiser: Human histocompatibility systems).

2. *Alloantigens* are lymphocyte membrane antigens which are detected by alloantibody sera gained by immunizing individuals of identical species.

Thymus leukaemia antigens (TL) can be observed on spontaneous leukaemia cells of mice and on thymocytes of some mice strains (for example of strains AKR,

Table 2

Surface features of T and B lymphocytes and the methods for their detection

Features	Methods	T cell	B cell
Antigens			
in mouse lymphocytes			
H−2	Specific alloantibody		
	Cytotoxicity	+	+
TL	Cytotoxicity	+	−
θ	Cytotoxicity	+	−
Ly A−D	Cytotoxicity	+	−
MSLA	Specific heteroantibody		
	Cytotoxicity	+	−
MBLA	Cytotoxicity	−	+
MSPCA	Cytotoxicity	−	±
in human lymphocytes			
HL−A	Specific alloantibody		
	Cytotoxicity	+	+
T cell specific	Specific heteroantibody		
	Cytotoxicity or immunofluorescein test		
	anti-foetal thymus serum	±	−
	anti-brain serum	+	−
B cell specific	Specific heteroantibody		
	Cytotoxicity or immunofluorescein test		
	anti-chr. ly. leuk. serum	−	+
Receptors			
in mouse lymphocytes			
Sheep red blood cells	Spontaneous SRBC rosette test	+	+
Immunoglobulin	Hetero Ig serum	−	+
	Immunofluorescein test		
Fc	Antigen-antibody complex	−	+
	Aggregated Ig		
	Immunofluorescein test		
C3	Antigen-antibody complex +		
	complement (incomplete) rosette test	−	+
in human lymphocytes			
Sheep red blood cells	Spontaneous SRBC rosette test	+	−
Immunoglobulin	Hetero anti-Ig serum		
	Immunofluorescein test	−	+
Fc	Antigen-antibody complex on RBC		
	surface	−	+
	Rosette test		
	Aggregated Ig	−	+
	Immunofluorescein test	−	±
C3	RBC + alloantibody + complement		
	(incomplete) rosette test		
Mouse RBC	Spontaneous mouse RBC rosette test	−	+
Eppstein−Barr virus	Alloantibody		
	Immunofluorescein test	−	+

NZB, C58) [25, 120]. The 4 specificities of the antigen are determined by the TL gene located distally to the H-2D locus [27].

GIX antigen, a structure identical to the antigen induced by Gross leukaemia virus. This is also determined by a gene bound to the H-2 complex region. This antigen is generally detected in thymus cells but when the animal is infected by the virus it is present in most lymphocytes [162].

Chronic lymphoid leukaemia-associated antigen has been the most definitely recognized antigen of those associated with human leukaemia [82, 175]. According to studies by Do Trun Phan et al. [136] made in our Institute, this antigen can be distinguished from other T and B lymphocyte specific antigens. In addition to chronic lymphoid leukaemia it occurs on malignant cells of other lymphoproliferative diseases. During remissions the antigen cannot be detected or is present at low concentrations.

Theta antigen (θ). Reif and Allen described this as the antigen most characteristic of T lymphocytes in mice [142]. A specificity of this antigen can be observed in the mouse strains AKR and C3H [139]. These specificities occur in cells of the nervous system, epidermal tissues and in some leukaemia cells [63, 73, 148]. The θ antigen occurs in all T lymphocytes but its amount is lower in peripheral lymphocytes [139].

θ specific antibodies are obtained by both allo- and heteroimmunization [63, 169]. They are suitable for the identification of T cells of murine and human origin. Generally, immunofluorescence techniques or lymphocytotoxic antibody titration are applied for detecting T lymphocytes [20, 72].

Other lymphocyte and plasma cell specific antigens and autoantigens also occur in mice; LyA, LyB, LyC, LyD and PC-1. They are detectable in various amounts [23, 24, 76, 163]. Analogous antigens in humans have not been found.

3. *Heteroantigens* are identified by antisera obtained by immunization performed in xenogenous circumstances. Mouse specific lymphocyte antigen (MSLA) probably has T specific features [153]. However, the most significant heteroantigen is the mouse specific B lymphocyte antigen (MBLA), as its analogue has been detected also in humans [1, 14, 139]. This antigen is the characteristic marker of B lymphocytes and thus they can be differentiated from T lymphocytes [1, 71]. Most leukaemic cells in human chronic lymphoid leukaemia are B lymphocytes, thus human B specific antiserum is produced by immunizing rabbits with chronic leukaemia cells and subsequently the HL-A and T specificities of the serum are depleted by thymus or brain tissue [69, 72].

Besides the above heteroantigens, mouse specific peripheral lymphocyte antigens (MPLA) and mouse specific plasma cell antigens (MSPCA) are also known [140, 164, 165].

Modulation of lymphocyte surface antigens and their *movement* on the membrane surface are features of great interest. According to the stage of cell division and other functional properties, various amounts of antigens induced by histocompatibility genes and viruses are revealed on the membrane of the lymphocyte. Cikes and Friberg [33] have shown that the amount of antigens was highest

in the G1 phase of growth. The amount of θ antigen in lymphocytes is lower after phytohaemagglutinin stimulation [103]. A characteristic feature of surface antigens are the "patch" and "cap" formations, further on their pinocytosis. These phenomena are induced by antibodies used for detecting these antigens [4]. Patch formation, i.e. the redistribution of antigen in patches is independent of cell metabolism and occurs at 0–4 °C. "Cap" formation and antigen pinocytosis are phenomena attributed to the lymphocyte uropods, occurring only at 37 °C [143]. These phenomena indicate that these antigens are present in varying amounts and at different sites on the lymphocyte surface, depending on the constant dynamic movement of antigens and the important life-functions of the cell [6].

Antigen modulation is the cause of some lymphocyte (TL, H-2) antigens being unverifiable both *in vivo* and *in vitro* [26, 121, 166]. The opposite has also been reported, when "extra HL-A" antigens occurred in leukaemia cells or in lymphocytes cultured *in vitro* [123, 130]. The occurrence of so-called "extra" antigens does not seem to be explained by the modification of gene effects or by antigen transformation. It seems more likely that the affinity of cells to antibodies or their sensitivity to antibody complement complex is increased. Our own work also supports this presumption. E. Gyódi et al. have demonstrated that the "extra" antigens can be dispersed by changing the ratio of components in the serological reaction (for example, by the dilution of the complement). This observation indicates that the specific test sera used for antigen detection contain a low titre of antibodies of other specificity, which are not detected by the less sensitive conventional (NIH) cytotoxicity reaction. However, antigens of pathological cells with altered sensitivity and affinity will react with these, indicating "false" or "extra" antigens [80]. In cultured lymphocytes of healthy subjects, the above described change in HL-A antigens can be observed from the 2nd day onwards.

Lymphocyte surface immunoglobulins. A well-proved fact in immunology is that lymphocyte surface immunoglobulins or receptors formed by antibody analogous molecules are responsible for antigen-binding on the cell surface. Details of this subject are discussed elsewhere in this volume [59]. We only wish to mention a few data important for characterizing T and B lymphocytes. All modern techniques of detecting surface antigens (extraction, elution, immunofluorescence, autoradiography using anti-immunoglobulins, histochemical and serological reactions, electron microscopy with ferritin-marked antibodies or viruses, etc.) unanimously demonstrate surface immunoglobulin in most B lymphocytes. (For review, see [76].) All types of immunoglobulins are detectable on these B lymphocytes. However, in part of them no immunoglogulin is detectable. These cells either participate in active antibody production or belong to the so-called "null" cell population (see later) [79, 93]. The surface immunoglobulin content is the most characteristic property of B lymphocytes serving as a means of differentiation from T lymphocytes with immune-fluorescein techniques [138].

Immunoglobulins forming receptors on T lymphocytes are hardly detectable with conventional methods. Most investigations have determined IgM or light

chain specificity [12, 74, 78, 83, 105, 116, 144]. Thus, due to the difficulty of detection, the presumed immunoglobulin receptor of T cells was denoted IgX [110].

Immunoglobulin Fc receptor

It has been known for a time that lymphocytes are capable of binding antigen-antibody complexes [28, 171]. Recently, Basten et al. [11] have shown that these lymphocytes are B cells. Immunochemical investigations have clarified that the receptor responsible for the binding of immunoglobulin is linked to the Fc part of the molecule [127]. The aggregated immunoglobulin is also bound *via* Fc receptor to the lymphocyte surface [28]. Most B cells have Fc receptors, the majority of these are resting cells. On the other hand, antibody-secreting cells have no such receptors. Recent studies have demonstrated that besides B cells a number of T cells from human tonsils have also Fc receptors [69].

Complement receptor

Bianco et al. [16] and Nussenzweig et al. [118] have shown that lymphocytes form rosettes when binding antibody and complement-coated sheep red blood cells (SRBC) [16, 118]. Only B lymphocytes have receptors for C3 complement component, whereas T cells have none. Monocytes and granulocytes have, however, such a receptor [15]. The latter cells are capable of binding complement only in the presence of Ca and Mg ions [15]. C3 receptor has also been detected in human lymphocytes [156], although some data suggest that not all B lymphocytes have C3 receptors [89].

Other "non-specific" T and B cell receptors

A large proportion of human and animal lymphocytes bind SRBC "non-specifically" [37, 97]. Cells with receptors for SRBC are T lymphocytes [56, 90, 156]. This is a property so characteristic of T lymphocytes that it can be used for their identification. SRBC rosette formation is a test which due to its simplicity is easily applied in clinical medicine.

Recently, a receptor with Epstein–Barr virus-binding capacity has been found in B lymphocytes [91]. This receptor was not present in T lymphocytes. Similarly, the mouse red blood cell-binding capacity of chronic lymphoid leukaemia cells has been described as a phenomenon specific for B lymphocytes [159]. Five to 15% of the lymphocytes in the peripheral blood of healthy individuals have mouse red blood cell-binding capacity; this ratio is in proportion to the number of B lymphocytes.

T and B sub-populations

Among T and B lymphocytes, there are always some cells which do not display the characteristic features. It was therefore assumed that besides the classical T and B lymphocytes, sub-populations must exist.

T lymphocytes are divided into two groups on the basis of θ antigen positivity. The first group consists of mature, cortisone-resistant cells occurring mostly in peripheral blood, with only a small amount of θ antigen and no TL antigen on its surface [4, 126]. A similar distribution pattern has been described for cells forming SRBC rosettes. Part of these θ positive cells are extremely sensitive to anti-theta antilymphocyte serum and to azathioprine. Only light chains were detected on their surface [9, 74]. The other group of T lymphocytes is resistant to the mentioned treatments and their surface has immunoglobulin heavy chains also.

T cells also differ in respect of PHA and Con-A response [161]. All these observations point to the existence of at least two sub-populations. The T_1, T_2 sub-populations probably indicate the functional maturation phase of T cells, where T_1 denotes the less mature non-committed "virginal" state.

Recently, cells with lymphocyte features have been detected without the characteristics of either B or T lymphocytes. As most of these cells were found in the bone marrow and spleen, they are assumed to belong to the B axis [161]. These θ negative cells without complement and Ig determinants, the so-called "null" cells are extremely resistant to antilymphocyte serum and X-rays. There is no exact evidence, but it is assumed that null cells are members of less mature population of B cells [139].

In analogy to T_1 and T_2 sub-populations, B_1 and B_2 sub-populations are also assumed. These are the two groups of antibody-secreting cells, the immune-competent "virginal" cells and the "primed" memory cells [109, 152]. B_1 and B_2 (B_x, B_y) cell groups differ in their cortisone sensitivity and tissue distribution pattern [12, 177].

Functional properties of T and B lymphocytes

The basic difference between the two cell types is in their role in antibody production and cell-mediated immune response. Although the primary role of B lymphocytes is antibody production and the primary role of T lymphocytes is a participation in the cell-mediated response, the two functions cannot be separated. T cells are assisting in antibody production while the cell-mediated response needs cells recruited from the B axis and the help of macrophages.

The functional features of T and B cells are determined partly by the activity of mitogens and antigens and partly by the so-called "spontaneous" lymphocyte functions.

Mitogen-induced blast-transformation of T and B lymphocytes

In 1960 Nowell described the mitogenic effect of phytohaemagglutinin (PHA) on human lymphocyte cultures which resulted in blast transformation [117]. PHA enhances RNA and protein synthesis in lymphocytes and induces cell division. The activated lymphocytes are transformed into blast-like cells with increased DNA synthesis. The latter is used for measuring the degree of transformation.

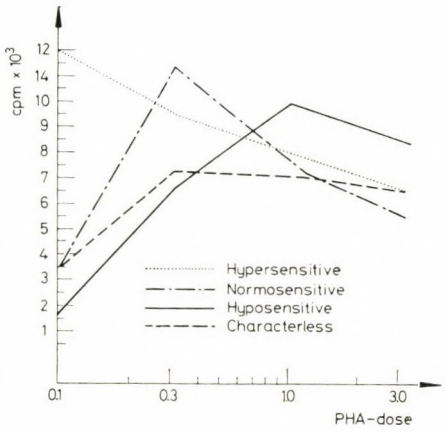

Fig. 1. Characteristic dose-dependent reactivity types of human peripheral lymphocytes after PHA stimulation

Intensive research has led to the recognition of other mitogens: concanavalin-A (Con-A) [60], lentil [185] and pokeweed [52] are of plant origin, while LPS stimulant is a bacterial lipopolysaccharide *(Proteus vulgaris, Shigella flexneri, E. coli)* [129]. Besides lectins of plant origin and bacterial mitogens, anorganic ions [150] and antibodies to the lymphocyte surface antigens are also mitogenic [151].

Investigation of purified T and B lymphocyte suspensions for mitogenic response revealed that the response to mitogens was a selective one [86, 87]. The specific effect of PHA, Con-A and PWM has been observed also in humans [45, 75].

The pattern of selectivity is as follows.

T axis specific mitogens: PHA, lentil, Con-A, PWM and antilymphocyte serum.

B axis specific mitogens: PWM, LPS, anti-immunoglobulins, and aggregated tubercle protein.

This selectivity does not always occur in a pure form. For example, Con-A and PHA also stimulate B cells, if applied in an insoluble form bound to sepharose [2, 70]. Also, lymphocyte sub-populations do not respond uniformly to mitogen [154, 161]. In our Institute, Onódy's investigations revealed 4 types of mitogenic

response in human lymphocytes [122]. Stimulation of lymphocytes of healthy individuals was performed using different amounts of PHA. Five per cent of hypersensitive, 28% normosensitive, 37% hyposensitive and 30% non-characteristic responses were obtained (Fig. 1). It remains to be clarified whether subpopulations or other factors are responsible for the differences in the response.

Mitogens as stimulators of lymphocyte functions serve as excellent assays of these [50, 149].

Antigen-induced proliferation

Lymphocytes from sensitized individuals stimulate the proliferation of the relevant antigen *in vitro* [48, 124]. The phenomenon can be induced with various antigens (tubercle protein, fungi, virus antigens, drug sensitivity, etc.) but the response is invariably weaker than in the case of mitogen-induced stimulation. The reaction is dependent on the presence of macrophages [34]. Opinions are controversial whether T or B lymphocytes participate in the process. According to Greaves et al. [76] the proliferation of T cells is primarily responsible for the increased DNA synthesis in this cell-mediated reaction *in vitro*, but the B lymphocytes cannot entirely be dismissed either.

A special form of antigen-induced lymphocyte transformation is stimulation with allogeneic cells (transplantation antigens) (for review, see [178]). The reaction is slower than the PHA-induced transformation. The degree of transformation is directly proportional to histoincompatibility and the difference in MLC (mixed lymphocyte culture) locus of the stimulating cell. The allogeneic cell-induced *in vitro* transformation termed MLC is extensively applied for donor selection in kidney transplantation. Most evidence suggests that T lymphocytes take part in the MLC [7, 21, 49, 104, 174]. According to recent observations B cells may, however, play a greater role in inducing the stimulatory effect, while T cells recognize the alloantigen and play a role in the proliferative response [21, 101].

In a special form of MLC denoted as mixed lymphocyte interaction test, the lymphocytes are stimulated by autochthonous or syngeneic tumour or other cells (autoantigens) [172, 173]. This reaction would prove response to the tumour-associated antigen. It is, however, an open question whether this *in vitro* test corresponds to the processes *in vivo*.

Cytotoxic activity of T and B lymphocytes

Lymphocyte-initiated tissue-damaging effect, a cell-mediated response *in vivo*, can be demonstrated *in vitro* by the killing effect of sensitized lymphocytes for xenogeneic, allogeneic or syngeneic target cells. Lymphocytes either kill the relevant target cell or inhibit its growth. The cytotoxic activity may develop first, subsequent to stabilization *in vivo* or *in vitro*, or secondarily, as a result of mitogenic stimulation, specific antibodies or mediators (see below). In the first case,

cytotoxicity is specific to the sensitizing antigen, in the second case the effect can be observed on various target cells, with the exception of the antibody-induced response where the specificity of the antibody determines the aim of lymphocyte activity [19, 131, 179].

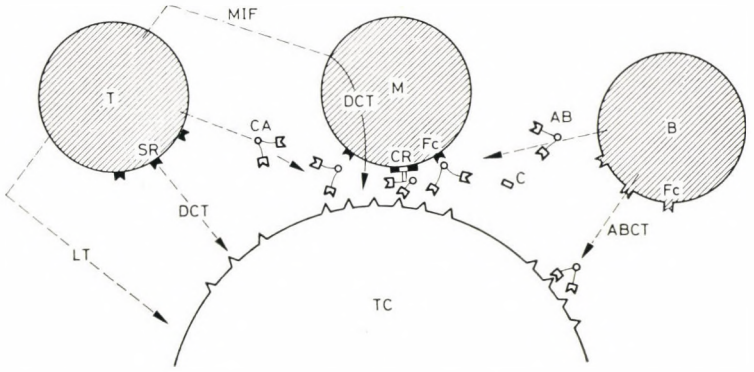

Fig. 2. Cytotoxic reactions of lymphocytes. T = T lymphocyte; B = B lymphocyte; M = macrophage; TC = target cell; LT = lymphotoxin; SR = specific membrane receptor; CA = cytophilic antibody? (macrophage arming factor); MIF = macrophage migration-inhibiting factor; CR = complement receptor; Fc = Fc receptor; AB = antibody; C = complement; DCT = direct cytotoxic activity; ABCT = antibody-mediated cytotoxicity

The role of cells cooperating in the complex cytotoxic reaction is difficult to follow. Participation of aspecific macrophages besides lymphocytes in the reaction has been shown [51, 67, 100]. It has been established in model experiments free of macrophages and B lymphocytes that T lymphocytes are responsible for short time (3–16 hours) cytotoxicity [29, 61, 62, 176, 183], whereas B lymphocytes play a primary role in antibody-mediated cytotoxicity. In models where mainly the growth of target cells is affected the response takes about 24 hours to develop [125, 132]. *In vivo*, the two mechanisms are probably linked and completing each other, supporting the development of the cell-destructive effect. Figure 2 displays the various possibilities of cytotoxicity and cells participating in the reactions.

Lymphocytes stimulated by some mitogens are also cytotoxic. This effect is, however, aspecific in that it affects all target cells without any previous antigenic stimulation [13]. In this mechanism, mainly T lymphocytes take part [11, 75]; cytotoxicity could not be induced by B cell specific stimulators [111].

Much attention has been paid to investigations into the spontaneous cytotoxic effect of lymphocytes. Takasugi et al., investigating the cell-mediated immune response to tumours, found that the lymphocytes of healthy individuals displayed different degrees of cytotoxicity on various lymphoblastoid target cells [79, 99, 167]. This activity, not mediated by mitogen or antibody, did not therefore result from sensibilization. Our own studies on the spontaneous cytotoxic activity

of human lymphocytes were detected on DBA/2 mouse fibroblasts and applied as a general immune-function test for studying the genetic background of immune reactivity (HL-A and Immune Reactivity Workshop, Budapest 1972). In accordance with those investigations, spontaneous cytotoxicity is, at least partly, genetically bound to the HL-A complex system. Namely, this function was missing in individuals with HL-A 3.7 antigens [133, 134]. The effect may also be found in other mouse target cells, particularly in P-815 mouse tumour cells. The reaction is probably released by lymphocytes with C3 receptors [137].

Factors and mediators produced by lymphocytes

Factors and mediators produced by lymphocytes belong to two groups (Table 3), *viz.*

(1) Antigen specific, biologically active, factors extracted from lymphocytes.

(2) Antigen aspecific soluble mediators; their release is the result of an antigen-induced reaction.

ad 1. These specific mediators extracted from antigen-reactive lymphocytes injected into non-sensitized individuals, induce a cell-mediated immune response to the relevant antigen. With the exception of the most significant "transfer" factor, these factors are sensitive to RNAase enzymes [96]. Despite the clinical

Table 3

Lymphocyte factors and mediators

Designation	Abbreviation	Effect
Factors extracted from lymphocytes	Transfer factor (TF)	Induction of cell-mediated immune response
	RNA analogous factor	Induction of cell-mediated and humoral immune response
	Antibody analogous factor	Induction of cell-mediated immune response
Factors released by lymphocytes	Migration inhibiting (MIF) factor	Inhibits macrophage migration
	Chemotactic factor (CF)	Attracts monocytes, granulocytes, eosinophils
	Mitogenic factor (MF)	Stimulates lymphocyte proliferation
	Lymphotoxin (LT)	Cytotoxic effect without complement
	Antigen recognizing product (PAR)	Granulocyte inflammative reaction
	Skin reactive factor (SRF)	Inflammative response
	Interferon	Increases resistance against virus
	Proliferation inhibiting factor (PIF)	Inhibits clone growth
	Immunoglobulins	Specific antibody effects

and theoretical importance of these mediators, it is not known whether T or B lymphocytes are producing them [92].

ad 2. Lymphocytes repeatedly meeting antigens or stimulated by mitogenic agents release soluble mediators inducing various immune phenomena. These factors were termed "lymphokines" by Dumonde et al. [46]. The biochemical properties of macrophage migration inhibition, chemotactic factor and lymphotoxin have been determined [40, 58, 68, 143]. At the same time, the biochemical properties and significance, and the correlations with the other factors remain to be clarified. Several indirect data suggest that these factors are produced by T lymphocytes [40, 66, 119, 157]. Considering that in some cases the effect of these factors cannot be separated from the function of cytophilic cells or IgM type antibodies, the role of B cells must not be dismissed [36], especially in view of convincing data in support of antibodies being produced by B lymphocytes [3, 47, 53, 77].

Other Functional Properties of Lymphocytes

There are some factors connected with the cell membrane and cell receptors.

1. *Adherence.* Some lymphocyte types will adhere to glass, plastic and nylon. This adherence is transitory and temperature-independent [154]. In general, blast cells or dividing cells and antibody-secreting cells adhere to a greater extent. Among resting cells, B lymphocytes are capable of adherence to surfaces [16, 84, 146, 168]. This property is used in the preparation of pure human T lymphocyte suspensions, when B lymphocytes are made to adhere to a nylon fibre [72].

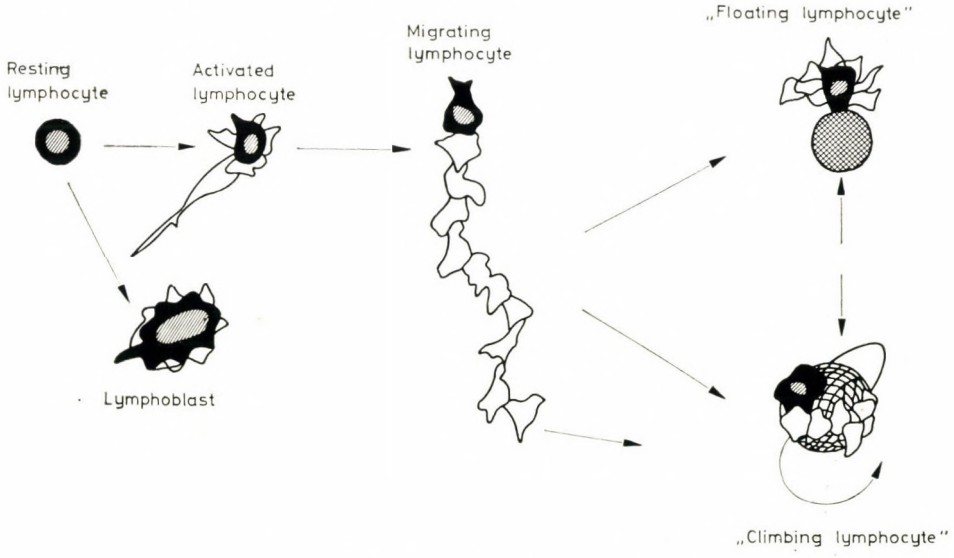

Fig. 3. Types of lymphocyte movement

2. *Migration in electric field.* The negative surface charge of lymphocytes varies and so in the electric field they migrate at different rates. Microelectrophoretical observations have shown that B cells migrate towards the positive pole at a higher rate than do T cells. T cells consist of two subpopulations [181]. Thus, T and B lymphocytes can be separated by continuous electrophoresis [115, 186].

3. *Active movement.* Microcinematographic observations demonstrate that there are resting, moving and migrating cells. According to our own studies, cortisone-treated mouse thymus cells hardly move, while spleen and lymph-node lymphocytes from the immunized animal show active movements (unpublished data). Benczur [14] induced an allogeneic cell-mediated immune reaction in a diffusion chamber and observed a high percentage (70–80%) of moving lymphocytes. Analyzing the forms of active movement, two characteristic features were noted (Fig. 3), *viz.* "floating": the lymphocyte touching a cell (own RBC, macrophage, foreign cells) or other substances with its uropod performs active pumping and irregular floating movements for several minutes. The second type of movement is when the lymphocyte adheres to the target cell with its whole surface and climbs on it. This latter type of movement is often seen in the course of cytotoxic reactions [135].

The data on the functional properties of T and B lymphocytes have extensively enriched our understanding of immunology. Still, many questions remain to be answered, among others the most important one, whether observations made *in vitro* correlate with the occurrence *in vivo*.

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Lymphocyte Transformation Induced by Autologous Platelets in Thrombocytopenic Patients

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Lymphocyte stimulation by autologous platelets in short-term cultures was examined in patients with idiopathic and secondary thrombocytopenia. Stimulation of lymphocytes by autologous platelets was statistically significantly higher ($P = 0.01-0.001$) in the patients than in healthy subjects. Differences among single groups of thrombocytopenias were not significant statistically. No correlation was found in the results of lymphocyte stimulation by autologous platelets and the presence of anti-platelet antibodies, immunoglobulin levels, platelet survival, i.e. between humoral and cellular immune mechanisms. The results suggested that autoimmune cellular mechanisms were involved in the pathogenesis of thrombocytopenia of diverse aetiology.

Lymphocyte transformation induced in short-term cultures by various specific and non-specific antigens is widely used for testing donor-recipient compatibility in organ transplantation, examination of cellular immunity in primary and secondary immunodeficiency [5, 6, 7, 17], testing allergic states *in vitro* [2, 3, 9, 18, 20], and for diagnosing some virus diseases [4, 11] and malignant tumours [8]. Attempts have also been made at testing by the method some autoimmune diseases [16, 19, 21].

Some papers on lymphocyte stimulation by autoantigens in Hashimoto's thyroiditis, Guillain—Barré syndrome [10] and on lymphocyte stimulation by autologous thrombocytes in thrombocytopenic purpura [14] have made us to study the autoimmune character of some thrombocytopenias.

Methods

Lymphocytes and platelets were isolated from heparinized venous blood by differential centrifugation in iodamide solution, according to Otto and Schmid [13]. To 5 ml of 60% Verografin (Léčiva) the specific gravity of which was adjusted by distilled water to 1.076, 5 ml of heparinized blood was added. After centrifugation at 1500 r.p.m. (260 *g*) we obtained three layers: Erythrocytes at bottom of the vial, supernatant plasma and in it a ring consisting of lymphocytes and platelets. This ring was removed by a Pasteur-pipette and used for cultivation where lymphocytes were stimulated by autologous platelets. For allogeneic mixtures,

lymphocytes were separated from platelets by further centrifugation at 500 r.p.m. (30 *g*) for 20 min, so that the lymphocytes fell to the bottom and the platelets stayed in the supernatant. These cell suspensions were washed three times in Eagle's medium for 15 min. The purity of lymphocyte concentrates was 96–98%, their viability as assessed by trypan-blue staining was 82–99%.

Lymphocytes (10^6/ml) were cultivated with adequate amounts of platelet suspensions in Eagle's medium with penicillin, streptomycin and calf serum added for 5 days at 37°C. Washed cell sediments after hypotonization and fixation were used for smears. These were stained panoptically and evaluated morphologically. Each culture was examined three times and 500 or 1000 cells were estimated each time. Results were expressed in per cents of transformed cells (partial transformation, complete transformation and occasional mitotic figures together). Cell viability after cultivation was at or above 80%.

Lymphocyte transformation has been studied in mixtures of lymphocytes and autologous platelets of healthy subjects, patients with thrombocytopenia, in mixtures of healthy persons' lymphocytes with patients' platelets and *vice versa*, finally the stimulation of control persons' and patients' lymphocytes by PHA (Bourroughs–Wellcome), and spontaneous lymphocyte transformation in culture medium.

Materials

Seventy patients with thrombocytopenia were examined, some of them repeatedly. Of these, 36 were in remission (spontaneous or induced by drug treatment or splenectomy). Others were recently diagnosed, before or during therapy (corticosteroids, immunosuppression) or in relapse.

Table 1

Lymphocyte transformation in thrombocytopenic patients

Diagnosis	Number of patients	Per cent of lymphocytes transformed by autologous platelets	Patients with negative lymphocyte stimulation		Patients with positive lymphocyte stimulation	
			number	per cent	number	per cent
Idiopathic thrombocytopenia	46	3–70 r = 25.8	14	30.4	32	69.6
Thrombocytopenia in hypersplenism	9	10–87 r = 21.3	4	44.4	5	55.6
Thrombocytopenia induced by drugs, infection or haemoblastosis	7	10–58 r = 28.8	2	28.5	5	71.5
Thrombocytopenia in bone marrow aplasia	8	6–65 r = 38.8	3	37.5	5	62.5

The first group consisted of 46 patients with idiopathic thrombocytopenia, predominantly adults with a history from several months to more than 30 years. All of them but 3 were treated with corticosteroids, some after splenectomy or azathioprine or cyclophosphamide treatment. At the time of diagnosis the platelet count was about 10,000/μl, at the time of examination in half of the patients 50,000/μl or normal. Anti-platelet antibodies were positive by complement fixation in 2 patients. Immunoglobulin levels (mostly IgM and IgA) were often elevated. In the majority of patients, survival of ^{51}Cr-tagged platelets was shortened, with prevalent platelet destruction more often in the spleen than in the liver.

The second group consisted of 9 patients with thrombocytopenia due to hypersplenism of various aetiology. The third group consisted of 7 patients with thrombocytopenia induced by drugs, infection or haemoblastosis, and the fourth group of 8 patients with idiopathic bone marrow aplasia. In all these groups, duration of the disease, therapy, results of examinations and laboratory findings were comparable to those in the group of idiopathic thrombocytopenia (Table 1).

Results

Lymphocyte transformation by autologous platelets in healthy subjects was 5–13% (mean, 7%) and did not differ significantly from the 8–13% (mean, 10%) rate of spontaneous lymphocyte transformation in the culture medium.

Table 2

Lymphocyte transformation induced by autologous and allogeneic thrombocytes

Spontaneous lymphocyte transformation in culture	8–13 per cent	r = 10.2 per cent
Lymphocyte transformation by autologous thrombocyte (in healthy controls)	4.5–13 per cent	r = 7.3 per cent
Lymphocyte transformation by allogeneic thrombocyte (in patients and healthy controls)	10–91 per cent	r = 31.45 per cent

Lymphocyte transformation by allogeneic platelets in mixtures of patients' lymphocytes with platelets from healthy persons or *vice versa* scattered between 10 and 91% (mean, 31%) and differed with a high statistical significance from spontaneous lymphocyte transformation and lymphocyte transformation by autologous platelets of healthy subjects ($P = 0.01$) (Table 2).

Lymphocyte stimulation by PHA was over 85% in both the patients and the healthy subjects.

In the first group of patients with idiopathic thrombocytopenia, lymphocyte stimulation by autologous platelets ranged from 3 to 70% (mean, 26%), with a positive result, i.e. one above 13%, in nearly 70% of the patients. In the group of thrombocytopenias accompanying hypersplenism the values changed from 10

to 87% (mean, 21%), with a positive result in 44% of the patients. In thrombocytopenias induced by drugs, infection or haemoblastosis, autologous platelets stimulated lymphocytes in 10–58% (mean, 29%), in 72% of the patients. In the last group (bone marrow aplasias) lymphocytes were stimulated from 6–65% (mean, 39%) with a positive value in 63% of the patients.

Table 3

Lymphocyte transformation in patients in remission

Diagnosis	Patients in remission (spontaneous, after splenectomy, corticosteroid or immunosuppressive treatment)				
	Total	Negative lymphocyte stimulation		Positive lymphocyte stimulation	
		number	per cent	number	per cent
Idiopathic thrombocytopenia	23	6	26	17	74
Thrombocytopenia in hypersplenism	7	4	57	3	43
Thrombocytopenia induced by drugs, infection, haemoblastosis	5	2	40	3	60
Thrombocytopenia in bone marrow aplasia	1	—	—	1	

Among the 36 patients in remission, 12 (31%) showed no lymphocyte stimulation by autologous platelets while in two thirds a positive stimulation occurred in spite of remission. All the patients in every examined group displaying a negative autostimulation were in remission. This means that no patient in the florid state gave a negative stimulation of his lymphocytes by autologous platelets (Table 3).

Discussion and Conclusions

The results demonstrated in every group of thrombocytopenia a statistically highly significant stimulation of lymphocytes by autologous platelets as compared to the results obtained in healthy subjects. The statistical significance *vs.* the healthy controls was $P = 0.01$ when comparing thrombocytopenias induced by drugs, infection or haemoblastosis, and $P = 0.001$ when comparing idiopathic thrombocytopenias and bone marrow aplasia. The group of thrombocytopenia secondary to hypersplenism did not differ from the healthy controls ($P = 0.1$). Differences among single groups of patients were not significant statistically.

Stimulation of lymphocytes by autologous platelets in thrombocytopenias showed practically the same values as lymphocyte stimulation by alloantigens (for instance allogeneic platelets), without statistically significant differences.

The influence of donors' or patients' own serum or allogeneic plasma on lymphocyte stimulation was slight and did not reach higher values than in spontaneous lymphocyte transformation, in accordance with the results of Alford [1].

Humoral and cellular immunological reactions in thrombocytopenia, similarly as in other diseases accompanied by antibody production, do not run parallel, as there was no correlation in the degree of lymphocyte transformation by autologous platelets and the positivity of anti-platelet antibodies and immunoglobulin levels; neither was there a correlation with shortened platelet survival.

MacLennan and Harding [12] as well as Perlmann et al. [15] showed that lymphocyte transformation and cytotoxicity to target cells are not parallel phenomena since blocking of DNA synthesis by antimitotic drugs does not abolish the cytotoxicity. In contrast, some transformed lymphocytes are not cytotoxic.

Our observations on lymphocyte stimulation by autologous platelets do not allow to draw conclusions concerning their behaviour *in vivo*. Still, we believe that in both idiopathic and secondary thrombocytopenia changes are taking place in the cellular immunological mechanisms. As we found a positive autostimulation in some patients in remission, in these cases a compensated thrombocytolytic state may be assumed to exist.

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Preventing the Incompatible Blood Transfusion

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There is a long chain, built up of many links, between the collection of blood from a donor and its transfusion to the patient; a failure of any of these links may result in an incompatible blood transfusion. The chain starts with identification of both donor and recipient. It continues with the correct labelling, grouping and matching of the blood samples and concludes with a bed-side check that the blood about to be transfused is that intended for this particular patient. Fortunately, during the years that have passed since Professor Susan Hollán had become associated with blood transfusion, most of these links, and especially those concerned with grouping and cross-matching in the laboratory, have been considerably strengthened. Despite the considerable increase in the amount of blood transfused, an incompatible blood transfusion occurs therefore less frequently and, thanks to therapeutic advances such as haemodialysis, the case mortality has been significantly lowered. Nonetheless, the transfusion of incompatible blood is almost completely preventable and it is because of this, and to try to strengthen some of the weaker links, that I offer the following personal observations in this Festschrift in honour of Professor Hollán.

Providing care is taken to avoid errors of labelling and identification, the hazard of transfusing incompatible blood is largely eliminated by the accurate ABO and Rh (D) grouping of the donor and recipient. All that is then required is to identify those exceptional patients whose serum contains irregular blood group antibodies and to prevent the transfusion of any potentially incompatible red cells by a well chosen direct matching procedure. There is no doubt that, whenever time permits, the screening of the recipient's serum for irregular antibodies against a panel of typed red cells is a valuable supplement to the direct matching test. Immune antibodies such as anti-c, for example, often show a "dosage" effect. They are therefore more certainly detected by testing the recipient's serum against a cell panel which contains homozygous c-positive red cells (i.e. rr, cde/cde) than by relying on their reaction with, e.g. heterozygous R_1r(CDe/cde) cells in a direct matching test. Our own practice in Bristol, whenever time permits, is to test each recipient's serum against (i) a pool of group O R_1R_1 and R_2r cells and (ii) group O rr, Kell positive cells by enzyme and indirect antiglobulin techniques. When antibodies are detected, their specificity is determined and blood negative for the

corresponding antigen(s) is selected. Our procedure for direct matching has been detailed in a Technical Broadsheet [1].

There will inevitably be an increased risk of transfusing incompatible blood when blood grouping tests, screening for antibodies and direct matching have to be fore-shortened or dispensed with altogether because of a deemed clinical urgency. We have to accept this situation, for example whenever our obstetric flying squad undertakes a domiciliary transfusion. Although every attempt is made to restrict such transfusion to a plasma volume expander, this is not always clinically desirable and, recognizing that the most dangerous hazard when transfusing non-matched blood is ABO incompatibility, the only blood carried by our flying squad is group O. This blood has been checked by the laboratory for absence of anti-Ap haemolysins and high-titre agglutinins [2], but also, because we recognize that despite every reasonable care it is virtually impossible to prevent an occasional mislabelling, we test each pilot sample with a group O ($\alpha\beta$) serum before consigning any particular group O donation for issue to the flying squad. All such donations are therefore grouped twice before issue: once as a part of our normal routine testing before labelling and, secondly, before issue for their possible transfusion to a non-matched recipient.

Having thus eliminated ABO incompatibility, the remaining hazard to be overcome is to minimize the possibility that the blood is transfused in emergency to a recipient whose serum contains incompatible immune blood group antibodies. Our selection of blood for such transfusions is based upon an analysis of 3,707 blood samples referred to our laboratory during a five-year period because they contained irregular blood group antibodies. 3,002 of these samples had come from patients who were Rh (D) negative and the antibodies present were anti-D, with or without anti-C, anti-E or anti-Kell. In the remainder, most of whom were Rh positive, the immune antibodies were: anti-C in 28, anti-E in 231, anti-c in 154, anti-e in 20, anti-K in 174, anti-Fy^a in 72, anti-Jk^a in 17 and anti-S or anti-s in 9. When blood has to be transfused without crossmatching, the group O blood we issue is therefore Rh negative (cde/cde) and Kell-negative if the recipient is Rh negative or has not been grouped, and c-negative, Kell-negative if the patient is known to be Rh positive. Reference to the above data shows that the Rh negative blood will be compatible for all recipients known to be of this group with the exception of the very small minority whose serum contains anti-Fy^a, anti-Jk^a, anti-S or anti-s. Also that by selecting group O c-negative, Kell negative blood, nearly all of which will be R_1R_1 (CDe/CDe), for the known Rh positive patient we avoid transfusing incompatible blood to those whose serum contains anti-K (174) or anti-c (154), and almost all of those sensitized to E (231). In brief, therefore, by these measures we can prevent up to 96 per cent of potential incompatible transfusions in the emergency situation.

We are fortunate in being able to group our donor blood samples by an automated technique using a Technicon Autoanalyser. In this procedure it is technically simple to type each sample for c and Kell antigens and we experience no difficulty in obtaining sufficient anti-c and anti-Kell for this purpose. Where

supplies of these reagents are limited and/or an automated technique is not available, we would recommend that at least sufficient group O donations are typed each week for c and Kell to provide an adequate reserve for emergency use. Grove-Rasmussen and Huggins [3] have arrived at an essentially similar conclusion when they advise that blood of these types should constitute the majority stock in banks of frozen blood. It will be some years yet, however, before such banks are developed on any nation-wide scale.

Despite all such advances in laboratory procedures, incompatible blood continues to be transfused and in the majority of instances this is the direct result of a human error in identification. A recipient sample is mislabelled at the bedside; a unit of blood correctly grouped and matched is labelled with a crossmatch tag intended for a different recipient; there is failure to check the identity of the donor blood or of the recipient sufficiently carefully before transfusion. An analysis of such accidents indicates that many can be prevented if, as a final check before blood is transfused, it is confirmed that the ABO and Rh (D) group of both the donor and recipient are identical. I became convinced of this when a patient about to be transfused, and mercifully conscious, looked up at the label on the container as it was being suspended above her bed and said to the transfusionist, "Excuse me, doctor, what is the group of that blood?". He replied, "Group A, Mrs. P. . ." Fortunately this patient had been a blood donor and knew her group, so promptly said, "But doctor, I am group O". By her own action, she had prevented an incompatible transfusion. Our lesson, surely, is that we ourselves should include this check as a part of our own routine whenever a transfusion is about to be administered or a used container is about to be replaced by a fresh one.

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The Clinical Use of Plasma Fractions

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On the basis of experience at the Blood Transfusion Service of the Swiss Red Cross, the following problems are discussed. 1. The clinical use of albumin solutions in comparison with plasma substitutes and electrolyte solutions. 2. The available and intravenously tolerated gamma globulin preparations are compared and critically evaluated. 3. The value of the coagulation factor concentrates is discussed.

During the Second World War, the team of E. J. Cohn in Boston developed a series of methods which permitted the fractionation of plasma on an industrial scale. They introduced concentrated albumin solutions, gamma globulin and fraction I-preparations into clinical medicine. The albumin solutions were heated to 60°C for a period of 10 hours and the risk of hepatitis transmission was thus completely abolished. The gamma globulin preparations proved *a priori* to be hepatitis-safe. During the fifties these methods established themselves in Europe, too. The salt-poor, 25% albumin solutions were, however, rarely used by the clinicians. The situation changed after PPL or PPF (plasma protein fraction) had become available. The original preparation, which was developed in our Institute by Nitschmann and Kistler [1, 2] consisted of totally desalted plasma, the heat-labile proteins being removed. This solution tolerated heating to 60°C for 10 hours and was thus hepatitis-proof. Technical problems connected with the sterility of the desalting columns and the isolation of the gamma globulins made it expedient to replace this product with crude albumin solutions obtained by the classical fractionation procedures. Since then, PPL or PPF is defined as a 3.5—5% albumin solution with a minimum albumin content of 85%. The current trend is towards more highly purified albumin solutions, so that one may pose the obvious question whether the term PPL or PPF is still justified. At any rate, the dilute, isotonic human albumin solutions and the PPL/PPF preparations may be rated as equal with regard to their therapeutic use. Since all these products are hepatitis-proof, it is no wonder that they should have driven the dried plasma and the pooled serum preparations out of the field. The tolerance is excellent; the incidence of febrile reactions being far below one per thousand. The consumption of albumin solutions has increased enormously over the years. In 1972, 29,440 liters of the 4% PPL solution and 1042 liters of 20% albumin were used in Switzerland, or the

equivalent of some 64,000 liters of plasma. Switzerland had, at this time, 6.4 million inhabitants.

Dilute isotonic albumin solutions are ideal preparations for initial blood volume support in states of shock. Their drawbacks lie in their limited availability – due to their human origin – and in the cost. At this moment, albumin is in world-wide short supply. The question therefore arises when and to which extent albumin may be replaced with colloid plasma substitutes. This problem has engaged us intensely for many years. At the time of the Korean War, our Army Medical Service asked for our opinion on the various plasma substitutes. We compared the data then at hand on the solutions of polyvinyl pyrrolidone, dextran and gelatin. We were impressed by the effectiveness and the safety of the gelatins and undertook to produce them in our Institute. We developed the preparation registered as Physiogel, which is manufactured under licence in Japan by the Toyo Jozo Company and marketed as Gelofusin.

In the sixties, the relative merits of the dextrans and the gelatins became the subject of a violent dispute in the German-speaking countries [3]. The conclusions of this discussion may be summarized as follows. The "clinical" dextrans such as Macrodex® are supplied as a 6% solution. The average molecular weight is 32,000. Physiogel®/Gelofusin® contains 4% modified fluid gelatin with a number average molecular weight of 22,000. The oncotic activity per gram of colloid relative to serum is 2.2 for the dextran and 3.6 for the gelatin preparation [4]. Because of their higher molecular weight, the dextrans have a longer intravascular half-life and thus a more protracted volume effect than the gelatins. In clinical practice this difference is as a rule irrelevant, since it can easily be compensated for by a somewhat larger and if necessary repeated dose of gelatin. As to the side effects, the dextrans suffer from the serious disadvantage that an infusion volume exceeding 1000 ml per 24 hours significantly increases the bleeding time. Still larger volumes may cause a genuine bleeding disorder which is highly undesirable in patients needing their intact haemostatic mechanism. By contrast, up to 10 litres of gelatin solution may be infused within 24 hours without fear of haemostatic, renal, pulmonary or other side effects. Allergic reactions to the gelatins are very rare, being in the order of one per thousand units infused.

Looking back upon the development of plasma substitutes over a period of 20 years, we are to-day convinced that the future belongs to the gelatins, the use of which for the initial blood volume support in hypovolaemic shock will enable us to save large quantities of the dilute, isotonic albumin solutions. At this time, Physiogel® is the emergency substitute of choice in many Swiss hospitals.

When balancing the indications for the use of albumin and of plasma substitutes against each other one must, however, keep in mind that the latter remain exogenous colloids, which the body strives to eliminate from the circulation as rapidly as possible. Erythrocytes, plasma proteins, electrolytes and water are the only genuine substitutes in hypovolaemic shock states. It appears, however, that plasma protein metabolism is quite resilient in a previously healthy individual who suffers a major acute blood loss which is treated with erythrocytes and plasma

substitutes only. The deficit of circulating plasma proteins is well compensated for by the extravascular reserves. Lundsgaard-Hansen and Pachlopnik [5] have recently performed a series of studies to assess the benefits of albumin solutions administered postoperatively according to a widespread custom in our country. To summarize their results, for a start, they carried out a prospective, controlled clinical study of 315 patients subjected to major elective abdominal surgery. Half of these patients received 4×500 ml 4% albumin solution postoperatively, the other half serving as controls. The number of days in hospital and the rate of clinical complications was the same in both groups. The treated patients had higher serum albumin, but lower serum globulin levels than the controls. As a result, the total protein concentration and the colloid osmotic pressure of serum did not differ significantly. The study demonstrated that dilute albumin solutions are poorly effective in the treatment of postoperative hypoproteinaemia. It seems preferable to confine the postoperative infusion of albumin to patients with severe serum protein depletion, who need massive amounts; it is futile to dissipate small doses of albumin in patients with minor deficits. The well-known sequels of severe hypoproteinaemia affect, above all, the absorption of fluid from the intestine and pulmonary gas exchange [6, 7]. It seems appropriate to forestall these complications in patients at risk by the timely and adequate provision of albumin. Information is lacking, however, on the degree of hypoproteinaemia at which such incidents will occur, and it probably varies from case to case. It may therefore be advisable to check the serum protein level and to initiate treatment if it goes below a certain "critical threshold". Let us assume that the interstitial volume should not increase more than 50% above normal, i.e. that the average patient should not retain more than 5 litres of water. This equals a decrease of the serum colloid osmotic pressure of 6.5 mm [8], i.e. about 21 mm of mercury. Departing from the relationship between colloid osmotic pressure and total serum protein concentration, we arrive at a critical protein level of between 5.0 and 5.5 g per 100 ml. In the course of their disease, 15 to 20% of patients with major abdominal surgery and 60% of septic intensive care patients reach a serum protein concentration of less than 5 g per 100 ml. Large amounts of albumin are required to correct in these cases the hypoproteinaemia, and thus the serum colloid osmotic pressure. An important factor contributing to this is the large extravascular deficit which is always present in albumin depletion states. The capillary walls being permeable to albumin, the interstitial space absorbs a substantial portion of the dose administered.

Altogether, the blood transfusion services are faced with the task of suggesting to the anaesthesiologist and the surgeon that for blood volume correction the dilute isotonic albumin solutions of the PPL/PPF type should initially be replaced by the gelatin plasma substitutes as far as possible. When bleeding has been controlled, the remaining protein deficit should then receive specific treatment. Furthermore, the available concentrated albumin solutions should be reserved for severe cases of hypoproteinaemia and not be dissipated in treating minor deficits. The guiding principle has been formulated by Lundsgaard-Hansen: Do not give a little to many, but much to a few! Summing up, we assume that the demand for albumin

will continue to increase during the next decade, despite the efforts of the blood transfusion services to propagate its well-aimed and economic use by the clinicians. This obliges the responsible institutions to ensure the timely provision of adequate capacities for the fractionation of plasma.

Besides albumin, the fractionation of plasma yields large quantities of gamma globulin, i.e. a 10 to 16-fold concentration of the IgG-globulins contained in the donor plasma. The standard gamma globulin preparation, which is usually dispensed as a 16% solution, contains large amounts of antiviral antibodies. In the beginning, the preparation accordingly served for the prophylaxis of viral diseases such as measles, poliomyelitis, hepatitis, etc. With rare exceptions, the prophylaxis of hepatitis A is all that remains of these indications, because effective vaccines were in the meantime developed against the other viral infections. A small dose of standard gamma globulins confers a sufficient protection against hepatitis A for 4 to 6 months, of which too little use is still being made. When one considers that vaccinations of minor or questionable effectiveness, e.g. against salmonellosis or cholera, are still compulsory in many countries for anyone visiting tropical or subtropical countries, it is hard to understand why the reliable effect of gamma globulin against hepatitis A is not exploited just as frequently.

In the years between 1955 and 1962, Barandun, Cottier, Riva and I [9, 10] devoted much time and effort in Bern to the study of immunodeficiency diseases. One aim of these studies was to shed more light on the biological functions of the immunoglobulins. As it turned out, bacterial infections of the respiratory and gastrointestinal tracts and the septic complications stemming therefrom were dominating events in patients suffering from agammaglobulinaemia. It thus became clear that the humoral immunity conferred by the immunoglobulins must be an important part of man's antibacterial defence mechanisms. The success of an intermittent substitution regimen in largely preventing and mitigating such infections in this type of patient showed that our assumption was correct. Evidence quickly accumulated, however, that these patients were extremely sensitive to even small doses of gamma globulin by the intravenous route, often showing violent incompatibility reactions. Proceeding to unravel the nature of these incidents, we found the reactants to be highly sensitive to the minute amounts of complement-binding IgG-aggregates which are present in the standard gamma globulin preparations [11]. The basic mechanism is comparable to a generalized Arthus phenomenon, the gamma globulin aggregates behaving like antigen-antibody immune complexes. It was thus necessary to develop gamma globulin preparations devoid of complement-fixing aggregates. This objective was attained by treating the native gamma globulin with pepsin, plasmin, or acid. The Behringwerke in Germany developed Gammavenin®, which consists of pepsin-fragmented IgG, mainly in the form of bivalent $F(ab)_2$ fragments. The Fc-fragments are largely broken down to small subfragments. The *in vivo* half-life of the antibody-containing fragments is about 18 hours. The $F(ab)_2$ fragments do not activate complement when fixed to the corresponding antigens. From the above it is clear that this preparation can be expected to fulfil the native gamma globulin functions only in part. Whereas it may have detoxifying

properties in bacterial infections with toxic symptoms, its short half-life makes it unsuited for the prophylaxis of infections in patients with agammaglobulinaemia. The preparation introduced by the Institut Mérieux in France under the name of Veinoglobulin® is an IgG fragmented with plasmin of human origin. It consists of monovalent Fab- and intact Fc-fragments. Thirty to 40% of the IgG molecules resist the action of plasmin; however, subclass analysis reveals them to differ qualitatively from normal IgG. The *in vivo* half-life of the Fab-fragments is 5 to 6 hours, that of the Fc-fragments 8 to 10 days. The half-life of the non-fragmented molecules is 20–22 days, like that of normal IgG. The biological effects of the product essentially rest on the antibody activity of the non-fragmented IgG moiety. In our Institute, we develop a modified gamma globulin (Intravenous Gammaglobulin SRK) by acid treatment at pH 4 with addition of pepsin in trace amounts. This procedure does not fragment the IgG molecules. The activation of complement upon the aggregation of the molecules is abolished by a change in the tertiary structure of the Fc-fragments. The *in vivo* half-life of the IgG molecules in this preparation is shortened to 12–14 days. Among the gamma globulins adapted for intravenous administration and available at this moment, the last-named product is the one best suited for the prophylactic treatment of agammaglobulinaemic patients. The Biotest Serum Institute in Germany has recently marketed an IgG preparation treated with β-propiolactone under the name of Intraglobin®. Though its molecules are not fragmented, the procedure has modified them so as to shorten their *in vivo* half-life to a moderate degree. Moreover, the isoantigenicity of this preparation has not been sufficiently explored.

This brief review makes it obvious that all intravenous gamma globulin preparations currently available have their particular drawbacks. We may, therefore, deem it a fortunate circumstance that Dr. Miekka in our Institute recently succeeded in demonstrating that the formation of complement-fixing aggregates during the isolation of gamma globulin is due to surface phenomena, which are suppressed by the presence of surface-active colloids like albumin, polyethylene glycol and gelatin during the fractionation process. These experiments might soon make it feasible to develop fractionation procedures on an industrial scale, which would yield an intact gamma globulin retaining the full biological effect of the IgG molecules and are devoid of side effects when given by the venous route. The potential importance of such an accomplishment is underlined by the fact that no one has so far succeeded in administering a sufficient quantity of biologically faultless gamma globulin to a patient who needed it. It is well-known that only 40 to 50% of the dose reaches the blood stream after an intramuscular injection, and what is more, these molecules have been altered and in part fragmented by the action of plasmin and tissue cathepsins. We are convinced that the possibility of giving large quantities of intact gamma globulin by the venous route will substantially improve the treatment of various immunodeficiency states and that we may succeed in controlling life-threatening infections resistant to antibiotics even in the absence of a demonstrable antibody deficiency. Another field which may open up to the intravenous gamma globulins would be those frequent infections which are

due to a partially defective immune mechanism in neonates, young children, and old subjects.

Apart from standard gamma globulin with an unspecified antibody content, immune globulins with specified properties play an ever increasing role. In our country antitoxic horse serum has been completely supplanted by human anti-tetanus immune globulin. We obtain it from the plasma of army recruits vaccinated against tetanus. From the plasma of this type of donor, we also obtain an anti-vaccinia immune globulin which is now in general use for the prophylaxis of encephalitis after late primovaccinations. By boostering and plasmapheresis of women having delivered children with haemolytic disease, large amounts of anti-Rh immune globulin are obtained which are used for prophylaxis in Rh-negative women in childbed. We have recently introduced an anti-zoster immune globulin for the prevention of varicella in children receiving immunosuppressive treatment. A specific anti-hepatitis B immune globulin will probably assume an important role in the prophylaxis of professional infections in laboratories and particularly in haemodialysis units.

When our Institute was founded in 1949, the production of dried plasma constituted its major activity. Like everybody else, we soon found pooled dried plasma to carry an intolerably high risk of transmitting hepatitis. Since 1954, we therefore strove to reduce that risk to the level of a standard blood transfusion by giving out single donor dried plasma exclusively [12]. As a corollary of this trend towards "small pool" or "single donor" products, Nitschmann et al. [13] developed a lyophilized fraction I preparation comprising two donations only. This preparation contains about 3 g of protein per unit, of which approximately 1 g is coagulable material. Under the name of "Antihaemophilic Globulin SRK", it served for substitutive treatment in cases of haemophilia A, and is still used as a broad spectrum haemostyptic under the name of "Fibrinogen SRK". When J. Pool demonstrated, in 1964, that the activity of factor VIII may be concentrated by cryoprecipitation, we introduced a small pool, lyophilized AHF-preparation containing the cryoprecipitate from 8 to 2 donations (2 liters or 500 ml fresh plasma), respectively [14]. The 8-unit cryoprecipitate is dissolved in 150 ml, the 2-unit precipitate in 40 ml of solvent. The infusion of 150 ml of this solution may be expected to increase the factor VIII activity in the recipient's plasma by $11-17\%$ ($0.11-0.17$ units per ml). The activity increase provided by the same infusion volume is thus about double that of the former 2-unit fraction I preparation. This lyophilized cryoprecipitate has given complete satisfaction in achieving haemostasis in patients with haemophilia A, as well as in cases of von Willebrand's disease. It has become the standard preparation for this type of patient in our country; the frozen cryoprecipitates and the large-pool cryoprecipitates corresponding to the ANRC preparations of intermediate purity [15, 16] have never been used in Switzerland. Lately we have made an AHF concentrate similar to the ANRC high-purity preparations available to our clinicians. Our concentrate contains about 250 units of AHF in 10 ml solvent with a protein content of approximately 1.2%. This concentrate has failed to supplant the established small-pool

cryoprecipitate. On the contrary, a number of clinicians has judged its haemostatic activity to be inferior to that of the older product.

Concentrates of the vitamin-K dependent coagulation factors II, VII, IX and X have recently become increasingly important. They serve not only for the treatment of haemophilia B, but particularly to achieve haemostasis following an overdose of anticoagulants and in liver disease. Our preparation is obtained by initially adsorbing the coagulation factors in question to DEAE-Sephadex A 50 and is named "Factor IX-Complex SRK". The subsequent alcohol precipitation largely equals the specifications for the original PPSB introduced by Soulier and Steinbuch [17, 18]. The DEAE-cellulose or Sephadex method permits the use of standard ACD or CPD plasma. This constitutes a major advantage as compared to the original French PPSB method for which fresh EDTA plasma was indispensable.

How does the Swiss Red Cross Blood Transfusion Service procure the plasma needed for its fractionation program? The main source continues to be the blood collected by the mobile teams of the Central Laboratory. To avoid conflicts with the activities of the local centres responsible for the whole blood program, the mobile teams restrict their activities to the rural districts. Last year, 205,310 units were collected, from which full use was made of the plasma. Of the packed red cells, however, only a modest portion could be utilized. The second source of plasma is the partially deplasmatized blood (PDB) program, which I described in my review of blood component therapy. This program yielded 15,314 liters of plasma for fractionation in 1972. The third, but almost insignificant source is outdated blood and blood from donors with a known history of hepatitis, both provided by the regional centres. In 1972, we received 3065 liters of plasma from this quarter. For the future we consider it essential to supplement our present sources with plasma obtained by plasmapheresis, and we feel confident that, in so doing, it will be feasible to conserve the principle of voluntary blood donations.

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Предмет и задачи трансфузиологии как научной дисциплины и ее место среди других медицинских наук

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Современная научно-техническая революция сопровождается бурным развитием всех областей человеческих знаний и техники. Рождаются новые научные направления, происходит их дифференциация и интеграция. Этот процесс захватил комплекс медико-биологических наук, в том числе науку о крови.

В течение всей своей истории учение о переливании крови пополнялось новыми фактами, арсеналом средств и методик. В настоящее время медицина располагает большим количеством эффективных средств, предназначенных для инфузионной терапии. Обосновать применение этих средств только учением о переливании крови стало невозможно. Возникла необходимость в более широком анализе методов терапии с использованием трансфузий различных средств, корригирующих состав крови больного. Так, постепенно складывалась трансфузиология — важнейшая составная часть современной медицинской науки и практики. Становление и развитие трансфузиологии способствовало прогрессу хирургии, терапии, педиатрии и других клинических наук. Вместе с тем эта наука всегда имела определенную самостоятельность, обусловленную особенностями предмета исследования и организации работы в системе здровоохранения.

Обширный материал, характеризующий содержание трансфузиологии, ее задачи, методы и цели, в настоящее время обобщен в книге Б. В. Петровского и Ч. С. Гусейнова «Трансфузионная терапия в хирургии». Впервые в отечественной литературе авторами сделана попытка дать определение трансфузиологии как научной дисциплины.

«За последнее годы, — отмечают авторы, — учение о переливании крови вышло за рамки своего первоначального представления. Современная трансфузиология представляет собой сложную проблему, включающую не только вопросы переливания и консервирования крови, но и сложные вопросы разделения крови на ее многочисленные лечебные и диагностические препараты, их дифференцированного применения в клинике, получения и клинического использования ряда кровезаменителей, серологии и совместимости, типирования форменных элементов крови и толерантности» (стр. 3). В этом определении четко сформулированы проблемы,

составляющие предмет исследования трансфузиологии. Перечень проблем может быть дополнен, но авторы и не стремились включить в свое определение абсолютно все проблемы трансфузиологии. Основная их цель — показать, что современная трансфузиология уверенно вышла за рамки только учения о переливании крови и гемотерапии.

В другой также недавно (1972) опубликованной монографии сделана попытка охарактеризовать иные стороны трансфузиологии. Книга А. Н. Филатова и Ф. В. Баллюзека «Управляемая гемодилюция» посвящена обоснованию главного ее положения, заключающегося в том, что «Качеством и количеством циркулирующей крови можно и нужно управлять в интересах более быстрого, более эффективного, более стойкого устранения или профилактики тех тяжелых нарушений, которые составляют основу подавляющего большинства форм патологии организма в самых различных клинических состояниях». Авторы справедливо подчеркивают значение одной из важнейших задач трансфузиологии — направленное изменение основных характеристик крови, представляющее собой сложное и глубокое вмешательство в процессы регуляции системы гомеостаза.

В литературе о содержании современной трансфузиологии важное место знимают труды акад. АМН СССР Н. А. Федорова. В его работах представлены задачи трансфузиологии как научной дисциплины. По мнению Н. А. Федорова, современная трансфузиология, опираясь на законы управления системой крови, должна разрабатывать методы целенаправленного регулирования состава и функций этой системы. Н. А. Федоров считает необходимым использование методов кибернетики и математического моделирования в изучении процессов гомеостаза.

Можно было бы продолжить анализа данных литературы, в которых так или иначе, более или менее подробно трактуются различные стороны современной трансфузиологии. Однако в этом нет большой необходимости, так как приведенные материалы в достаточной степени свидетельствуют о возможности формулирования полного и всестороннего определения современной трансфузиологии как научной дисциплины. Обобщая эти материалы, можно сформулировать определение трансфузиологии как **науки об управлении функциями организма путем целенаправленного воздействия на морфологический состав и физиологические свойства системы крови и внеклеточной жидкости с помощью парентерального введения органических и неорганических трансфузионных сред.**

Как и любая научная дисциплина, современная трансфузиология имеет конкретный предмет исследования, свои задачи, цели и наконец, свои методы исследования. Рассмотрим их в самой сжатой форме.

Предметом исследования современной трансфузиологии являются органические и неорганические трансфузионные среды и механизм их действия на организм человека. **В качестве трансфузионных сред** изучаются кровь, ее дериваты, костный мозг и другие кроветворные ткани, некоторые биологические субстраты (продукты гидролиза растительных и животных

белков, различные гормоны, ферменты, интерферон и др.), растворы солей, коллоидов, аминокислот, полипептидов, нуклеотидов, щелочей и других химических соединений, жировые эмульсии, антисептики и т. д.

Исследование **механизма действия** трансфузионных сред заключается в установлении закономерностей их влияния на организм в целом и на отдельные его системы. При этом изучаются особенности воздействия, направленность, ближайшие и отдаленные последствия при введении различных трансфузионных средств в зависимости от тактики их применения, дозировок, сочетаний и т. д., а также от исходного состояния организма.

Задачи трансфузиологии к настоящему времени хорошо определялись. Среди них можно выделить три группы, три направления.

Первая группа включает задачи по разработке теоретических основ трансфузиологии, в том числе обоснование требований к современным трансфузионным средам в зависимости от потребностей клинической медицины; моделирование, синтез, получение и изучение новых моно- и полифункциональных по свойствам трансфузионных средств; обоснование способов применения трансфузионных средств, обеспечивающих их эффективное лечебное действие и коррекцию нарушенных функций организма; разработка актуальных теоретических проблем консервирования крови и ее дериватов; изучение проблем, пограничных между трансфузиологией, трансплантологией, реаниматологией, анестезиологией, гематологией и многими фундаментальными биологическими науками (физиология, молекулярная биология, иммунология, иммунохимия, генетика и др.); прогнозирование направлений развития трансфузиологии в зависимости от состояния, уровня и тенденций развития биологии, медицины, физики, химии и других областей человеческого знания и техники.

Таким образом, задачи первой группы связаны с исследованием основных теоретических проблем, находящихся на стыке очень многих научных дисциплин. Диалектика развития трансфузиологии, как это нередко отмечается в условиях научно-технических революций, в том и состоит, что она родилась и прочно утвердилась в арсенале человеческих знаний и практики на стыке многих наук.

Задачи трансфузиологии, составляющие второе направление, определяют содержание исследований клинического плана. Они включают в себя определение показаний и противопоказаний к применению трансфузионных средств; обоснование методов и тактики трансфузионной терапии в различных условиях, при разных патологических состояниях; разработку требований к современным техническим средствам и аппаратуре для проведения трансфузионной терапии; научное обоснование мер профилактики посттрансфузионных осложнений в клинике; разработку рациональной организации трансфузиологической службы в системе здравоохранения.

Задачи трансфузиологии, отнесенные нами к третьей группе, носят научно-производственный характер. В их число входят: научная разработка принципов организации донорства и заготовки крови, научная разработ-

ка методов и организации заготовки дериватов крови и костного мозга, совершенсвование методов консервирования и хранения крови, гемодериватов и костного мозга, определение потребностей здравоохранения в трансфузионных средах и аппаратуре для проведения трансфузионной терапии, разработка технологической документации на промышленное производство трансфузионных средств, разработка методов контроля трансфузионных средств в процессе их производства и хранения.

Кратко подытоживая задачи и суммируя приведенные выше данные, можно отметить, что современная трансфузиология изучает, изыскивает и создает трансфузионные средства, обосновывает принципы массовой заготовки этих средств и разрабатывает способы их применения в клинике.

Подобно многим медицинским наукам, современная трансфузиология, как это видно из ее задач, состоит из трех основных частей: теоретической (общей) трансфузиологии, клинической (частной) трансфузиологии и производственной трансфузиологии. Каждый из этих разделов имеет свои научные направления, обеспечивающие решение основных задач трансфузиологии. Так, например, теоретическая (общая) трансфузиология содержит следующие разделы: а) химия, биохимия, биофизика крови; б) иммунохимия, изоиммунология и иммунология крови; в) цитология крови; г) химия и биохимия соединений, моделирующих структуру и функции крови; д) учение о консервировании тканей и органов; е) трансфузиологическая криобиология; ж) патологическая физиология с трансфузиологическим направлением; з) трансфузиологическая бактериология.

Клиническая же (частная) трансфузиология имеет в своем составе хирургическую трансфузиологию, трансфузиологию клиники внутренних болезней, трансфузиологию в педиатрии, акушерстве, гинекологии и т. д. Сюда же включается трансфузионная терапия в реаниматологии, а также в нефрологии (гемодиализ на аппарате «искусственная почка» и экстракорпоральное кровообращение с помощью аппаратов искусственного кровообращения).

Неконец, производственная трансфузиология включает ряд разделов технологического и организационного плана — технологию производства трансфузионных средств, организацию и тактику донорства и службы крови, научные основы государственного контроля за трансфузионными средствами.

Содержание трансфузиологии и ее задачи **подчинены главной цели** — разработке средств, методов и рекомендаций для управления функциями организма путем целенаправленного воздействия на морфологический состав и физиологические свойства системы крови и внеклеточной жидкости. **Вторая цель** трансфузиологии заключается в разработке наиболее совершенных форм организации трансфузиологической службы в системе здравоохранения, обеспечивающих своевременное использование ее достижений в лечебных учреждениях. Трансфузиологическая служба в СССР представляет собой систему специальных учреждений здравоохранения или струк-

турных подразделений в медицинских учреждениях, призванных обеспечить потребности больниц и клиник кровью и ее дериватами для трансфузионной терапии.

Таким образом, современная трансфузиологии является теоретической базой важной части советского здравоохранения — его службы крови. Вместе с тем современная трансфузиология является теоретическим фундаментом мощной промышленности трансфузионных средств.

Современная трансфузиология, будучи комплексной наукой, включает в себя ряд разделов из смежных наук. Поэтому трансфузиологи в своих исследованиях пользуются методами физиологии, физики, аналитической неорганической и органической химии, иммунологии, криобиологии, гематологии, хирургии, терапии и т. д. Однако наряду с методиками смежных наук учеными-трансфузиологами разработаны специфические методы исследования, к которым относятся общая, вспомогательная и изолированная перфузия, фракционирование крови (фракционирование плазмы, цитофорез, плазмофорез), управляемая гемодилюция, экстракорпоральное облучение крови, физиологическое, математическое, химическое моделирование трансфузиологических ситуаций, различные методики гемодиализа, культивирования клеток и их консервирования. Важную роль среди специальных трансфузиологических методов исследования играет клиническое испытание трансфузионных средств, осуществляемое по согласованным, заранее разработанным программам.

Краткая характеристика предмета, задач и содержания современной трансфузиологии позволяет определить ее место среди теоретических и клинических медицинских наук. Становление трансфузиологии было вызвано потребностями клинических дисциплин. Ттансфузиология «обслуживала» сначала акушерство, гинекологию, хирургию, гематологию, терапию, затем педиатрию, анестезиологию, реаниматологию, трансплантологию. В настоящее время трансфузиология имеет не меньшее значение чем рентгенология, физиотерапия, реаниматология, анестезиология. Служебная подчиненная роль трансфузиологии очевидна, но так же очевидно исключительное значение этой служебной роли. Без трансфузиологии невозможен прогресс хирургии, нельзя представить себе хирургические вмешательства без использования современных средств и методов трансфузиологии. В равной степени программа терапии многих патологических состояний не может бытъ полноценной без использования достижений трансфузиологии. **Во всех случаях трансфузиология** выступает как средство (весьма эффективное) для достижения **целей, определяемых современными клиническими науками.**

Следует отметить особое значение связей трансфузиологии с тремя разделами медицины — хирургией, гематологией и физиологией. Если рассматривать эти связи в историческом ракурсе, то не трудно убедиться, что трансфузиология родилась на стыке этих трех наук. Современная трансфузиология успешно развивается прежде всего совместно с гематологией

и хирургией и очень часто на базе объединенных клинических и научно-исследовательских учреждений. Подобное объединение необходимо.

От **«выравнивания крайностей»,** провозглашенных 50 лет назад А. А. Богдановым в качестве главной цели трансфузиологии, до управления функциями крови с использованием компьютеров — таков путь современной трансфузиологии. Сложность и специфичность методов трансфузиологии, разнообразие современных трансфузионных средств требуют от клиницистов хорошего знания особенностей состава каждой трансфузионной среды и метода ее использования, умения оценить целесообразность и целенеправленность ее лечебного действия, а также возможность комбинированного применения с целью повышения эффективности трансфузионной терапии. Именно в этой связи после рассмотрения вопроса о месте трансфузиологии среди других медицинских наук необходимо остановиться на проблеме трансфузиологической специализации врачей, работающих в этой области медицины. Дело в том, что иногда вдруг возникают вопросы о специальности врачей, занимающихся проблемами теоретической, клинической и производственной трансфузиологии в научно-исследовательских лабораториях, на станциях переливания крови или в клиниках. Настало время четко регламентировать специальность этих медицинских работников. Речь может идти об основной специальности — трансфузиолог — и ее разновидностях.

Трансфузиолог-клиницист должен хорошо владеть тактикой современной трансфузионной терапии, уметь избежать шаблона в выборе трансфузионных средств, применять их в соответствующих ситуациях со всесторонним учетом свойств, в том числе присущих им недостатков. Он должен уметь активно влиять трансфузиологическими средствами на механизм патологического процесса и механизм саногенеза. Трансфузиологи-клиницисты обязаны управлять аппаратурой для экстракорпорального кровообращения, гемодиализа, владеть способами искусственного поддержания заданного режима циркуляции крови и газообмена в организме. Поэтому трансфузиолог-клиницист должен хорошо понимать сущность своих действий и ответных реакций организма, должен быть грамотным физиком, химиком и даже бухгалтером, способным строго учитывать «приход» и «расход» переливаемых сред, их ингредиентов и изменений в распределении этих ингредиентов между секторами водных пространств организма (А. Н. Филатов).

Трансфузиологи-теоретики могут иметь различные направления в своей работе — биохимическое, иммунологическое, физиологическое, цитологическое и т. д. Они решают проблемы теоретической трансфузиологии в плане как фундаментальных, так и прикладных исследований, в том числе в плане создания новых трансфузионных средств.

Наконец, трансфузиологи, занимающиеся научно-производственными проблемами трансфузиологии, должны хорошо знать службу крови, уметь организовать заготовку крови, работу станций и отделений переливания

крови, лабораторий по производству препаратов из плазмы крови, компонентов крови, осуществлять контроль за качеством трансфузионных средств в процессе их производства, хранения и применения. Это большая группа практиков службы крови, специалистов по работе с донорами, по консервированию крови, эксфузионистов и т. д.

Таким образом, все специалисты, работающие в области трансфузиологии, должны именоваться трансфузиологами. Некоторые из них имеют смежные специальности, необходимые для решения задач, выдвигаемых основной дисциплиной.

В заключение следует напомнить, что современная трансфузиология прошла длительный путь исторического развития. Потребовалось несколько веков, в течение которых были периоды подъема и застоя, периоды блестящих открытий и постепенного эволюционного развития. В разные периоды и этапы развития трансфузиологии доминировали определенные идеи и тенденции. Каждому этапу были свойственны конкретные задачи. Настоящий этап развития трансфузиологии, свидетелями и участниками которого мы являемся, начался с середины текущего столетия. Он связан с гигантской научно-технической революцией. Основная задача этого этапа — соединение достижений научно-технической революции с потребностями современной трансфузиологии и новый качественный скачок в ее развитии.

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Subject and Tasks of Transfusiology as a Scientific Subject and its Place among other Medical Sciences

Transfusiology is a science of the control of functions of the organism by directed action on the morphological composition and physiological properties of the blood system and extracellular fluid by parenteral injection of organic and inorganic transfusion media. Organic and inorganic transfusion media and their action on the human organism are the subjects of study of modern transfusiology. Tasks of transfusiology, contents, methods of investigation and their inter-relationship with other medical sciences are presented in this paper.

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Prevalence of HB Ag and HB Ab in the Canadian Blood-donor Population*

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It is an unfortunate fact that, having tested almost two million blood donations for HB Ag, we have no proof of the effectiveness of all this work in reducing the incidence of post-transfusion hepatitis. Whether the use of methods capable of detecting lower concentrations of HB Ag will materially decrease the infectivity of our blood products appears debatable (e.g. [6]). The most direct approach is still greater discrimination in the prescription of blood components and products, a point with which I feel sure that my good friend Dr. Susan Hollán — to whom this paper is dedicated — will agree.

Post-transfusion hepatitis (PTH) is a viral infection the incidence of which is not accurately known, the aetiological agent or agents of which have not been isolated, and the diagnosis of which must usually be inferred; it continues to provide problems for all connected with blood-replacement therapy. Discovery of the hepatitis B antigen (HB Ag) and antibody (HB Ab) and of their presence in apparently healthy blood donors provided transfusion services with the first scientific tool for reducing the incidence of PTH.

In the Canadian national blood transfusion service, all blood donations have been tested simultaneously for HB Ag and HB Ab since the beginning of 1972. The particular technique of counter-immunoelectrophoresis (CIEP) used by all 16 regional transfusion centres has been described elsewhere [1]. Antiserum and antigen are pooled and dispensed in a central laboratory after suitable dilutions have been found as a result of tests with strong and weak examples of HB Ag (both ad and ay) and HB Ab. The particular lot of agarose used by all centres at any one time is selected on the basis of the above tests and upon measurement of electro-endosmotic flow [1]. In a recent international proficiency survey [2], this technique compared favourably with those used by other laboratories in other countries.

Data based upon the prevalence of HB Ag among blood donations have been reported from many countries including Canada [3]: such data, unfortunately, may include more than one donation from the same donor. Once a screening programme has begun and HB Ag carriers are being eliminated from the donor

* Based upon a paper presented before a national Conference on Serum Hepatitis, Toronto, 7th September, 1973.

panel, the prevalence of HB Ag among donations drops. Table 1 illustrates what has occurred in Canada to date.

Of greater value as an indication of the reservoir of infection are estimates of the prevalence of HB Ag among first-time and "regular" donors. Since our

Table 1

Reduction in prevalence of HB Ag-positive blood donations in Canada after one year of HB Ag screening by CIEP

Geographic location	Period of time and prevalence of HB Ag				Per cent change in prevalence
	Before May, 1972		April–August, 1973		
	sample size	prev-alence*	sample size	prev-alence*	
British Columbia	133,224	127	32,232	28	−78
Alberta	49,067	73	32,469	43	−41
Saskatchewan	18,630	32	19,818	25	−22
Manitoba	59,018	78	20,979	29	−63
Ontario	270,015	131	98,805	103	−21
Quebec	166,204	473	75,066	322	−32
Atlantic Provinces**	37,562	106	40,354	52	−51

* Prevalence per 10^5 blood donations

** Includes data from New Brunswick, Nova Scotia and Newfoundland

Table 2

Prevalence of HB Ag among volunteer blood donors in Canada in a sampling between May and July, 1973

Geographic location	First-time donors*		Regular donors*	
	sample size	prevalence**	sample size	prevalence**
British Columbia	5,133	117	28,192	28
Alberta	2,913	103	15,100	40
Saskatchewan	2,094	96	13,123	30
Manitoba	3,074	65	19,080	42
Ontario	23,894	306	75,111	39
Quebec	17,732	502	51,594	283
Nova Scotia & Newfoundland	2,634	152	19,182	16
Ontario				
London	2,566	195	9,243	32
Hamilton	2,745	182	11,703	17
Toronto	13,520	377	44,499	49
Ottawa	5.063	237	9.666	21

* Prevalence per 10^5 donors

** By definition, a "regular" donor is one who is *not* donating blood for the first time in contrast to the newcomer to the blood-donor clinic, the "new" donor, who has never donated before.

records are not yet fully computerized, the sampling shown in Table 2 is relatively small. Among first-time donors, the prevalence of HB Ag carriers in Canada is, with the exception of Ontario and Quebec, similar to that found in many other parts of the world (Table 3).

Within Ontario, the highest rates were found in the metropolitan – and now, cosmopolitan – area of Toronto and in Ottawa, which is near the Quebec border. The high frequency of HB Ag among new and "regular" donors in the province of Quebec is striking; whether this is due to environmental, economic or ethnic factors, or some combination of these, is not known. Duravetz and Valet [4] found that the ratio of ad : ay subtypes was 1 : 0.92 in Toronto, but 1 : 0.064 in Montreal; this suggestion of a difference in host response [5] is now under study.

The fact that HB Ag is still to be found among "regular" donors after almost two years of testing may seem peculiar. Many donors, however, donated last before the screening programme began. A few known carriers may have decided to disregard the warning not to attend donor clinics. Some may have a weak concentration of antigen missed when they donated last. And some may have devel-

Table 3

Prevalence of HB Ag carriers among first-time voluntary blood donors tested by CIEP

Country	Sample size	Prevalence (per 10^5)
Australia (W)	25,636	113
Scotland (W)	123,102	118
Denmark	16,626	144
Sweden	2,737	146
U. S. A.	825,690	157
Austria	167,000	458

From data kindly provided by Drs. M. G. Davey (Perth, W. Australia), J. Wallace (Glasgow), E. Freisleben & E. Dybkjaer (Copenhagen), J. Säfwenberg (Uppsala), T. J. Greenwalt (Washington D. C.), and Prof. F. Wewalka & Dr. F. Pesendorfer (Vienna).

Table 4

Per cent distribution of HB Ag carriers in Canada by age and sex

Age	Males	Females
18–25	46	52
26–30	22	21
31–40	23	15
41–65	11	12

Ratio, M : F = 5 : 1 (among all donors 2 : 1)

oped demonstrable HB Ag only since their last donation. In short, test reproducibility and timing on the one hand, and surveillance of carriers on the other, are both important factors in reducing the incidence of HB Ag in the regular-donor pool.

The distribution of HB Ag carriers by age and sex is shown in Table 4.

Table 5

Presence of HB Ag or HB Ab related to history of previous transfusions

Previously transfused	HB Ag+		HB Ab+	
	O	E*	O	E*
Yes	9	3.2	5	0.76
No	71	76.8	14	18.24
P		<0.001		<0.001

O = Observed. E = Expected
* Mean of 4% of Manitoba blood donors admit to previous transfusions
From data kindly provided by Miss C. Anderson (Winnipeg)

Table 6

Prevalence of HB Ab among blood donations in Canada and other countries as determined by CIEP

Geographic location	Sample size	Prevalence/10^5 HB Ab
British Columbia	80,433	80
Alberta	70,363	14
Saskatchewan	34,481	11
Manitoba	56,943	28
Ontario	282,253	51
Quebec	157,473	38
New Brunswick	22,828	4
Nova Scotia	40,847	24
Newfoundland	15,403	7
Denmark	16,626	198
England (S. E.)	151,166	146
W. Berlin	46,700	133
Scotland (W)	265,407	61
Poland	21,742	37
Venezuela	5,940	17

Data from other countries kindly supplied by Drs. E. Freisleben & E. Dybkjaer (Copenhagen), T. E. Cleghorn (London), W. Hasse (Berlin), J. Wallace (Glasgow), K. Madalínski (Warsaw), and C. Q. Gamboa (Caracas).

In Manitoba all blood donors are asked whether they have ever received a blood transfusion. Table 5 shows that, of those donors with a history of transfusion, a significantly higher proportion than expected were carriers of either HB Ag or HB Ab. On the other hand, the great majority of carriers gave no such history; this raises the question of what factors were responsible for their presumed infection.

The decision to test for HB Ab as well as for HB Ag was based upon the need to uncover examples of HB Ab for use as test reagents and upon the lack of certainty that donations containing HB Ab were not capable of causing PTH. For this purpose, CIEP is relatively insensitive. The low CIEP titres of most HB Ab detected – of 347 samples, 53% had titres of 1 – coupled with the subjective method for reading the test results lead to a lack of reproducibility. It is not surprising, then, that the prevalence of HB Ab detected by CIEP should fluctuate widely from centre to centre so important is the "personal equation" (Table 6 and [2]). The mean prevalence is 42 per 10^5, which is much lower than that reported by many European countries.

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Pathophysiological Implications of the Hepatitis Associated Antigen (HAA)*

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With the discovery of an antigen associated to the hepatitis B virus (HAA), a new tool has become available for the investigation of the relationship between this antigen and a number of pathological conditions. Depending on whether one is focussing on a given change in the liver or directly on the presence of HAA (Australia antigen), different patterns of association appear. Both approaches seem to be necessary to obtain a better insight into the actual connection between the presence of HAA, eventual antibodies to it, and a given liver change. In this paper, we are summarizing the present knowledge in an attempt to clarify some pathophysiological implications of the Australia antigen.

Liver changes in subjects with persistence of HAA in the circulating blood

A number of studies have been focussed on this subject with different results. While some authors found few changes in the liver [2, 13, 25], others found the contrary [23, 27, 29]. We have conducted a study on 10,000 blood donors in which we correlated a number of haematological parameters to the presence or absence of the Australia antigen [10]. Forty-eight of the investigated subjects, i.e. 0.48%, were found to be HAA positive. The distribution according to age

Table 1

Laboratory data of 46 HAA+ and 55 HAA— blood donors

	Elevation of transaminase	Thrombocytopenia $\leq$ 130,000	Diminution of C3 $>$ 1.5 S.D.
46 HAA+ blood donors	19 (41%)	16 (35%)	19 (41%)
24 with liver biopsy	11 (46%)	11 (46%)	10 (42%)
55 HAA— blood donors	2 (3.6%)	2 (4%)	0

* Supported by the Dinu Lipatti—Dubois Ferrière Foundation.

showed: up to 60 years, 0.36–0.66%, with a sudden increase after 60 years to 1.22%. The incidence of elevated SGOT was 41.3% in the HAA+ group as opposed to 3.6% in the HAA− group. There was a prevalence of thrombocytopenia of 16.7% with less than 100,000 platelets as opposed to 0% in the HAA− blood donor group. The complement assays showed a diminution of complement in 41% of the HAA+ subjects.

Table 2

Incidence of thrombocytopenia

	Number	Thrombocytopenia ≤ 130,000
HAA+ donors/elevated transaminases	20	70.0%
Acute viral hepatitis HAA+/elevated transaminases	18	11.1%

Table 3

Liver biopsy findings

	24 HAA+ blood donors		70 Controls	
Hepatitis	0		1	
Inflammatory foci	5		1	
Persistent hepatitis	5	(50%)	0	(2.8%)
CPA	1		0	
Postnecrotic cirrhosis	1		0	
Nutritional disorders (steatosis, fibrosis)	4	(16.7%)	14	(20%)
Miscellaneous findings	0		3	(4.2%)
Normal tissue	8	(33.3%)	51	(73%)

Tables 1 and 2 summarize the pattern of reactivity with regard to transaminases, thrombocytopenia and complement. Liver biopsies were carried out in 24 HAA+ subjects. The incidence of thrombocytopenia and diminution of C3 was similar in the two groups. Seventy biopsies in patients with arteriosclerotic Parkinson's syndrome served as a control. These 70 subjects were otherwise normal. The results of this study are summarized in Tables 3 and 4. Representative histological changes are given in Figs 1, 2 and 3.

The majority of HAA carriers exhibited, under light microscopy, hepatocytes with abundant eosinophilic ground-glass-like cytoplasm, a finding which was originally made by Hadziyannis et al. [12]. In cases with normal liver or only mini-

Table 4

Liver histology and elevation of transaminase SGOT in HAA+ blood donors

Histology	No.	Presence of hepatocytes with "ground glass" appearance	Elevation of serum transaminase
Normal tissue	8	7	3
Inflammatory foci	5	3	1
Chronic persistent hepatitis	5	4	5
Chronic aggressive hepatitis	1	0	1
Inactive post-necrotic cirrhosis	1	0	1
Nutritional disorders (steatosis, chronic alcoholic disorder)	4	1	1

mal changes, many hepatocytes had this ground-glass appearance, while in cases with more important liver involvement (persistent hepatitis), only a few cells with focal distribution could be found in 4 out of 5 cases. According to the study by Hadziyannis et al., these cells are HAA + when liver sections are assayed by immunofluorescence. The finding corroborates previous reports that if there is a significant amount of liver damage, there will not be many HAA-containing hepatocytes, i.e. the amount of liver damage is inversely proportional to the number of

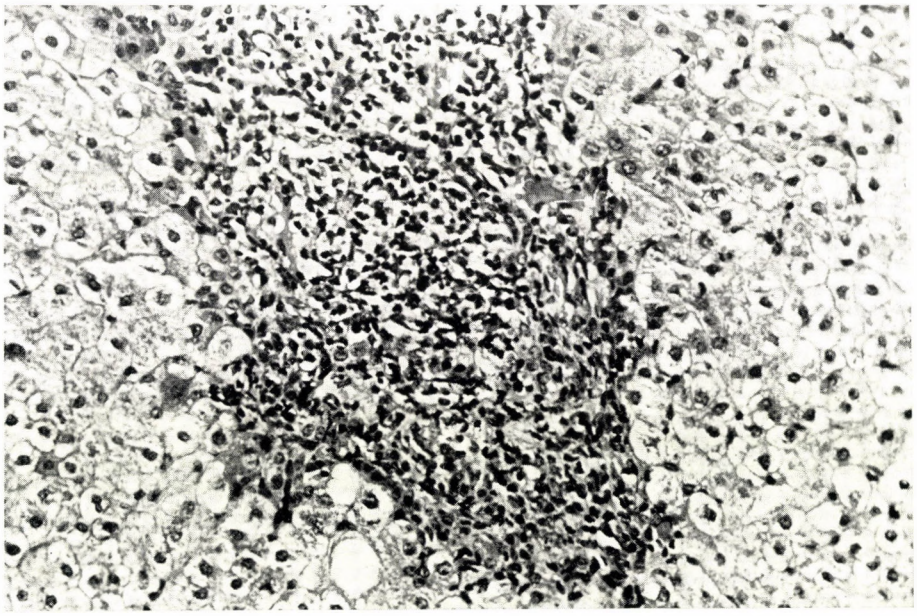

Fig. 1. Liver biopsy in HAA+ blood donor exhibiting typical changes associated with persistent hepatitis

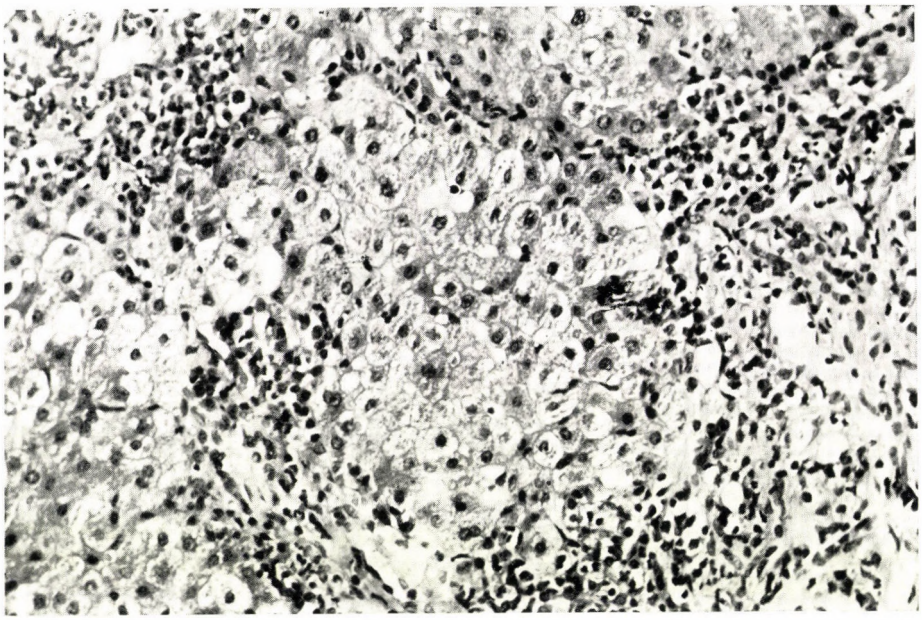

Fig. 2. Liver biopsy in HAA+ blood donor with the histological findings of chronic aggressive hepatitis. There are very few lymphocytes and plasma cells within the piecemeal necroses. This patient did not have hypergammaglobulinaemia, nor antibodies to smooth muscles nor to cell nuclei

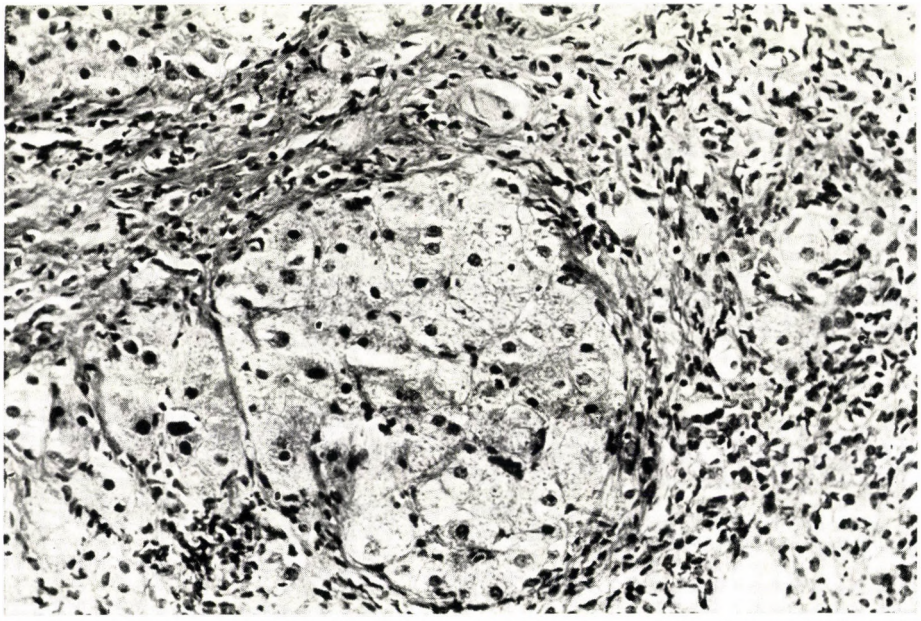

Fig. 3. Liver biopsy in HAA+ blood donor exhibiting features of post-necrotic cirrhosis

HAA-containing hepatocytes. Few HAA-containing hepatocytes, usually associated with the presence of HAA in Kupffer cells, are reported in patients with chronic or acute hepatitis [11, 12].

From this study it appears that the continuous presence of HAA is associated with a significant prevalence of liver changes. It is interesting to find changes which appear to be directly connected with hepatitis (discrete inflammatory infiltrations, chronic persistent hepatitis). In the small series there was one case of chronic aggressive hepatitis and one of post-necrotic cirrhosis. The prevalence of changes which may be secondary to nutritional disorders was similar in the control and in the study group.

HAA positive polyarteritis nodosa

The association of a persistence of HAA in the peripheral blood and polyarteritis nodosa has been found repeatedly [8, 9, 24, 28]: HAA was even found within the polyarteritis nodosa lesion. Whether the association is fortuitous or pathogenetically related has been much disputed. However, the fact that the incidence of liver damage is about six times higher in HAA+ polyarteritis nodosa than in HAA− polyarteritis nodosa, speaks in favour of a direct relationship [28]. Furthermore, Nowoslawski et al. [20] and Madalinski [15] found an immune complex type lesion in the spleen of patients who died in the course of HAA+ hepatitis. It thus appears that the simultaneous existence of HAA positivity in the blood of patients suffering from polyarteritis nodosa is neither fortuitous nor does HAA appear to be a complication of polyarteritis nodosa. It would seem logical to assume a causal relationship between the two facts.

HAA positive aggressive hepatitis

Chronic aggressive hepatitis is a histological and not a clinical diagnosis. It is characterized by a slightly irregular structure with a few intralobular inflammatory foci and, most typical, by a loss of the linear structure of liver plates. The portal tracts are enlarged and more or less heavily infiltrated by histiocytes, lymphocytes and plasma cells, as well as with occasional eosinophilic cells. These cells frequently penetrate the liver plates, forming small intrusions into the liver parenchyma, where necrotic liver cells are seen between chains of histiocytes, lymphocytes and plasma cells. These foci have been termed by Popper as "piecemeal necroses". The liver plates look like the edge of a cheese which has been attacked by mice. Within the portal space, there is frequently a proliferation of bile ducts, with an increase of collagen fibres. Intralobular infiltrations of histiocytes, lymphocytes, plasma cells and eosinophils, with an occasional necrosis of a single hepatocyte, may be found, especially within the centre of the lobules. Cholestasis, bile thrombi and bile droplets in liver cells, may be either absent or

prominent. There is always a polymorphism of nuclei with areas of increased mitotic activity. The slighter the parenchymal and mesenchymal damage, the more conspicuous are the piecemeal necroses and the histiocytic granulomas.

Chronic aggressive hepatitis comprises a spectrum of diseases with basically two well-defined conditions [5].

Nowoslawski et al. [20] and Madalinski [15] found in a number of HAA+ patients who succumbed to HAA+ hepatitis, a picture of chronic aggressive hepatitis. Piecemeal necroses usually only contained a few lymphocytes and plasmacellular elements. Immunofluorescence studies allowed the detection of immunoglobulin between piecemeal necroses but not within them. With the same patients, the authors sometimes found depositions of immunoglobulin and complement around blood vessels mainly in the spleen. For obvious reasons, HAA could be detected in the peripheral blood of practically all these patients.

At the other end of the spectrum, there is chronic active hepatitis. This condition is defined in clinical, biological and histological terms. In the active phase, piecemeal necroses are usually very rich in lymphocytes and plasma cells. The condition is characterized by the presence of high titre antibodies against smooth muscle antigen. In addition, one may also find antibodies against mitochondria and against nuclei. Smooth muscle antibodies are explained by the fact that liver cells contain actin, an antigen very concentrated in smooth muscle. In the initial phase, but frequently for a period of many years, these patients present a considerable hypergammaglobulinaemia. The clinical course is rather slow and usually lasts several years. In this condition, a combined 6-mercaptopurine/prednisone therapy usually reverses the histological, biological and clinical pattern of the disease within a few months, whereas the entire condition usually reappears upon discontinuation of therapy [18]. In three of our patients, relapses occurred after discontinuation of three to four years' therapy. In chronic active hepatitis as defined above, the incidence of HAA in the peripheral blood varies from one geographical location to another. In Australia, London and Geneva, very few patients proved to be HAA+ , while in New York City the HAA is detected in approximately one-third of patients with chronic active hepatitis [4, 5, 17].

It is interesting that HAA+ chronic aggressive hepatitis can gradually lead, sometimes after a few years' course, to typical chronic active hepatitis.

The role of HAA in the pathophysiology of disease

In a patient contracting hepatitis B, the Australia antigen appears early in peripheral blood and disappears after a period of one to three months. With the disappearance one may detect antibodies against this antigen, usually for a limited time [29].

The first question deals with the mechanism of liver damage in hepatitis, as well as with the immune mechanism necessary to eradicate the virus. Is the virus toxic for the liver cells in which it can be detected? Based on our knowledge of

HAA carriers exhibiting only mild signs of liver damage but with a large amount of hepatocytes containing the Australia antigen, the direct cytotoxicity must be weak. For this reason, a mechanism in which the T cell immune response against virus is responsible for elimination of the virus, at the price of producing a cytotoxic reaction of the cells carrying the virus, has been proposed to explain liver cell damage in hepatitis. In this connection, the experiments carried out by Kohler et al. [14, 27] in Denver are of special interest. These authors had the opportunity of transferring histocompatible lymphocytes to a chronic HAA carrier. The transfer produced on the one hand a temporary increase in the circulating HAA; on the other hand, the patient developed signs of liver damage with an increase in transaminases and bilirubin. It thus appears that the T cell immune response does lead to liver cell damage while liberating and possibly killing the virus [6].

The role of antibody in the pathogenesis of viral hepatitis is not fully understood. From clinical experience it appears that the antibody may prevent a subject from contracting hepatitis if given at the moment of infection. Accordingly, the antibody may prevent the virus from penetrating the liver cell, presumably by complexing the virus, followed by its sequestration within the reticuloendothelial system. It is also apparent that once the liver cell is infected with the virus, antibodies are no longer of much use to the patient. It appears that patients with agammaglobulinaemia contract viral hepatitis initially the same way as normal subjects. It has even been suspected that antibodies to the virus may become pathogenic in producing immune complex mediated damage. Indeed, a number of signs in patients with hepatitis has been attributed to the action of immune complexes, such as arthralgia [4, 27], thrombocytopenia and diminution of the serum complement level [10, 26], as well as kidney involvement (glomerular deposits of immunoglobulin and complement [5a]). With this potential danger in mind, Kohler et al. administered large amounts of antibody to a patient with chronic hepatitis B infection. They observed a temporary decrease in the serum HAA level, but there was no change in liver function, and no adverse clinical reactions were observed [14].

With this basic concept in mind of a hepatocellular infection which is overcome by a T cell immune response at the price of liver cell toxicity, hepatitis very much resembles chronic lymphocytic choriomeningitis of mice (CLCM). In this condition, the primary manifestations represent the consequence of T cell immune response against virus localized within the central nervous system [16]. In fact, the symptoms and signs of CLCM can be avoided either by rendering the animals immunotolerant to the virus at birth or by giving them immunosuppressive therapy, for example cyclophosphamide. Animals thus treated usually do not become fully immunotolerant since the antibody response is not suppressed. Much to the surprise of the investigators, the animals thus protected against the main expression of CLCM disease gradually develop a different type of disorder: a disseminated vascular condition which could be defined in terms of chronic immune complex disease with deposition of immunoglobulin and complement in the glomeruli as well as in various blood vessels [21]. The similarity between CLCM and hepatitis B

thus not only comprises the T cell immune response, but seems also to comprise the danger of an immune complex condition when the T cell response appears depressed. As a matter of fact, the increasing number of patients with typical polyarteritis nodosa, as mentioned before, may be explained along these lines.

Table 5

Probable immune response in various HAA related conditions

	T cell response	Antibody response
Uncomplicated hepatitis	+++	transient
Persistent hepatitis	++	transient
HAA carrier	(+)	+
Chronic aggressive hepatitis	+	++
HAA+ polyarteritis nodosa	0	+++

With these extreme conditions in mind, one might attempt to understand better other less well defined conditions associated with HAA. In Table 5 we have assessed the immune response of a number of HAA+ conditions in terms of T cell/antibody balance.

In the light of the above discussion, patients contracting uncomplicated hepatitis would have an excellent T cell response with just a temporary antibody response. The clinical signs and symptoms of hepatitis would be a consequence of the T cell encounter with virus-containing liver cells. The virus may be killed during this event or eliminated by antibody complexing upon liberation into the circulation. Viral antigen/antibody complexes may produce a number of disease manifestations such as arthralgia and thrombocytopenia [1, 4, 10, 27].

In the conditions known as persistent hepatitis, the T cell response may be insufficient to overcome the infection, yet sufficient to produce continuing damage by interacting with virus-containing liver cells [3].

"Healthy" HAA carriers would be individuals with a diminished T cell response to hepatitis virus B. In patients completely lacking a T cell response, there may be minimal liver damage, whilst the individuals exhibiting little liver damage may show a very mild T cell response. In this connection it is interesting to note that Frei et al. found a negative migration inhibition test in HAA+ normal individuals [7].

Chronic aggressive hepatitis [5] is a more complicated condition which may be associated with hepatitis B antigen. The condition may either be severe with a fatal outcome, or it may produce only slight changes such as an elevation of transaminases and/or bilirubin, with no clinical manifestations. The pathogenesis of the piecemeal necroses remains unknown. Since Nowoslawski et al. [20] found depositions of apparent immune complexes and even, in some patients, an immune complex type vasculitis, one might suggest an increased and prevailing antibody response with a decreased T cell response.

In this connection, we should briefly discuss patients with the histology of chronic aggressive hepatitis and the immunobiological characteristics of chronic active hepatitis. In these patients, piecemeal necroses are usually rich in lymphocytes and plasma cells. However, we have seen patients with typical post-hepatitis B chronic aggressive hepatitis with piecemeal necroses containing very few lymph and plasma cells, who have undergone gradual changes to post-necrotic cirrhosis, and who, a few years later, developed hyper-gammaglobulinaemia, a high titre antibody against smooth muscles and a histological picture of increased lymphoplasmocytic activity within the piecemeal necroses.

It has been suggested that piecemeal necrosis is the consequence of an autoimmune response to liver specific antigens [19]. Since any condition leading to liver necrosis may lead to liver specific immune phenomena, chronic active hepatitis may be a condition in which such phenomena have become self-perpetuating. The possibility cannot be ruled out that the pathological substrate of piecemeal necrosis is the consequence of an autoimmune response to liver cell specific antigens. However, it may be also the consequence of a prolonged T cell immune response to hepatitis B antigens. More investigation is necessary to define the pathogenesis of the piecemeal necrosis and also the development of the condition called chronic active hepatitis.

Finally, HAA polyarteritis nodosa may be considered a condition with insufficient T cell response and a continuing antibody response. The localization of vascular involvement in patients with polyarteritis nodosa does not reflect the distribution pattern seen in experimental serum sickness. It is possible that the varying vascular pattern of polyarteritis nodosa is due to a primary localization of the viral antigen with subsequent antibody reaction. More investigation is necessary to define precisely this intriguing condition.

Conclusion

A number of pathological conditions are known to be associated with HAA. With the discovery of the Australia antigen, it is now possible to assess the presence of antibodies to HAA. However, it is still questionable whether one can also reveal cellular immunity to the hepatitis B virus. In particular, conflicting results have been reported with lymphocyte cultures. While one group of investigators reported positive results in terms of increased thymidine uptake by lymphocytes upon addition of HAA [22, 30], others were not able to duplicate these results. In our own laboratory, we have been unable to stimulate lymphocytes with purified HAA, even in patients who had recovered from B hepatitis. More recently, the leucocyte migration inhibition test has been applied to this problem with promising results in different laboratories [7, 14]. However, with the use of human leucocytes, one is dealing with the migration of polys and not monocytes. Whether this inhibition is due to a T cell immune response, remains to be decided. Yet, with the new technique one has obtained a dissociation between antibody formation as detected

by radioimmunoassay and leucocyte inhibition. Indeed, patients recovering from B hepatitis usually display a positive migration inhibition test and no antibodies, while apparently healthy HAA carriers may have small amounts of antibody but usually show a negative migration inhibition test.

If one takes into account all other parameters, it may be permitted to propose the presumptive relationship summarized in Table 5. At one extreme, a violent T cell response would be able not only to kill the virus but also to destroy the host cells of the virus, i.e. the liver cells, at the risk of a fatal outcome for the patient. In the middle, there would be the patient with a minimum T cell response and a minimum antibody formation, with plenty of virus in the liver but few changes. The other extreme would be characterized by a lack of T cell response with continuing antibody formation resulting in an immune complex vasculitis, which again may lead to a fatal outcome. This scheme is certainly a gross over-simplification, it may, however, constitute a useful basis for future research.

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Preparation of Specific Immunoglobulin for the Prevention of Hepatitis B

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Isolation of IgG from the blood plasma with a high content of HBAg was made and the final product was tested by sensitive methods both immunochemical, radioimmunological and also by electron microscopy. HBAg was not detected in the final product. These findings suggest the possibility of preparing hyperimmune IgG containing a high titre of HBAb for preventive use in persons with a high risk of HB infection.

Introduction

Virus B-induced hepatitis (also referred to as "serum" or "inoculation" hepatitis) has become a subject of intensive studies since the discovery of HBAg (the so-called Australia antigen). It seems that a HBAg-positive factor is present in the plasma of most hepatitis B patients. It is not known whether HBAg is a real infective agent, or just an accompanying phenomenon. Many of the recent papers suggest [1, 2, 9] that some forms of the antigen, the so-called Dane's bodies in particular, could be listed among the picornaviruses and the presence of DNA in particles has also been reported [5].

The importance of screening for HBAg was especially increased after the discovery that the blood of antigen-positive donors induced hepatitis in the recipient [3, 6]. The high incidence of hepatitis B in renal dialysis units and after major surgery together with the fact that, by observing the incidence of HBAg, it was possible to prove that hepatitis virus B can persist in the organism for a long time, suggested the possibility of a virus carriership [4, 7].

Since the discovery of the two groups of hepatic antigen, HAAg and HBAg, the lack of cross-immunity between the two forms of the disease — hepatitis A and B — has been apparent, as the normal gamma globulin currently used for the prevention of hepatitis A in persons exposed to the infection failed to offer satisfactory protection against hepatitis B.

For some years we have studied the possibility of preparing an immunoglobulin from the plasma of patients convalescing from hepatitis B and having a specific antibody against HBAg. The idea was even more engaging when, by the detection of HBAg, it has become possible to find relevant evidence of HB antibody in the blood by means of immunochemical methods.

First of all it was necessary to prove that IgG prepared from convalescent plasma did not contain hepatitis virus B either free or in an antigen complex. To this end several studies have been conducted and our efforts have recently been supported by the analogous attempts in some other laboratories.

In 1966, plasma contaminated with Motol virus (identified later as ectromelia virus) inducing severe hepatitis in mice was fractionated [8]. Immunoglobulins obtained by standard ethanol fractionation according to Cohn, or with rivanol [9], were tested in mice and tissue cultures. Virus was detected in neither of them. In 1972, after more sensitive techniques of HBAg detection had been described, plasma containing this antigen in considerable quantity was fractionated by rivanol or column chromatography on Sephadex [9]. This also failed in revealing HBAg in any of the prepared immunoglobulins.

It was then decided to use a plasma pool obtained from HBAg-positive patients recovering from hepatitis B. The plasma was processed by ethanol fractionation and the products obtained were tested for HBAg.

Material and Methods

Two fractions were performed of two different plasma pools.

Experiment No. 1

The following fractions were prepared from the pool: Fibrinogen (packed per 1 g), 10% gamma globulin solution and 10% albumin solution. Initial plasma and isolated fractions were screened for HBAg by means of immunoelectrophoresis [direct as well as indirect (inhibitory) method] and by means of a Rapidophor set, Immuno Vienna (29 V/1 cm/30 min).

Experiment No. 2

Using ethanol fractionation the following products were obtained from the HBAg-positive plasma pool:

Fibrinogen
Immunoglobulin (gamma globulin)
20% Albumin
Fraction III
Supernatant F II
Supernatant F V

The products were tested by immunoelectrophoresis (as in Exp. No. 1) and by radioimmunoassay (RIA). Finally, a sample of the contaminated plasma and immunoglobulin prepared from it were analyzed electronoptically at Wallenberg Laboratory.

Results

Experiment No. 1

Immunoelectrophoresis of concentrated gamma globulin was performed with polyvalent AHSEL 1 (ÚSOL – Institute of Sera and Vaccines), to detect non-gamma globulin-like admixtures. On immunoelectrophoresis (Fig. 1), gamma globulin migrating towards the cathode prevailed, formed mostly by IgG, then IgA, a small portion of IgM and an unidentified complex with IgA-like mobility. As admixture there were transferrin (beta-1-S), 2–3 alpha$_2$ globulin complexes and an albumin-like migrating complex (most likely orosomucoid). These non-gamma globulin admixtures were present in traces.

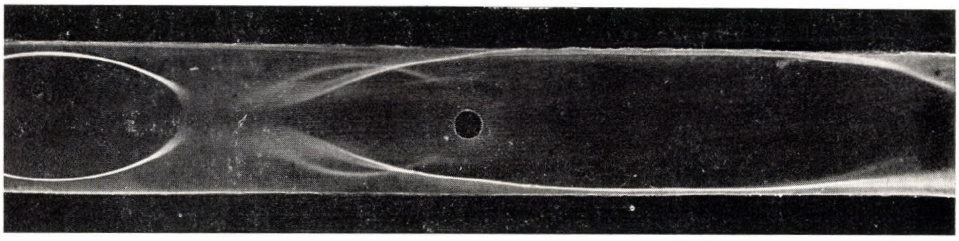

Fig. 1. Immunoelectrophoresis of IgG isolated by ethanolic fractionation from the plasma of a patient recovered from hepatitis B. (Details see in text)

Analysis for the presence of HBAg was carried out by direct counter-immunoelectrophoresis, by an inhibitory method [10] using two different HBAg sera (ÚSOL) and with a homologous Va serum (giving a good immunoprecipitation with HBAg) and eventually with HBAg antiserum Austragen (Immunodiagnostica, Vienna). HBAg was negative in the gamma globulin preparation at different dilutions ranging from 1 : 1 to 1 : 32, irrespective of the method used. The initial plasma and albumin fraction contained HBAg in high titre.

Table 1

Preparation	Non-diluted	Dilution			
		1 : 2	1 : 4	1 : 8	1 : 16
Initial contaminated plasma	+	+	+	+	–
Fibrinogen 25 mg/l ml saline sol. 0.9 %	–	–	–	–	–
Fibrinogen 25 mg/l ml AB plasma	–	–	–	–	–
Immunoglobulin	–	–	–	–	–
Albumin	+	+	+	+ (±)	–

Note: In each test series four controls were made; all of them were positive.

The initial material (plasma), albumin and gamma globulin were tested for the presence of antibody (HBAb). This was detected neither in the initial material, nor in albumin; when using counter-electrophoresis (which is more sensitive than the direct method) a precipitation line was formed in the gamma globulin range even at 1 : 4 dilution. With the Rapidophor set (immunoelfo at 29 V/1 cm/30 min), the results shown in Table 1 were obtained.

The negative result of the fibrinogen test should be evaluated with reservation, since the preparation tested was only partly soluble.

Experiment No. 2

The preparations obtained by fractionation of HBAg-contaminated plasma (fibrinogen, immunoglobulin, 20% albumin, F III, supernatant F II and supernatant F V) were analyzed by RIA (Abbott Pharmaceuticals) and by counter-immunoelectrophoresis using the Rapidophor set (Immuno, Vienna).

Detection of HBAg by RIA occurs by means of two antibodies (HBAg), one spread over the bottom of a test tube and the other labelled with ^{125}I. If present in the serum tested or in any of the plasma fractions, HBAg is fixed to the antibody (HBAb) spread over the tube bottom within 16 hrs; ^{125}I-labelled antibody which is added later reacts directly with the fixed HBAg forming an antibody-antigen-antibody complex (hence the sandwich principle); finally, the impulses per 100 sec are counted by means of a gamma-counter.

Table 2

HBAg-positive plasma	Impulses/100 sec	Rapidophor
ÚHKT	2,092	negative
Plzeň	2,111	+
Karlovy Vary	2,144	+
Klatovy	1,787	+
Rokycany	1,883	+
Hradec Králové	136	−
Plasma pool	2,320	+

Technically the tests are carried out as follows. 0.1 ml of serum or fraction tested is pipetted by an automatic pipette into a test tube with HBAb and allowed to incubate at room temperature for 16 hrs. The content is then sucked off and washed 5 times with 2 ml buffered solution, 0.1 ml of ^{125}I-labelled antibody is added and left to incubate for further 90 minutes, suction and washing is repeated as above, and the number of impulses is counted.

First, the number of impulses given by the negative controls is determined; this number, multiplied by 2.1, then represents the number of impulses indicating the presence of HBAg. The mean number of impulses given by 7 negative controls

Table 3

Preparation	Impulses/100 sec, mean (samples)	HBAg-positive	Pos. : Neg. (background V. = 13, in fbg 27)	Rapidophor
Fibrinogen				
Normal	147 (7)	309	16.5	neg.
Contaminated	2,009 (3)			pos.
Immunoglobulin				
Normal	461 (7)	968	1.9	neg.
Contaminated	862 (6)			neg.
Albumin 20%				
Normal	443 (7)	930	16.2	neg.
Contaminated	6,996 (3)			pos.
F III				
Normal	359 (7)	754	3.5	neg.
Contaminated	1,231 (3)			neg.
Supernatant F V				
Normal	409 (7)	859	1.2	neg.
Contaminated	498 (3)			neg.
Supernatant F II				
Normal	388 (7)	815	1.6	neg.
Contaminated	598 (3)			neg.

Table 4

HBAb-positive donor	Impulses/100 sec	HBAg-positive from	Pos. : Neg. (background V. = 13)	Rapidophor
No. 3	403		1.9	neg.
No. 4	488	737	1.5	neg.
P.	367		2.0	neg.

Table 5

Diagnostic serum with HBAb	Impulses/100 sec	HBAg-positive from	Pos. : Neg. (background V. = 27)	Rapidophor
Immuno 1	176		1.5	neg.
2	148		1.9	neg.
ÚSOL 1	173	254	1.6	neg.
2	187		1.4	neg.

in one of our tests was 121; therefore, 254 impulses in this case spoke for the presence of HBAg. Then the number of impulses in 3 HBAg-positive controls is measured and their mean value is calculated; in our case it was 756. This preliminary control trial is suitable only when the number of positive control impulses minus the background value of the counter (in our case 27) divided by the number of negative control impulses minus the background value of the counter is at least 2.1 (in our case it was 7.8).

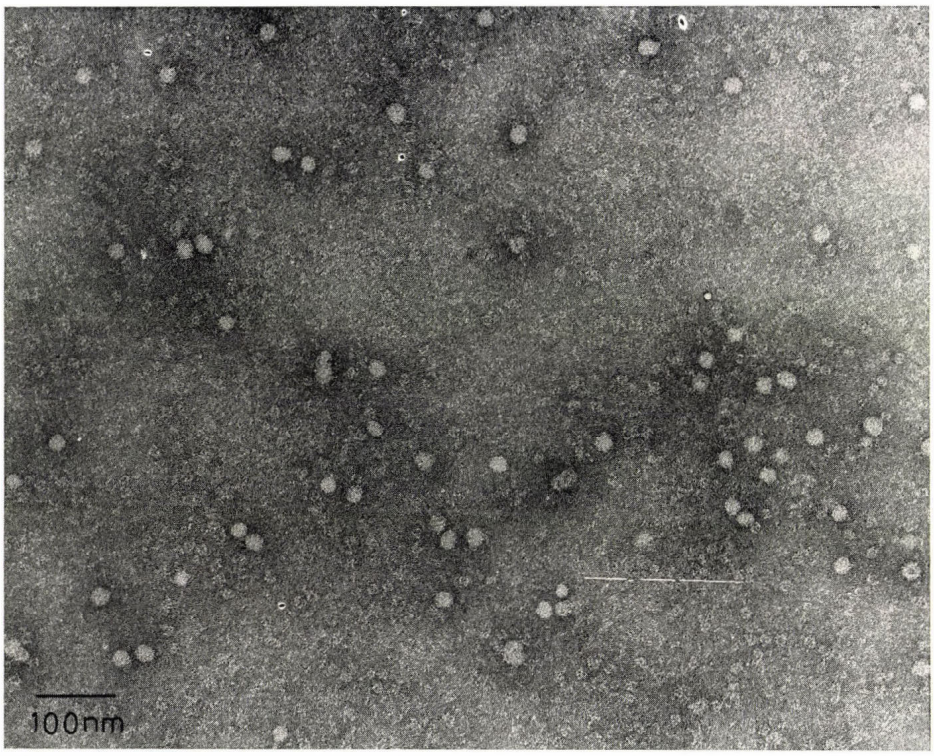

Fig. 2. A negatively stained preparation of the original contaminated plasma at pH 7.1 studied by transmission electron microscopy (TEM). A great number of virus-like particles are observed among the small protein molecules

Table 2 shows the results for various contaminated plasma specimens used for the fractionation pool.

Further, the results of individual fractions are given. With each preparation obtained from the contaminated plasma, an analogue sterile preparation (released for clinical application) was tested (see Table 3).

It has been studied whether HBAg was present in individuals whose plasma contained HBAb antibody, since, if so, they could be used as prospective donors

of plasma for the preparation of specific immunoglobulin. The results are summarized in Table 4, from which it is apparent that HBAg was never found in the plasma of any of the individuals tested.

Finally HBAg diagnostic sera of two firms (Immuno, Vienna; and ÚSOL, Prague) were analyzed for the presence of HBAg, with negative results (Table 5). Nevertheless, it was detected by RIA in fractions I, V and III, provided these had been prepared from HBAg-contaminated plasma. By counter-immunoelectrophoresis, HBAg was detected only in albumin (Table 3).

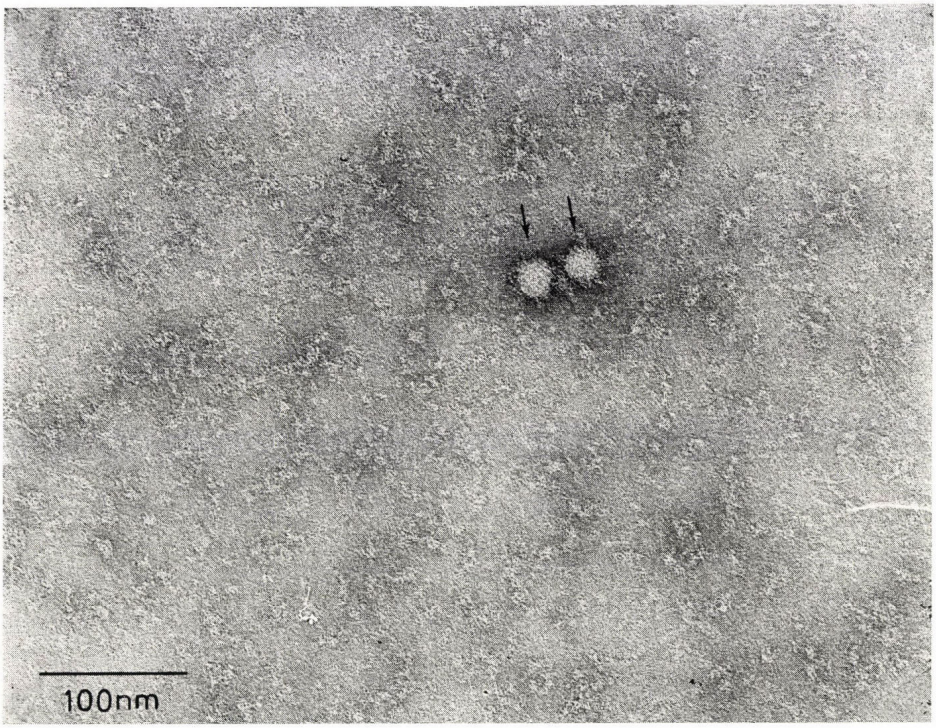

Fig. 3. TEM of an immunoglobulin fraction obtained by rivanol treatment occasionally demonstrating a few virus-like particles (indicated by arrows) among the immunoglobulin molecules

Electron microscopic examination of the different fractions, by means of transmission electron microscopy of negatively stained specimens, demonstrated a great number of particles with a mean diameter of approximately 210 nm, resembling small virus particles, in the original contaminated plasma (Fig. 2). In the immunoglobulin fractions obtained by rivanol treatment the small virus-like particles occurred occasionally (Fig. 3, indicated by arrows), and most of the samples comprised small molecules (Fig. 3). No particles resembling HBAg in Fig. 2

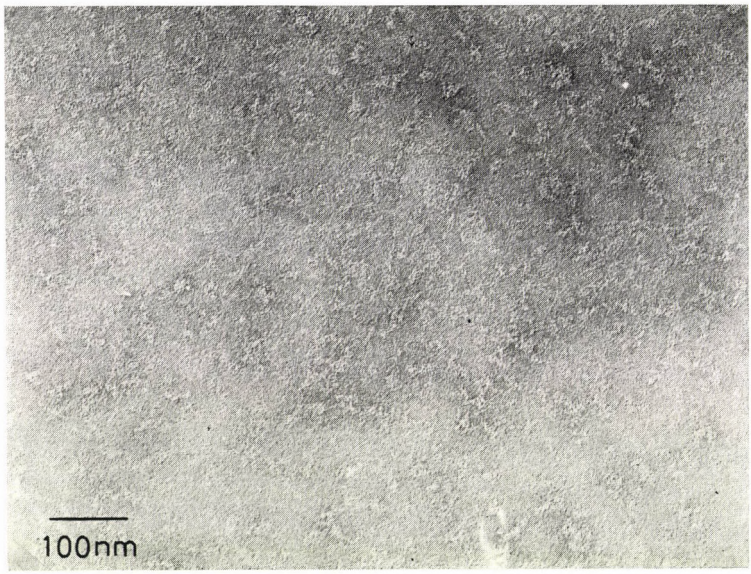

Fig. 4. A gamma globulin sample prepared by ethanol fractionation freed of any virus-like material

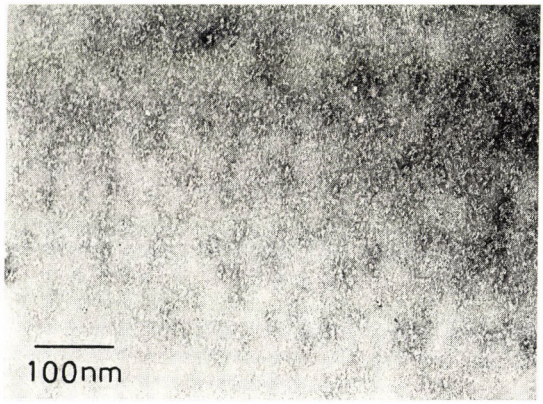

Fig. 5. An albumin sample prepared by ethanol fractionation only comprising small molecules

could be visualized in the preparations of gamma globulin, albumin, or fibrinogen produced by ethanol fractionation. The projections of small, negatively stained molecules of gamma globulin (Fig. 4) or albumin (Fig. 5) occasionally aggregated due to high protein concentration in the specimens.

Discussion

It is apparent from the results that gamma globulin obtained by the standard ethanol fractionation procedure does not contain HBAg even if prepared from heavily contaminated plasma. This was confirmed by three independent techniques: Immunoelectrophoresis, radio-immunoassay and the electronoptical method. Among the three methods, RIA was by far the most sensitive one; it gave positive results even where immunoelectrophoresis failed.

By means of electron microscopic examination it is possible to exclude the presence of antigen-antibody complexes untraceable by immunochemical methods.

Our hypothesis has been fully confirmed [8, 9] and it was concluded that immunoglobulin obtained by different fractionation techniques cannot be a potential source of infection transfer as far as hepatitis B is concerned.

The first attempts at hepatitis B prevention have been made a long time ago. The efficiency of normal gamma globulin used for hepatitis A prophylaxis is doubtful and many authors reject it altogether. If, after all, some effect was observed, it was only after large doses (10 ml) of gamma globulin [13]. Early experiments with gamma globulin prepared from plasma of hepatitis B convalescents were also unsuccessful [12].

Since 1971, a series of studies has appeared [13–16] on the protective effect of immunoglobulin prepared from plasma of patients convalescing from hepatitis B. Krugman et al. [4] reported that immunoglobulin obtained in this way afforded protection in 70%. The optimum dosage of the preparation whose protective effect was apparent for 6 months, was estimated at 5 ml [15].

In 1972, Soulier et al. [11] published a report on the preparation of "anti-Australia" immunoglobulin, relating the first experience with its application in hepatitis B prevention. They reached favourable results after the application of blood from a preserve in which HBAg was later detected as well as in intensive care units with increased danger of hepatitis B.

It follows from our results that it is possible to prepare HB antigen-free gamma globulin even from HBAg-contaminated plasma. In fact, the risk of infectious agent transfer would be practically eliminated if convalescent plasma of high HBAb titre and without HBAg was used for the preparation of anti-HB immunoglobulin, the more so, as this agent was not detected by radioimmunological or electronoptical tests even in immunoglobulin prepared from HBAg-contaminated plasma.

Many further problems have to be solved before specific anti-HB immunoglobulin can be introduced into everyday practice. (a) The effect of exogenous antibody (passive immunization) may attenuate the immunologic response. Prince et al. [14] suggest that careful measures must be taken especially during the incubation period and shortly after exposition to hepatitis B. (b) A follow-up should be made with the aim of elucidating the effect on the virus-carrier and the possible development of an autoimmune reaction induced by the antigen-antibody complex.

We are, however, of the opinion that even now the application of this specific immunoglobulin should be considered where contaminated blood or blood derivatives are proved to have been administered; in this case a single application of a large quantity of the preparation would be required.

Conclusions

1. Ethanol fractionation of plasma with high HBAg contents was performed.

2. Immunoelectrophoretic, radio-immunochemical and the electronoptical methods failed to detect HBAg in the gamma globulin prepared by the above technique.

3. Antigen was not detected in gamma globulin from HBAg-free plasma of hepatitis B convalescents.

4. The therapeutic application of anti-HBAg immunoglobulin for the prevention of hepatitis B has been discussed. According to the world literature, this could be possible in terms of a single application in cases where HBAg-contaminated blood or blood derivatives have been transfused.

5. The problem of regular and repeated applications of the preparation in intensive care units remains open to further studies.

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Some General Remarks about Research in Spontaneous Arrest of Bleeding

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In a noteworthy monograph published a few years ago [1], Sir Harold Himsworth, former Secretary of the British Medical Council, pointed out that it was essential for the growth of scientific knowledge to promote steadfast exchange between specialized and basic research. This is well shown by the part played by the sympathetic nervous system, the catecholamines and some of their metabolites in the spontaneous arrest of bleeding, in other words, spontaneous haemostasis [2, 3].

In 1922, Naegeli wrote about bleeding time as follows. "Diese Methode ist natürlich sehr roh und die Tiefe des Stiches nie genau zu bemessen. Die Resultate sind selbstverständlich auch sehr abhängig von der Verletzung kleiner Gefäße. Trotzdem gibt die Methode gewisse Einblicke."

At that time we had started researches on the arrest of bleeding which led my collaborators and myself to an extensive statistical study of mean bleeding time (M. B. T.) in animals and human beings. The main conclusion was that the M. B. T. (i.e. the average value of a statistically sufficient number of individual bleeding times measured in an experimental animal or in a man under standardized conditions) is nearly the same at each ear (and this is also true for two narrow symmetrical cutaneous regions) provided that no factor had acted on the organism between the two series of determinations.

Thanks to our "correlation method" for studying spontaneous haemostasis strongly based upon such an amount of statistical data, we could discover that very small doses of a labile oxidized adrenaline compound called adrenochrome immediately and sharply reduce the M. B. T., but only when injected before the skin is pricked or cut.

Then we proposed to stabilize adrenochrome and consequently stable derivatives of this compound became available as patent medicines under the names of Adrenoxyl (Labaz), Adrenosem (Massengil) and Adona (Tanabe). On the other hand the evidence we obtained concerning multicausation in disturbed haemostasis prompted us to recommend synergistic administration of stabilized adrenochrome derivatives and of efficacious coagulants as preventive treatment of haemorrhages which cannot be treated specifically or locally.

When Axelrod discovered that orthomethylation to metanephrine by means of D-methyl-transferase is the main pathway of human catecholamine metab-

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olism, it became dubious whether adrenochrome could play a role in the arrest of bleeding. However, Axelrod's important discovery does not exclude that tiny parts of adrenaline oxidized into adrenochrome in the organism might be sufficient to reduce the bleeding time. This is the only possibility compatible with the bulk of our experiments concerning the role of adrenergic function in haemostasis.

In another connection, clinicians, physiopathologists and even statisticians have organized controlled clinical trials in order to see whether stabilized adrenochrome derivatives would reduce surgical blood losses in non-haemorrhagic people or, by chance, might be useful in spontaneous profuse haemorrhages.

After a survey of about thirty trials, I could only consider two of them as worthwhile. Their results as well as some personal data concerning the recurrence of bleeding in experimental conditions suggested a new problem, i.e. a possible correlation between length of bleeding times and haemostatic plug resistance.

In spite of many experiments, our knowledge is still poor of the relative importance and mode of action of the main factors involved in arrest of bleeding, namely blood clotting, adhesion of platelets to the injured vessel wall and their reciprocal aggregation, haemostatic plug resistance, disturbances of local blood circulation. This is especially true where the haemostatic properties of sympathomimetic amines are concerned. In this connection, von Euler's outstanding observations upon the submicroscopic structures of adrenergic axones might be the starting point of a new trend of research in the chapter of spontaneous haemostasis.

Thus we have seen in the field of spontaneous arrest of bleeding how exchanges between specialized and basic research may favour the development of scientific knowledge. But we want to add to this brief statement two remarks of practical interest.

1. As recommended by H. P. Himsworth, it is important for Governments and scientific Foundations in order to facilitate such exchanges to subsidized research according to the common field of interest of the investigators engaged in a same "province" of knowledge, for instance the biomedical or the bioagricultural one, instead of subsidizing specialized and unspecialized investigators.

2. The investigators should be sufficiently informed of the various trends of research in the "province" of knowledge they are working in. On the other hand, they ought to fear some personal lack of criticism in the planning and carrying out of their own research.

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Standardization of Thromboplastin Reagents and Control Plasmas

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Different thromboplastins give different results. The standardization of reagents and the development of means for comparing results is an urgent problem, because of the clinical problems involved. In order to assess their acceptability in clinical laboratories, different reagents have been compared concerning stability, specificity and sensitivity to separate clotting factors and group of factors and to the clotting defect caused by oral anticoagulants. Certain preparations have a too low sensitivity to factors they are supposed to measure. Control plasmas have recently been introduced as reference materials for the quality control of thromboplastin preparations and for establishing the therapeutic range in oral anticoagulation. It is found that most of the commercial plasmas are highly activated, containing activated factor VII, which produces abnormally short clotting times and false high percentage values, leading to an all too low and ineffective intensity of anticoagulation.

The introduction in recent years of poorly standardized thromboplastin reagents, of different ways of expressing results and of contact activated standard plasmas as reference materials, has created a chaotic and confusing situation. It has repeatedly been demonstrated that different prothrombin time methods give highly different results by testing of the same plasma from a patient on anticoagulant therapy (Table 1). The clinician, who is responsible for the patient, feels bewildered. Which of these results should he choose for guiding the treatment, which might mean life or death to the patient.

Improved standardization of the thromboplastin time methods has been an urgent problem for several years and has been extensively studied by the Thromboplastin Standardization Sub-Committee of the International Committee of Thrombosis and Haemostasis [3—10] and by the Sub-Committee on Coagulation Reagents of the Standards Committee of the College of American Pathologists.

It has now been generally agreed that before acceptance of a reagent to be used in clinical laboratories, it should fulfil the following minimum requirements: It should be stable, sensitive to the factors it is supposed to measure, and the results should be reproducible.

Table 1

Great differences are observed when testing the same plasma from a patient on anticoagulant therapy with different methods. Correlation curves for converting clotting times in seconds into per cent of normal activity, were made by the same normal non-activated plasma (the Oslo standard) for all methods. Adsorbed plasma was used as diluent for all thromboplastin used for Quick's method

	Results in per cent of normal		
	patient no. 1	2	3
Thrombotest	18	24	9
Manchester comparative reagent	28	42	17
Dade	26	34	14
Ortho	39	45	17
Geigy	52	35	22
Simplastin	56	70	26
Acuplastin	45	48	20
Difco	32	42	23
2-7-10	24	29	14
Human brain	30	44	19
Rabbit brain	31	46	21
P-P method	29	43	18
Normotest	38	52	20

Stability

Bangham et al. [1, 2] at the Division of Biological Standards, National Institution for Medical Research in London, a laboratory which also serves as a control laboratory for W. H. O., have found that certain thromboplastin preparations, which had been freeze-dried and sealed in evacuated ampoules under the conditions used for biological reference materials, were very stable on accelerated degradation. Very good stability was found for code 68/434 (which is Thrombotest [16]), code 70/115 (which is Normotest with rabbit brain [18]), code 67/40 (which is like Normotest with human brain) and also code 69/223 (which is human brain alone) and code 71/78 (which is rabbit brain alone). These five preparations now serve as standard or reference preparations for the control of other thromboplastins.

There are still preparations in use which are not stable. Dried thromboplastins, which are exposed to air, lose potency after a few weeks. Phenolized liquid extracts (such as the Manchester reagent) lose activity both on freezing and thawing and on storage at 4–6°C for a few months. Liquid extracts without phenol might resist freezing and thawing, but when stored at −20°C a loss of activity is seen after a few months.

Sensitivity

The sensitivity of a thromboplastin preparation is defined as the extent of prolongation of the clotting time of an abnormal test sample as compared to a normal sample. Thromboplastins displaying large differences are called sensitive, because they are easily discriminating preparations.

Sensitivity to separate factors

It is an obvious requirement that thromboplastins should be sensitive to the factor or factors to be measured. Most important in guiding anticoagulation and testing of liver function is the sensitivity to factors VII and X. One of the reasons for low sensitivity to these factors of certain reagents is a contamination with serum clotting factors or coagulation intermediates which originate from blood in the organ used for preparation

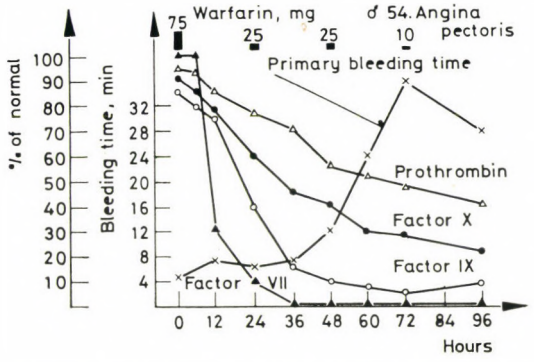

Fig. 1. On high coumarin dosage, the clotting factors decrease according to their survival time in the circulation. Factor VII decreases first (half-life of about 5 hours), then follows factor IX (half-life about 24 hours), factor X (half-life about 48 hours) and finally prothrombin (half-life 60—70 hours). The primary bleeding time becomes prolonged when both factor VII and IX are reduced below certain critical levels and the risk of clinical bleeding is then increased

During the initial period of anticoagulant therapy, factor VII, which has a half-life of only 4—5 hours, will disappear first. Then follow factor IX, factor X and finally prothrombin (Fig. 1). The disappearance of factor VII does not usually result in severe bleedings. The primary bleeding time remains normal. If factor IX is also reduced to below a certain critical level of about 8%, bleeding time becomes prolonged and bleedings are likely to occur. A similar situation occurs if the dosage is increased too much in a stabilized patient.

In order to detect these situations it is imperative that the reagent used be sensitive to factor VII, because thromboplastin methods as a rule are not sensitive

Table 2

Sensitivity ratios of different thromboplastin reagents

	Normal plasma			Dicoumarol		Factor VII		Factor X	
	100%, sec	10%, sec	ratio*	plasma, sec	T.T. 7% ratio	defi-cient, sec	plasma ratio	defi-cient, sec	plasma ratio
Calcium thrombo-plastin (Stago)	14.8	24.4	1.65	33.4	2.26	56.4	3.81	62.1	4.19
Hepato Quick (Stago)	30.0	195.0	6.50	125.0	4.17	102.0	3.40	146.0	4.88
Simplastin (rabbit brain and lung)	13.6	23.2	1.70	26.0	1.91	54.6	4.01	49.0	3.60
Simplastin A	25.8	87.0	2.37	93.0	3.60	57.4	2.22	113.2	4.37
Dade thrombo-plastin (rabbit brain)	11.8	21.4	1.81	23.4	1.98	32.4	2.74	48.3	4.09
Rabbit brain (labo-ratory prep.)	14.4	28.6	1.99	31.0	2.15	69.8	4.84	60.0	4.16
Human brain (labo-ratory prep.)	13.2	32.2	2.48	34.2	2.59	104.6	7.92	78.2	5.92
Thrombotest	41.8	114.0	2.72	146.6	3.51	168.4	4.03	234.5	5.61
Normotest (test vol. 0.025 ml)	20.4	81.6	4.00	88.2	4.32	143.8	7.05	100.0	4.90
Normotest (test vol. 0.010 ml)	27.4	178.1	6.50	—	—	222.0	8.10	180.2	6.56
Suggested minimum requirement			1.80		1.60		2.0 – 2.50		2.0 – 2.50

* Diluted with adsorbed plasma

to the depression of factor IX. The importance of a high sensitivity to factor VII has recently been stressed by several workers.

A high sensitivity to factor X is important under all circumstances in view of the essential role of factor X in both the intrinsic and the extrinsic clotting system [22].

Sensitivity to factors VII and X can be measured by testing of a normal reference plasma and plasma from patients who are congenitally deficient in one of these factors and then calculate the prolongation of the clotting time or the prolongation ratio (sensitivity ratio) as shown in Table 2. Among the reagents tested in this experiment, Normotest and human thromboplastin had the highest, and Simplastin A and Dade thromboplastin the lowest sensitivity to factor VII. The small difference in clotting times suggests that these latter preparations contain contaminations which compensate for the lack of factor VII.

On testing the sensitivity to factor X more equal sensitivities of all preparations were found.

Overall sensitivity to a combined reduction of factors II, VII and X

This sensitivity is reflected in the prolongation of the thromboplastin time by testing of plasmas with decreasing activities of factors II, VII and X, but with normal concentrations of the other clotting factors. The usual method is to test a normal standard reference plasma of 100% activity and a plasma with 10% activity of factors II, VII and X, prepared by a 1/10 dilution of the normal plasma in adsorbed plasma, containing the same citrate concentration as the normal plasma, and then calculate the prolongation ratio. It was found that Normotest and Hepato-Quick had the highest and Simplastin the lowest sensitivity (Table 2).

Sensitivity to the coumarin defect

Methods for anticoagulant control should measure all the essential changes which govern the haemostatic function on the one side and the antithrombotic effect on the other side. It was generally accepted for many years that the coumarin congeners act exclusively by reducing the vitamin K-dependent clotting factors, prothrombin and factors VII, IX and X. In 1963 a most important discovery was made by Hemker et al. in The Netherlands [11–13]; they demonstrated that anticoagulant therapy and vitamin K-deficiency, in addition to the effect mentioned, results in the appearance of a circulating anticoagulant, which they termed PIVKA (protein induced by vitamin K antagonists or absence). PIVKA was first assumed to be a pre-stage of prothrombin (pre-prothrombin). It is now quite clear that PIVKA represents pre-stages of all the 4 vitamin K dependent clotting factors. The antigenic properties of the PIVKA factors are the same as for the normal factors. By immunological techniques it has been found that the concentrations of normal plus abnormal factors are about the same before and during anticoagulation. The polypeptides which are responsible for the antigenicity, therefore seem to be synthesized and released in fairly normal amounts also in absence of vitamin K.

From these immunological studies it is assumed that the PIVKA inhibitors represent the difference between the original biological activity of the four factors involved and the remaining activity during anticoagulation. A patient with an initial value of 80% of prothrombin, factor VII and X, will have 65% as PIVKA at a treatment level of 15%. A patient starting at a level of 120% will have a PIVKA activity of 105% at the same therapeutic level. The amount of inhibitor consequently might vary quite much in anticoagulated patients. This has been confirmed by various methods of quantitation [19].

The sensitivity to the coumarin defect is reflected in the difference between the thromboplastin time of normal plasma and of plasma from patients on anticoagulant treatment. Several such studies have shown findings similar to those of Biggs (Table 3), that Thrombotest has the highest sensitivity and Simplastin the lowest. Simplastin had a difference of only 3–4 seconds between normal plasma

and anticoagulated plasma as against a difference of more than 40 seconds with Thrombotest (the two-seven-ten-reagent is the English version of Thrombotest).

From the study illustrated in Table 2, which includes Normotest and Boehringer's reagent, it will be seen that Normotest and Hepato-Quick have an even higher sensitivity than Thrombotest, whereas Simplastin again has the lowest. The high sensitivity of Normotest is a reflexion of its high sensitivity to factors VII and X, because it is insensitive to PIVKA.

Table 3

Results of testing a normal sample and one from a patient treated with anticoagulant, using various methods. From Biggs 1965 [3]

Method	Normal plasma	Anticoagulation plasma	Difference, seconds	Sensitivity ratio
Geigy	16.4	24.8	8.4	1.51
Dade	12.5	20.8	8.3	1.66
Stayne	15.6	21.8	6.2	1.39
Difco	13.0	20.0	7.0	1.53
Simplastin	13.8	17.2	3.4	1.24
Acuplastin	15.7	26.2	10.5	1.68
Human brain (laboratory)	16.6	26.0	10.0	1.56
2-7-10	34.5	77.0	43.5	2.23
Thrombotest	38.5	80.0	41.5	2.07

There is a great difference in the sensitivity of thromboplastin preparations to the inhibitory effect of PIVKA. Several studies have shown that Thrombotest has the highest sensitivity; PIVKA was in fact discovered by studies with Thrombotest. Normotest, however, was intentionally devised in such a way that it should be insensitive to both PIVKA and other inhibitors [18]. In patients stabilized on anticoagulant therapy, Normotest follows the actual concentrations of II, VII and X (Fig. 2). Thrombotest records are about half the value of Normotest. The difference which is caused by PIVKA, can be used as an indirect measure of the PIVKA inhibitors [19].

We can now explain the main causes for the differences found on testing the same dicoumarol plasma with different methods (Fig. 3). Methods which are sensitive to II, VII, X, but not to PIVKA, will give values close to Normotest. Thromboplastins which are sensitive to both II, VII, X and to the PIVKA inhibitor, will give values approaching Thrombotest, depending on the degree of PIVKA sensitivity. Reagents which have a low sensitivity to factors II, VII and X and to PIVKA will give values higher than Normotest does. Most studies have shown that Simplastin has the lowest sensitivity of all reagents. The Boehringer reagents were not tested in these studies.

There is still no consensus of opinion as to the clinical role of the PIVKA inhibitors in anticoagulant therapy. There is, however, much indirect evidence to suggest that the PIVKA inhibitors play an important role for both the bleeding tendency and the antithrombotic effect. There is a good correlation between the intensity of anticoagulation, as measured with a PIVKA sensitive method such as Thrombotest, and both the incidence of bleeding complications in large clinical

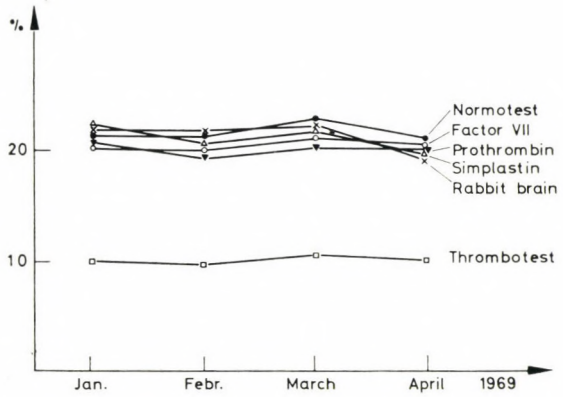

Fig. 2. In stabilized patients, Normotest reflects the level of II, VII and X. The difference between Normotest and Thrombotest is caused by PIVKA which inhibits Thrombotest but not Normotest

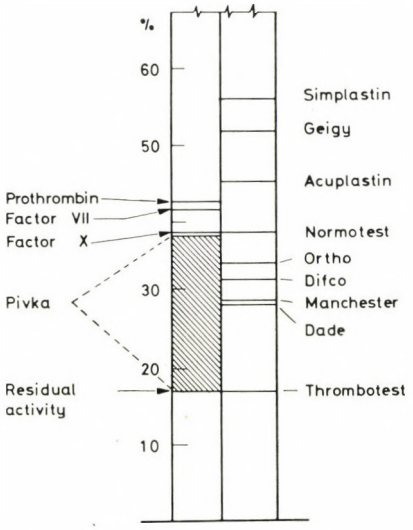

Fig. 3. Variations in results depending on whether or not the thromboplastin used is sensitive to factors VII and X and to the PIVKA inhibitor

materials [21], and the therapeutic results in patients with coronary disease as pointed out by Loeliger et al. [14].

When we want to measure the true concentrations of II, VII and X, we should use an inhibitor-insensitive method. This is particularly important when thromboplastin reagents are used for assaying the synthesis of clotting factors as a measure of liver function. Normotest was devised especially for that purpose [20].

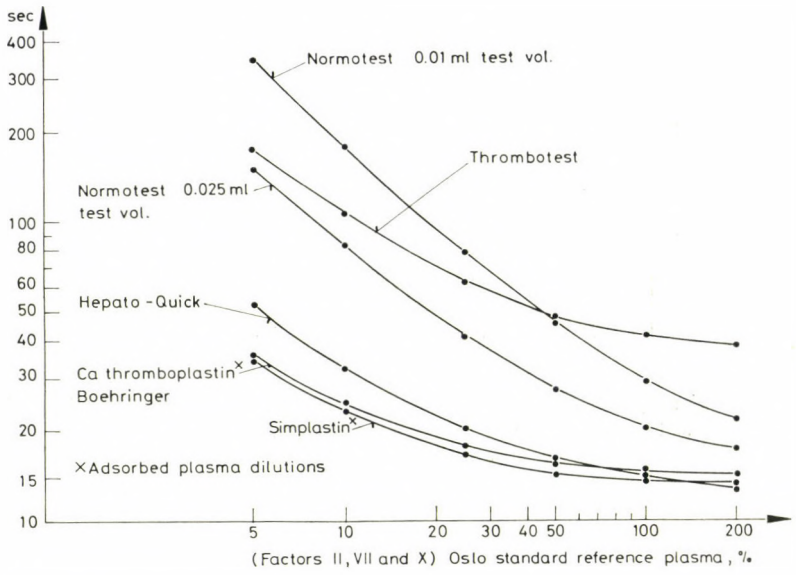

Fig. 4. Correlation curves for different reagents. A steep correlation curve results from a high sensitivity to reduction of factors II, VII and X (Hepato-Quick in this experiment was performed with Quick's technique in undiluted plasma)

The overall sensitivity of a thromboplastin reagent is reflected in the steepness of the correlation curves (dilution curves) used for converting clotting time in seconds into per cent of normal (Fig. 4). A certain difference between clotting times for different concentrations is necessary for exact quantitation. Quick's method with active thromboplastins such as Simplastin and Boehringer's thromboplastin gives a flat curve above 50% of normal. All concentrations above this level will usually be recorded as normal.

Control plasmas

For the quality control of thromboplastins it is very important to use only non-activated control plasmas. Many of the control plasmas which are commercially available today are highly contact activated and therefore give misleading

results. They contain activated factor VII which accelerates clotting and produces abnormally short clotting times and false high percentage values in most test systems.

The degree of acceleration depends largely on the final dilution of the test plasma in the clotting mixture applied. The effect is therefore much more dramatic when such plasmas are tested with Thrombotest, which has a final dilution of 1 : 10, than with Normotest (0.01 ml) where the final dilution is about 1 : 50. In fact, Normotest is very little influenced by activation of factor VII, and the difference between Thrombotest and Normotest therefore not only discloses the presence of accelerators, but can even be used as an indirect measure of the degree of activation. The situation is just opposite to that found in the presence of inhibitors such as PIVKA, where Thrombotest has a lower value than Normotest.

Table 4

Testing of different, so-called "normal" control plasmas. Contact activation of the plasmas is evidenced by the abnormal short clotting times with Thrombotest and high values for factor VII. (Contact activation has a slight influence on Quick's prothrombin time because of the insensivity of this method above 100%, and on Normotest because of the high dilution of the test plasma)

	Thrombotest		Normotest		VII*	V*
	sec	%	sec	%	%	%
Ortho plasma coagulation test lot No. 4D112	35.0	>200	29.9	86	145	75
Dade standardized normal plasma lot No. 694	38.2	>175	29.0	88	118	91
Warner Chilcott, verify normal lot No. 1135122	34.4	>200	29.1	88	136	96
Boehringer, Quick control normal lot No. N18	33.8	>200	27.5	120	120	115
Boehringer, Hepato Quick control lot No. N11	34.1	>200	28.6	90	115	70
Metrix prothrombin control lot No. 413	42.9	80	34.2	70	69	24
Oslo standard reference plasma	40.5	100	27.5	100	100	95

* Results for VII and V depend on dilution of test plasma

Table 4 shows results from testing some of the so-called normal control plasmas on the market. Most of them had been highly activated by contact or cold during preparation. By specific measuring of factor VII the activities were not overwhelmingly high, but this was because of a high final dilution (1 : 40) of the test plasmas. A dilution of 1 : 20 gave values above 200% for all the five activated plasmas.

Correlation curves prepared with activated plasmas have a slope which is quite different from that of non-activated plasma (Fig. 5). Most of the commercial plasmas behave like the glass-activated Oslo standard. Loeliger in Leyden has, however, prepared a normal standard plasma which behaves like the Oslo standard.

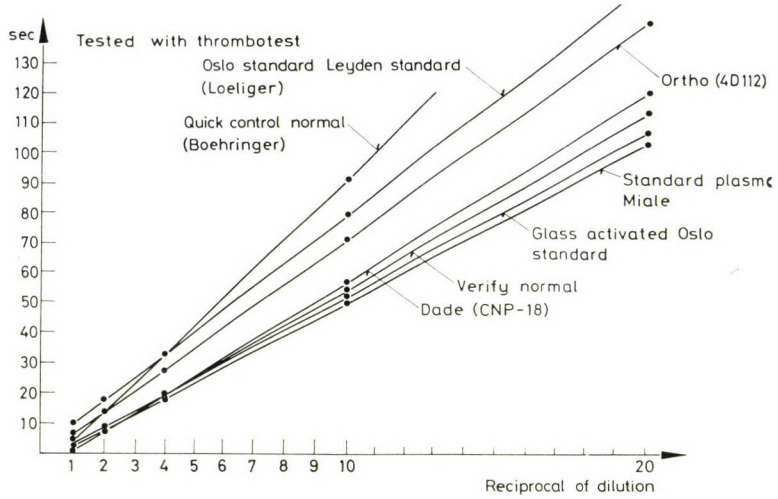

Fig. 5. Correlation curves for different "normal" plasmas. By plotting the reciprocal of dilution on the abscissa and clotting time in seconds on the ordinate, straight lines are obtained. Activation of the plasma is evidenced by shorter clotting times and reduced slope of the curves

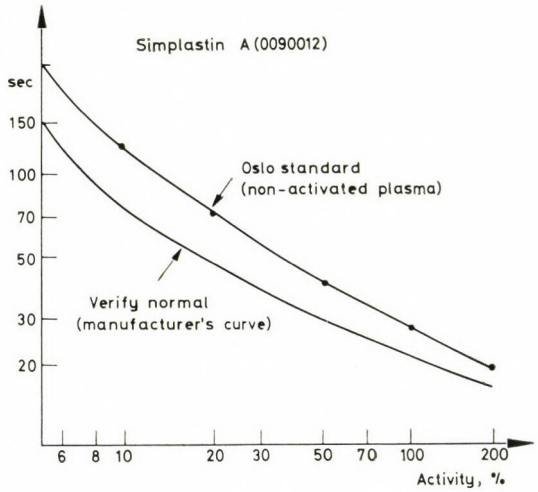

Fig. 6. Manufacturer's curve for Simplastin A, prepared by the use of activated plasma (Verify normal), results in values much lower than the actual concentration of factors II, VII and X

Table 5

Control plasmas of low activity (Warner Chilcott, Boehringer) are contact (or cold) activated as evidenced by abnormally high values for Thrombotest and factor VII

	Per cent activity stated			
	by manufacturer	Thrombotest	Normotest	factor VII*
Warner Chilcott:				
Verify abnormal I	12	100	23	55
Verify abnormal II	5.1	18	10	10.5
Boehringer:				
Quick control abnormal	20	25	10	34
Nyegaard & Co:				
Control plasma (10)	10.5	10.5	10	10.5
Control plasma (25)	25	25	25	25

* Tested in a final dilution 1 : 40

Boehringer's Quick control normal showed a peculiar behaviour suggesting the presence of an additional accelerator in addition to activated factor VII. Activated plasmas may give erratic results when used for preparing correlation curves for different methods and reagents.

Most unfortunate is the use of activated control plasmas for preparing the correlation curves used in the control of anticoagulant therapy. The manufacturer's curve provided for Simplastin A may serve as an example (Fig. 6). The values read from this curve correspond to only about half the correct percentage values for factors II, VII and X, as can be read from the non-activated standard plasma curve. Simplastin A, using the manufacturer's curve prepared with activated plasma, gives values which are close to those recorded with Thrombotest. The therapeutic range for Simplastin A, however, is given as 14–34% whereas the therapeutic range for Thrombotest is 5–12% [14, 15]. The use of activated control plasmas consequently results not only in false dilution curves and sensitivity ratios, but implies the serious consequences of an inefficient anticoagulant therapy. This is the case for all the activated control plasmas on the market. If Quick control normal is used for Hepato Quick, an actual concentration of 10% will be read as below 5% (Fig. 7). Consequently, only non-activated plasmas should be used for preparing correlation curves. If this is adhered to, and the thromboplastin reagent is perfectly uniform and stable, the reproducibility will be good both within the laboratory and between laboratories.

The dilution curves or correlation curves for normal plasma which are used for converting prothrombin times in seconds into per cent of normal should not be prepared with saline or buffer when testing thromboplastin reagents for Quick's method. The saline dilution curve gives a false prolongation of the pro-

thrombin time at higher dilutions because of the dilution of factor V and fibrinogen. There exists no clinical condition in which all clotting factors including factor V and fibrinogen are reduced in parallel. The saline or buffer dilution curves are therefore meaningless as a yardstick for quantitation. The saline or buffer dilution curves result in false high percentage values. A plasma which actually contains 10% of factors II, VII and X and normal factor V and fibrinogen, will usually be recorded as an activity of about 20–30% on the buffer dilution curve. Adsorbed

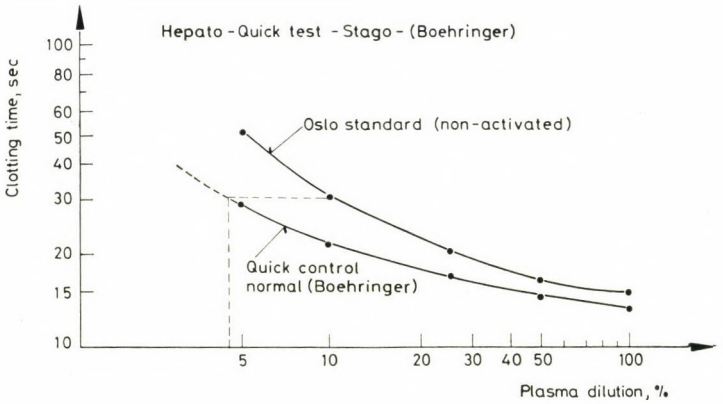

Fig. 7. Abnormal low values obtained with the Hepato-Quick test (Boehringer) if results are read from a correlation curve prepared with Quick control normal plasma, which is activated

plasma should therefore be used as diluent when using Quick's test in order to supply factor V and fibrinogen. The buffer or saline dilution curves have created much confusion, particularly in relation to the therapeutic range. For Thrombotest, Normotest, Simplastin A, and Hepato-Quick (Boehringer), citrated buffer or saline dilution curves can be used, because factor V and fibrinogen are included in the reagents.

What has been said about the "normal" control plasmas also applies to the abnormal plasmas with lower activities. Plasmas on the market are activated and testing in collaborative studies have concluded that they are not suitable for control [2]. This is evident as seen from Table 5. Because of highly activated factor VII, they give an enormous variation in the results when tested with different methods. Nyegaard & Co. A/S, Oslo has recently marketed non-activated control plasmas which we have found very useful for the routine inter- and intralaboratory control of reagents and technicians.

I believe that by careful standardization of reagents and control materials we would solve very important clinical problems to the benefit of our patients.

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The Progressive Antithrombin (Antithrombin-III, Heparin-cofactor)*

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Problems connected with the theoretical and practical aspects of progressive antithrombin are discussed and the characteristics of the functionally defective molecule antithrombin-$III_{Budapest}$ are presented.

Maintenance of the dynamic equilibrium is one of the basic properties of the blood clotting mechanism. A number of biochemical systems ensure the fluidity of blood. Among these factors, the role of the "antithrombins" is of a special importance since their action is manifold. For a long time, the antithrombins were classified according to Seegers et al. [1]. This categorization is now obsolete as it was proved to include factors the existence of which has not been confirmed (antithrombin-IV); also, antithrombin-II and III, considered earlier to represent two different factors, are now known to be identical [2, 3, 4].

According to the present concept, antithrombin is a protein that inactivates thrombin both irreversibly and progressively. From the 6 antithrombins classified by Seegers, this property applies to antithrombin III only. However, as shown by Lyttleton [5], even this factor is a complex one. While studying serum thrombin inactivation he found that the process cannot be described with a uniform reaction order. From this Waugh and Fitzgerald [6] have drawn the conclusion that thrombin activity in the serum disappears as a result of some complex process. Adopting van't Hoff's differentiation method to examine the order of the inactivation reaction, we found that the reaction is becoming of an increasingly higher order as the process advances [7]. Abildgaard [8] succeeded in isolating from antithrombin-III two different plasma fractions which were thought to be uniform. One of the fractions is $alpha_2$-macroglobulin, an 820,000 molecular weight protein. The other is a glycoprotein of 64,000 molecular weight. This latter is now called antithrombin-III. As to their electrophoretic mobility, both proteins are $alpha_2$-globulins.

Antithrombin-III plays a decisive role in the elimination of activated prophase factors. It neutralizes the activated contact product (factor XIa), the activated factors IX and X (IXa and Xa) and, finally, the thrombin.

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The process most intensively studied is the inactivation of thrombin. If the inactivation of normal plasma thrombin is taken for 100, then antithrombin-III is responsible for 70%, and alpha$_2$-macroglobulin for 30%. In 1 ml heat-defibrinated plasma, antithrombin-III inactivates 700 NIH units of thrombin in a few minutes, an amount which makes about 1000 ml of 300 mg per 100 ml of fibrinogen to coagulate in 20 sec.

Antithrombin-III acts by inhibiting the proteolytic enzymes. Its characteristic feature is that it inhibits thrombin activity as a function of time, progressively and irreversibly. According to data in the literature, antithrombin-III is inactivated at 80 °C and is active in the pH range 6 to 9.5. According to our own measurements, inactivation starts at a significantly lower temperature and at 65 °C the loss in activity at pH 7.3 amounts to 90% in 10 minutes.

The majority of contradictions and controversies concerning antithrombin are associated with the mechanism of action of heparin. According to the original hypothesis, a cofactor required for the action of heparin (antithrombin-II in Seeger's terminology) is a factor independent of the progressive antithrombin (antithrombin-III). At present, most researchers regard the two factors as identical [2, 3, 4, 9, 10].

Thrombin inactivation in serum is considerably accelerated by heparin [11]. Biochemical studies have revealed the exact mechanism of this phenomenon. Gerendás [12] believed heparin to be the co-enzyme of antithrombin-III. Monkhouse and Milojevic [13] presumed that antithrombin inactivated the thrombin in an enzymatic way and was itself inactivated in the course of the process. As shown by Rosenberg and Damus [9], antithrombin forms a 1 : 1 complex with thrombin. This complex cannot be dissociated, either by reducing or by alkylating agents. The binding is formed between serine, present in the active centre of thrombin, and the arginine residues of antithrombin. Heparin accelerates the formation of this complex. It binds to the epsilon-amino groups of lysines in the antithrombin [9].

As regards the relation between heparin and thrombin, heparin was shown to prevent the heat inactivation of thrombin [14]. The possibility that heparin may bind not only to antithrombin but also to thrombin was raised again by Markwardt and Landman [15]; it induces a change in the tertiary structure of the latter following which antithrombin III rapidly inactivates the coagulative enzyme. Recent experiments [16] have confirmed by gel filtration that thrombin and heparin form a complex that elutes in a range of a higher molecular weight than would either heparin or thrombin by themselves. Furthermore, certain cations with one or two positive charges (K^+, Na^+, Mg^{++}, Ca^{++}) highly affect the thrombin-antithrombin-heparin reaction [17]. Several authors seem to have performed the experiments *in vitro* in some non-optimal ionic milieu. It is well-known that heparin of a strong negative charge is able to bind aspecifically to a number of proteins [18]. Accordingly, it may be assumed that, even under physiological conditions, heparin is bound to some protein. We have, therefore, made some experiments with insoluble heparin and found that the effect of immobilized

heparin on the thrombin-antithrombin reaction does not differ from the effect of dissolved heparin [19].

Damus et al. [20], Marciniak [21], Yin et al. [22] and Osamo [23] studied the additional functions of antithrombin-III. According to their findings, antithrombin-III inactivates the activated X factor (Xa) and is one of the main inhibitors of activated XI factor (XIa). These results allow the assumption that in the coagulative cascade-enzyme system, inactivation of the serine protease factors (XI, IX, X thrombin) is based on the same mechanism. The serine protease inactivating activity of antithrombin-III is considerably increased by heparin [20].

An example of the practical bearings of biochemical studies was offered by a recently reported case [24]. Since the age of 18, this patient had had thromboses 21 times and pulmonary embolism twice. It was found that the clotting time of the patient's blood failed to lengthen upon the effect of intravenous heparin. Neither did the thrombin time change after repeated therapeutic doses of heparin added to his plasma *in vitro*. The thrombin inactivating ability in the patient's heat-defibrinated plasma and serum amounted to 30% only, and this was attributed to the effect of $alpha_2$-macroglobulin. In this case, thrombin inactivation could not be stimulated by heparin. When $alpha_2$-macroglobulin was neutralized by monospecific $alpha_2$-macroglobulin antiserum, the patient's serum failed to inactivate thrombin. The heparin cofactor activity, measured by Egeberg's method [2], amounted to 5% of the normal value. Hence, the frequent thromboses and the elevated heparin tolerance were assumed to be due to the low level of thrombin inactivation. Considering the high incidence of thromboses in the patient's family the disease was assumed to be hereditary, and this was supported by the confirmed consanguinity. No chromosome aberration was found.

Adopting Mancini's method [25], we used monospecific antithrombin-III antiserum, and found that antithrombin-III as antigen in the blood of both the patient and his relatives was present at normal concentration. This being a dummy-protein condition, we have termed the pathological molecule as antithrombin $III_{Budapest}$.

Examination of the antithrombin-III revealed that its other functions were also pathological. Its ability to inactivate the activated factor X (Xa) was only 25% of the normal control. In addition to antithrombin-III, Marciniak and Tsukamura [26] confirmed the existence of a further factor, belonging to the macroglobulin fraction, that inactivates Xa. It may, therefore, be assumed that in our case the capacity to inactivate the residual Xa factor was a result of the function of this macroglobulin. This corresponded to the numerical value (75 : 25 ratio), too. In this case antithrombin $III_{Budapest}$ has lost its capacity to inactivate either thrombin or factor Xa.

Measurement of the amount and activity of antithrombin-III furnishes information not only concerning hereditary thrombophilia. Changes in its activity were observed in several other clinical entities.

Ødegard et al. [27] reported on a case of diffuse intravascular coagulation (DIC) of fatal issue. In this case the antithrombin level was found to be 30%

immunologically. According to other authors [28] the regular use of oral contraceptives reduces the immunologically detectable antithrombin-III level as well as thrombin inactivation to about 80%. This phenomenon might explain the disposition for thrombosis in patients taking these drugs. Parallel to the reduced antithrombin-III activity, the activity of factor X increased. Gertler et al. [29] observed a moderate increase of thrombin inactivation in patients with ischaemic heart disease, becoming marked in myocardial infarction, confirming our earlier data [30]. According to Duckert [31], the synthesis of antithrombin-III decreases in chronic liver disease. However, this author failed to find a correlation between the decrease of prothrombin and antithrombin levels, and did not exclude the synthesis of some pathological protein.

Purified human or bovine antithrombin preparations have allowed to estimate the plasma heparin content by a method more sensitive than any former one. Since already 0.01 μg of heparin considerably increases the capacity of antithrombin-III to inactivate factor Xa, Yin et al. [32] developed a simple and highly sensitive method of plasma heparin assay based on this principle. The method has permitted the reliable tracing in the circulating blood of heparin administered for therapeutical purposes.

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Haemo-lymphocytopoiesis

A Review

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The old controversy generated by the polyphyletic and monophyletic theories concerning lymphocytopoiesis and haemopoiesis, based mostly on poor morphological criteria, has found its resolution by the results of new and more functional approaches to the problem. Even the position or potentiality of the lymphocyte(s) in the stem cell puzzle is now clearer. In fact, the spleen colony assay, the polycythaemic mouse assay, the granulocyte-macrophage colony assay, and the focus assay for antigen-sensitive cells, first provided evidence for the existence of multipotent stem cells capable of undergoing either self-replication, or multiple differentiation in such a way, that they may repopulate both the lympho- and haemopoietic tissues of an irradiated animal. It appeared that a single pluripotent stem cell (colony-forming unit) may undergo qualification either for a lymphoid committed stem cell, or for a haemic committed stem cell. Then, these specifically committed stem cells may differentiate into several unipotent progenitor cells, which are: 1. The antibody-forming cell progenitors, and the antigen reactive cell progenitors, for the lymphocyte series; and 2. for the haemic series, the erythropoietin-sensitive cells, the colony- and then cluster-forming cells (granulocyte-macrophage colonies), the eventual megakaryocyte progenitor cells. In their turn, the above-mentioned unipotent progenitors give rise to progenies of morphologically identifiable blast cells, which ripe to the different mature cell stages.

The old controversy generated by the polyphyletic and monophyletic theories concerning haemopoiesis and lymphocytopoiesis, based mostly on poor morphological criteria, has found its resolution by the results of new and more functional approaches to the problem. Even the position or potentiality of the lymphocyte(s) in the stem cell puzzle is now clearer. In fact, the spleen colony assay [16], the polycythaemic mouse assay [8], the granulocyte-macrophage colony assay [3, 14], and the focus assay for antigen-sensitive cells [7, 13] offered evidence for the existence of a multipotent stem cell (colony-forming unit), capable of undergoing either self-replication or multiple differentiation, both lymphatic and haemic (Fig. 1). It was in fact shown that transplantation of bone-marrow cells into lethally irradiated animals resulted in the presence of unique stem cells, which proliferate and form spleen macroscopic colonies containing new stem cells, as well as lympho-haemopoietic cell-clones. The clonal nature of the above-mentioned colonies was proven (a) by the observation of unique radiation induced chromo-

* Supported by The Blood Research Foundation, Washington, D.C., USA.

some markers within the colonies [17], and (b) by the observation that bone-marrow cells bearing a distinctive chromosome marker may undergo both myeloid and lymphoid differentiation [10].

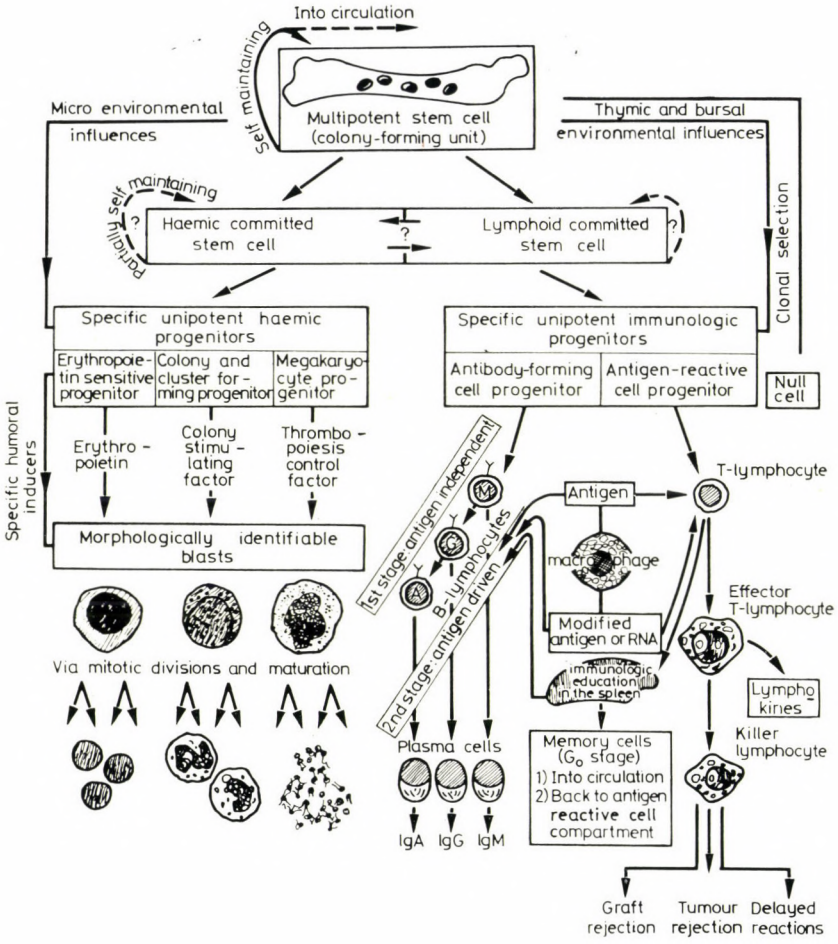

Fig. 1

In chronic granulocytic leukaemia, the Ph_1 chromosome is carried either by the granulocyte cell series or the erythrocyte and thrombocyte series, and by the monocytes as well, whereas lymphocytes are Ph_1-negative. This shows that the single pluripotent self-maintaining stem cell (colony-forming unit) may undergo qualification either as a haemic committed stem cell or as a lymphoid committed stem cell; these cells are still partially self-maintaining. In their turn, the above-

mentioned specifically committed stem cells may differentiate into several unipotent progenitor cells which, though unipotent, possess intensive proliferative capabilities. These cells are 1. the antibody-forming cell progenitor, and the antigen reactive cell progenitor, for the lymphocyte series; and 2. for the haemic series, the erythropoietin-sensitive cell, the colony- and then cluster-forming cell (granulocyte-macrophage colonies), the eventual megakaryocyte progenitor cell. In their turn, the above-mentioned unipotent haemopoietic progenitors give rise to progenies of morphologically identifiable blast cells (proerythroblast, myeloblast, megakaryoblast), which then mature to their final stage. During maturation, these cells may also show proliferation with decreasing extent sequentially to their maturation phase.

In contrast to the blast cells, both the colony-forming unit (stem cell) and the progenitors are not yet identifiable by morphological criteria. Obviously, cells with unknown characteristics are easier identified at high concentrations. In order to contribute to the problem, experimental studies were designed to obtain such concentrations by means of a discontinuous albumin density gradient centrifugation of bone-marrow cells [2]. By these procedures, the colony-forming units were concentrated up to 100-fold, i.e. the stem cells in these enriched fractions reached $10-20\%$ of the total amount of cells. Observations carried out in mice, monkeys, and humans, either by light or electron microscopy, indicated a lymphocyte-like cell as a possible candidate for the stem cell, but with structure and ultrastructure clearly different from those shown by the immune competent lymphocytes. The above-mentioned stem cell measured $10-11$ μ in the primates, and $8-10$ μ in the mouse [5].

As far as differentiation processes are concerned, apparently the critical factor which stimulates the colony-forming unit to enter into a particular line-progenitive cell is given by the microenvironment into which the above-mentioned stem cells migrate, including cell-contact interaction with adjacent stromal cells [9]. Commitment to a specific pathway of evolution of the colony-forming unit into a given lymphatic or haemic progenitor stage could depend either on a random self-generating differentiation process, or on intrinsic heterogeneity and a history of mitotic division. In contrast, differentiation of the progenitors into specific blasts seems to be regulated by humoral inducers and, particularly for haemic cells, by (a) erythropoietin, which is a specific humoral inducer of erythropoietic differentiation; (b) the colony-stimulating factor, which is a specific humoral regulator and probably inducer of colony-forming cell differentiation; (c) the thrombopoiesis control factor, which is a specific humoral stimulator of differentiation into megakaryocyte progenitor cells.

Lymphatic cell differentiation occurs via clonal selection. There is evidence that differentiation of the stem cell into the antigen reactive cell progenitor is influenced by the thymic epithelial environment. On the other hand, the equivalent bursal environment influences stem cell differentiation into the antibody-forming cell progenitor.

The antigen reactive cell progenitor, in its turn, gives rise to the T-lympho-

cytes. When these T-cells meet with antigen, they may 1. receive in the spleen an immunologic "education", and cooperate with B-cells in humoral antibody production, or exercise immunological memory and return to the compartment of the antigen reactive cells; 2. enter a prolonged G_0 stage and recirculate (lymphocyte traffic); 3. undergo transformation and growth, and become "killer" lymphocytes, thus providing for graft rejection, tumour rejection, delayed hypersensitivity. Activated T-lymphocytes may also produce a number of soluble biologically active factors, called "lymphokines": this is a generic name indicating a source of materials of non-antibody lymphocyte activation products which, even in small quantities, act as auto-pharmacological mediators of T-cells' responses to antigen. In short, lymphokines are functioning in the expression and regulation of cell-immune responses.

The antibody-forming cell progenitor undergoes maturation into several B-cell clones specifically committed for the production of selective classes of immunoglobulins. According to recent data [4], there is a first differentiation stage, which is antigen-independent. During this stage, there occurs (1) conversion of progenitors into lymphoid cells producing small amounts of IgM; (2) conversion of some of these IgM-producers into IgG-producing cells; (3) conversion of some of these IgG-producers into B-cells which produce IgA. The antibodies formed are incorporated into the cell membrane, and they form the B-cell markers. This scheme is supported by the fact that apparently it is the only one which completely agrees with clinical occurrences, i.e. with the pattern presented by the congenital immune deficiencies.

More recent data [15] have shown that a large number of the immune competent lymphocytes from the human umbilical cord blood carry only membrane-bound IgD-receptors. This suggested the interpretation that the very early conversion step from the antibody-forming cell progenitor into the B-cell line is given by the development of IgD-receptors, and therefore such a step should precede that of IgM production.

The second stage of the maturation and function of B-lymphocytes is antigen driven, and the following events occur during this stage: 1. cell-antigen interactions, with recognition of B-lymphocyte surface receptors; and 2. maturation of B-cells into antibody-secreting plasma cells, also via the modulation of T-lymphocytes, macrophages, and preformed antibodies.

It seems that besides T- and B-lymphocytes, a third lymphoid cell population also exists. Recent studies [1, 6] have indeed offered evidence for the existence of cells which are antibody-dependent, and endowed with cytotoxic properties (and hence termed K for killer), but they are neither Ig-bearing (B-cells), nor thymus-derived (T-cells). In other words, they form a group of lymphoid cells without any known surface markers, and are hence called null cells. Finally, mononuclear lymphoid cells belonging to the monocyte series could act as the cytotoxic effector cell.

The problem of the lymphocyte surface-specific receptors is rather complex, as (a) the normal cell population exhibits an extensive phenotypic diversity of cell

surface structure, owing to selective generation; (b) there probably exists a second order of diversity mediated by surface codes which enable the cell to draw upon a small number of genes to achieve an extensive range of surface display; and (c) the altered antigenicity of cancer cells could be envisaged as either a phenotypic or genotypic error.

In other words, the entire cell surface should be imagined as a grid in which each unit is precisely located. The surface specificity is then mediated by centrifugations of gene products rather than merely by their individual specificity. This may explain the versatility of surface display, which is essential from the point of view of cell membrane receptors both for immunological surveillance, and for tumour immunology.

Concerning T-cell markers, there are investigators who believe that the receptor immunoglobulins at the T-lymphocyte surface are responsible for the specific functions of these cells, but the basis of the specificity of T-cells still remains one of the controversies of our days. Several possibilities might be taken into account: (1) that a single immunoglobulin is present on the surface of T-cells (this is the so-called "Igx" hypothesis); (2) that the T-cell surface receptor is part of an immunoglobulin molecule, as a half light chain or half heavy chain containing an antigen-combining site; (3) that the surface receptor of the T-cells is a membrane-bound immunoglobulin, not secreted, and present at low concentration; (4) that the T-cell receptors do not fall into any of the above-mentioned three conventional immunoglobulin bases but rather into other molecular bases such as those operating for plant lecithin, invertebrate agglutinins, enzymes, etc.

Things are more clear for B-cells. Although not yet formally proven, it is uniformly believed that the immunoglobulins demonstrable at the B-cell surface represent the Ig-receptors of that cell population. After the development by Pernis et al. [12] of the immunofluorescence technique, much new information about B-cells has rapidly been obtained by the use of antisera specific for mu, gamma, and alpha heavy chains, and lambda and kappa light chains. More recently, Nussenzweig et al. [11] discovered another marker in several B-cells, the C-complement receptor, which could represent a surface marker of a subpopulation rather than of all B-lymphocytes. It is absent from the surface of plasma cells. It has been suggested [19] but is still under discussion that B-lymphocytes develop sequentially C_3-receptors, Ig-receptors, and start Ig-production. This order being imperative, we cannot have Ig-receptors unless we have C_3-receptors, and the B-lymphocyte will reach its Ig-production stage only when C_3- and Ig-receptors have developed.

Time will give the final answer to these questions as well as to many others.

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Haemopoietic Precursor Cells in Early Human Embryos: Light Microscopic Pictures

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Liver and yolk sac smears and/or histological preparations obtained from 6.5 to 22 mm crown-rump length (second month of intrauterine life) intact human embryos are presented. The morphological inhomogeneity of early, unprocessed free haemopoietic progenitor cells of the liver is emphasized and the peculiar location of apparently similar cells at the yolk sac entoderm is mentioned.

Light microscopic pictures of May – Grünwald – Giemsa stained, scattered, unprocessed, free haemopoietic precursor cells, too immature to be classified, found in liver smears of an intact, 6.35 mm crown-rump (CR) length (approximately 4 weeks old) human embryo are presented in Fig. 1. Unlike processed stem cells [1] these cells are larger than 10 microns. They amount to about 10% of all free, non-hepatocyte cells. The morphological inhomogeneity of these early progenitors is apparent. In view of the rarity of observing such an early, intact human embryo, its macroscopic picture is presented (Fig. 2). Although the earliest haemopoietic cells have been detected in the yolk sac [5], in the present case we

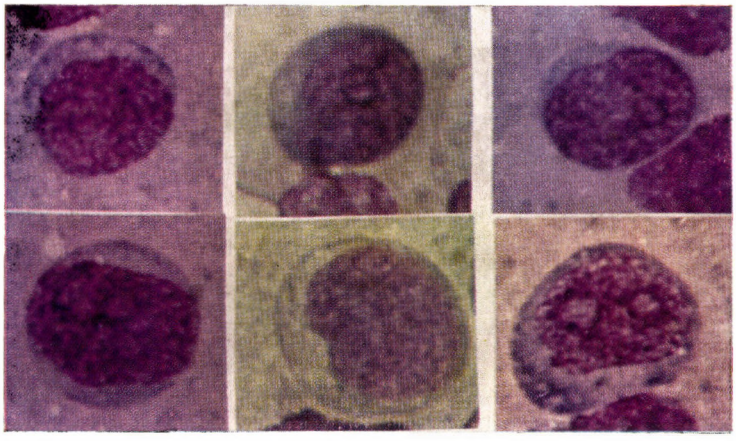

Fig. 1. May–Grünwald–Giemsa stained free haemopoietic precursor cells in liver smears of an intact 6.35 mm CR-length human embryo. For details, see text

have failed to discover the cells presented in Fig. 1 in yolk sac inner surface touch preparations. The presence of haemopoietic stem cells in the animal yolk sac has been demonstrated functionally by Moore and Metcalf [5]. In any event, it is of some interest that blood-containing vessels, so characteristic for the yolk sac, could not be seen in our preparation under the dissecting microscope.

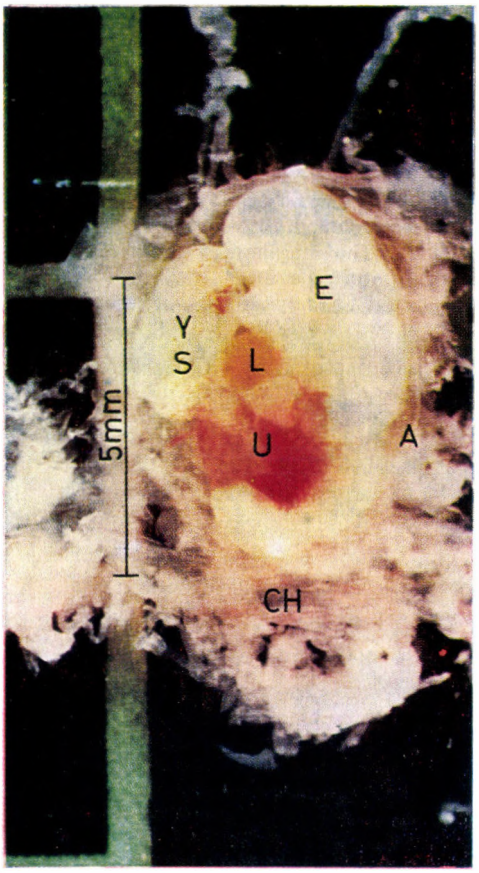

Fig. 2. Macroscopic picture of a 6.35 mm CR-length human embryo; E = cranial part, L = liver, YS = yolk sac, U = umbilical cord, A = amnion, CH = chorion (spread out)

Fig. 3 derives from an 11 mm CR-length (approximately 6–7 weeks old) embryo. It demonstrates size differences of the unclassified early progenitors as well as the occasional presence of irregularly shaped nuclei. Morphology of early human embryonic livers has been discussed earlier [3].

The histological picture of the yolk sac entoderm of a 16 mm CR-length (approximately 7–8 weeks old) human embryo is shown in Fig. 4. There are

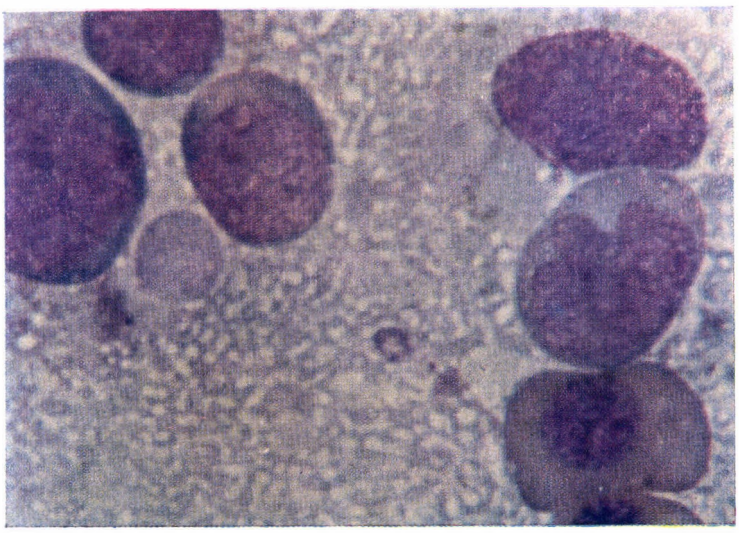

Fig. 3. May—Grünwald—Giemsa stained haemopoietic cells in the liver smear of an intact, 11 mm CR-length human embryo. Bottom right, embryonic erythroblast; on left margin, a large definitive proerythroblast

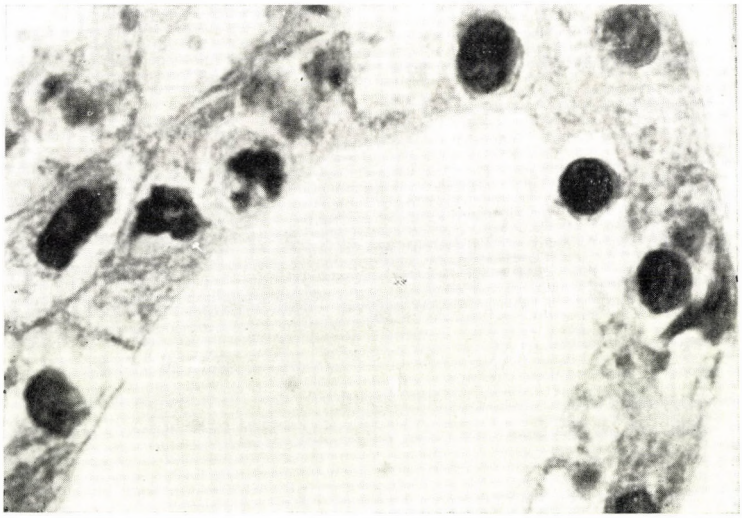

Fig. 4. Histological preparation from the yolk sac entoderm of a 16 mm CR-length human embryo. The apparently incarcerated cells (formalin fixation and haematoxylin-eosin staining) are 8—12 microns in diameter. For further details, see text

several, apparently outstanding, free haemopoietic precursors in an entodermal cell-box. This intimate contact between the entodermal epithelium and early haemopoietic precursor cells in the human yolk sac awaits explanation and is now investigated at the electron microscopic level in a cooperative study with the Institute of Forensic Medicine of our University (Dr. I. Balogh).

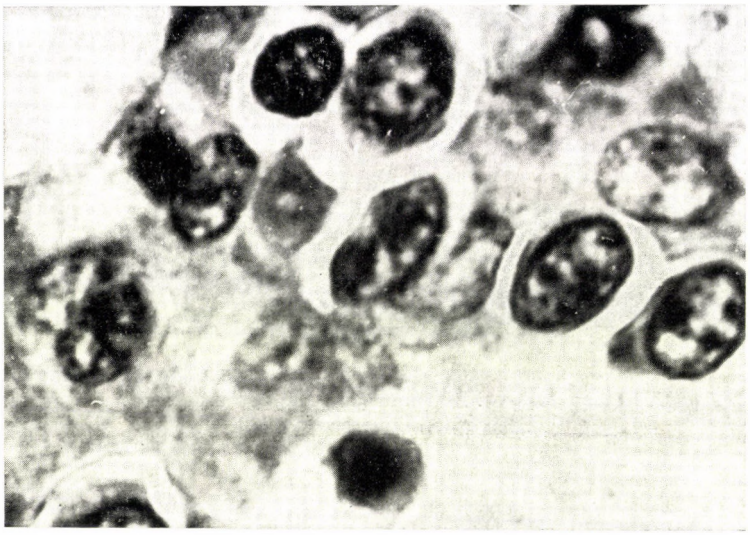

Fig. 5. Free haemopoietic progenitors apparently developing into definitive erythropoietic precursors, in a formalin-fixed haematoxylin–eosin preparation from the liver of a 22 mm CR-length human embryo. The longer diameter of free pre ursor cells is around 20 microns

Of course, we must consider that the presence of an occasional haemopoietic precursor in the cytoplasm of an entodermal epithelial cell of the yolk sac is an artifact. Cells in Fig. 4, such as the cell at the right upper angle, located at the outer surface of the photographed entodermal pseudotubule, especially speak against a simple artifact. The incarcerated cells appear to be uninjured in each case, and apparent mitotic figures could be discovered at the left middle part of the inner surface. Further analysis of this phenomenon, along with pertinent references, will appear separately [4]. Apparent cell boxes could be clearly demonstrated in our serial electron micrographs, but the villous structure of the yolk sac may be the simplest explanation.

The last histological picture (Fig. 5) derives from the liver of a 22 mm CR-length (approximately 8 weeks old) embryo. Free haemopoietic progenitors, apparently developing into definitive erythropoietic precursors could be recognized. Unprocessed liver material from this embryo has been injected intravenously in

1971 into the mother, who suffered from chronic idiopathic aplastic pancytopenia; results have been published in 1973 [2]. Although untreated since the intervention, subtotal clinical and haematological remission continues.

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Treatment of Polycythaemia Vera with Reinfusion of Autologous Blood Plasma Obtained by Phlebotomy

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In 37 out of 41 patients with polycythaemia vera, complete haematological remission has been achieved by phlebotomy and reinfusion of the autologous plasma. Improvement lasted from 7 to 48 months. Repeated application of the same procedure in 18 patients led to a new remission in 2 patients lasting more than 25 months. In the blood plasma a factor inhibiting erythropoiesis has been detected in normally fed or previously stimulated rats by testing the plasma for ^{59}Fe uptake. The nature of this inhibiting factor is discussed.

Treatment of polycythaemia vera with phlebotomy alone, although useful since the red cell mass and haematocrit levels can be normalized by one or two phlebotomies every 3—4 months [1], is being replaced by ^{32}P treatment which ensures much longer remissions. However, ^{32}P treatment of polycythaemic patients causes in 11% acute granulocytic leukaemia, while in patients not subjected to ^{32}P treatment therapy the incidence of leukaemia is below 1% [2]. On the basis of the fact that in the blood of animals made polycythaemic by hypertransfusion a specific erythropoietic inhibitor has been found [3, 4], and there being evidence of a similar erythropoiesis inhibiting factor [5] in the plasma of untreated polycythaemic patients, we have attempted to obtain remission by phlebotomy and reinjecting into patients their own blood plasma. Then the same plasma was injected into normally fed rats previously conditioned by a single dose of one unit per 100 g of standard erythropoietin preparation and the effect upon ^{59}Fe uptake has been determined. Comparing ^{59}Fe uptake in these rats with the uptake by rats treated with normal human plasma, in some cases we found a significant decrease pointing to the presence of an erythropoiesis inhibiting factor.

Such a treatment of polycythaemia (phlebotomy followed by autologous plasma reinfusion) was applied in 41 patients with polycythaemia vera in the period June, 1961, to June, 1974. The diagnosis was based on clinical tests including blood volume determination. None of these patients had had treatment for polycythaemia.

Table 1 shows the sex and age distribution of the patients.

The procedure consisted of withdrawing 350—700 ml of blood into standard transfusion flasks containing ACD solution. The blood was centrifuged immediately and the plasma was reinfused on the same or the following day. In most

Table 1

Sex	Age (years)						
	21–30	31–40	41–50	51–60	61–70	71–80	Total
Male	–	2	7	10	5	1	25
Female	1	1	4	6	4	–	16
Total	1	3	11	16	9	1	41

patients the haematocrit decreased abruptly below 50 and RBC below 6×10^6 per/cu.mm after 6–8 treatments. Haematological improvement set in after 18–24 days. Ten patients needed 15–30 phlebotomies for clinical remission. In 3 male and 1 female patient the treatment had to be given up because of markedly increased thrombocythaemia in 1 patient, and an unsatisfactory decrease of the haematocrit and RBC count in 3 patients.

Treatment was completely successful in 37 patients. The duration of the remission achieved is shown in Table 2.

Table 2

Duration of remission (in months)	7–8	9–10	11–12	13–14	24–25	48
Number of patients	18	9	5	3	1	1

Table 2 shows that in 18 patients remission lasted 7–8 months. In 2 patients, who had required 16 and 21 phlebotomies, respectively, improvement persisted for 25 and 48 months. In 18 patients relapses were treated in a similar fashion with full success; in 3 patients the reticulocyte count increased but remission lasted 7–8 months. The probability of remission follows a single exponential equation

$$Y = e^{-0.1t}$$

where Y is the probability in per cent calculated for all the treated patients and t represents the duration of improvement in months.

In our first animal experiments [6], female rats were injected with 0.3 ml per 100 g of plasma from polycythaemic patients daily for 3 days, and ^{59}Fe uptake was determined 19 hours after the last injection. While in the control animals uptake never decreased by more than 11%, animals injected with polycythaemic plasma incorporated significantly less ^{59}Fe (Fig. 1).

In our recent experiments performed on 35 rats, the animals were conditioned by a single injection of 1 unit per 100 g of a standard erythropoietin preparation prior to the administration of polycythaemic plasma. Such an inverse iron-uptake

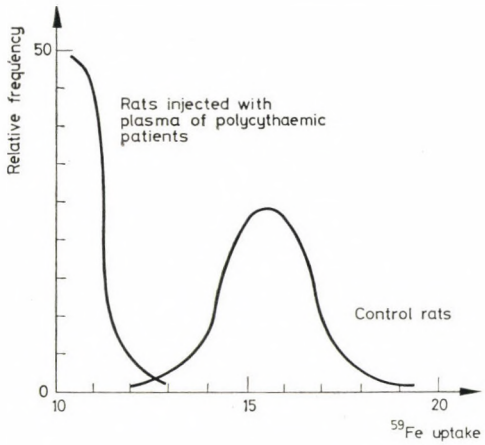

Fig. 1. Difference between ^{59}Fe uptake in control female rats (right) and female rats which received blood plasma from polycythaemic patients (left)

test was used, assuming that the erythropoietin incorporation will be suspended if the patient's plasma really contains some inhibiting substance. The difference between the control and experimental rats was evaluated by calculating the probability of null hypothesis (15–16), assuming that a failure to increase the 19-hour uptake by more than 8% indicates the presence of a specific inhibitor.

Discussion

In polycythaemia vera the increase in erythropoiesis is due to a production of red cells by the marrow without increased erythropoietin production [8–10], the plasma erythropoietin level being normal and the urinary level lower than normal [11]. After phlebotomy only a moderate increase in urinary erythropoietin is observed. It seems that two cell lines are present in the case of polycythaemic bone marrow erythropoiesis: an abnormal cell line that is relatively insensitive to erythropoietin [12] and a normal cell line which is regulated by erythropoietin and proliferates in the bone marrow after successful treatment of the disease with busulfan [13]. The presence of erythropoiesis inhibiting factor in the plasma of untreated polycythaemia vera patients [5] as well as in hypertransfused polycythaemic animals [3, 4] might be due to hyperoxia impairing erythropoietin to create normal erythropoietic cells in polycythaemia vera. This suggests that after phlebotomy the plasma should be preserved and reinjected. Bleeding alone, without reinjecting the plasma may cause an increase of the reticulocyte count and enhance iron turnover rate [14]. The latter implicates an increase of erythropoiesis due to stimulation of the normal erythropoietic cell line by erythropoietin in the bone marrow of polycythaemic patients.

The effect of our treatment seems to point to the presence in the plasma of an inhibiting factor. The nature of this factor has not been elucidated. It could be related to specific erythropoietic chalone, particularly in view of its negative feedback effect upon cell division and aging of the mature cell population having exerted respective shift in dichophase [17, 18]. The described method seems to present a successful therapeutical approach to polycythaemia vera. Complete remission, lasting in some cases for more than one year, significantly surpasses the effect obtained by phlebotomy alone. Thus, subsequent reinfusion of autologous plasma appears to represent the treatment of choice in polycythaemia vera.

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Major Surgery in Patients with Polycythaemia Vera

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In the period 1968 to 1973, 9 patients with polycythaemia vera were subjected to 11 surgical interventions at the Department of Surgery, Institute of Haematology in Warsaw. No postoperative complications, haemorrhagic or thrombotic, were observed. Successful surgical treatment of patients with polycythaemia vera depends on temporary control of the disease, i.e. reduction of the erythrocyte mass to haematocrit values below 50% and of the platelet count to below 500,000/cu.mm with maintenance of these reduced values for a possible long time prior to surgery.

Polycythaemia vera is a disease in which frequent complications in the form of thrombosis, bleeding or peptic ulcer require surgical considerations or even operative treatment. The results of surgical management depend to a considerable extent on the preoperative diagnosis of polycythaemia vera and on its control by appropriate treatment. In some cases the diagnosis must be established by the surgeon to whom the patient has been referred because of the special complications of the disease. Insufficient knowledge of the manifestations of polycythaemia vera and the indications for emergency surgery may cause error [1, 6, 8, 15, 17, 18, 19, 21].

A paradoxical phenomenon in polycythaemia vera is the inclination to thrombo-embolic complications as well as to bleedings. In the cases observed by Wasserman and Gilbert, disturbances of haemostasis occurred in 42% of the patients, spontaneous bleeding in 16% and thromboses in 26%, while after treatment resulting in remission, the frequency of spontaneous bleedings was 2.2% and that of thrombotic complications, 5.5% [18].

Despite the tendency for bleeding in polycythaemia vera, profuse and generalized haemorrhagic diathesis is rare; more common are spontaneous bleedings from the nasal mucosa, gingivae, genito-urinary tract or peptic ulcer, a frequent complication of the disease [21].

The causes of the disorders of haemostasis are complex. The increased volume of circulating red cell mass increases the viscosity of blood, slows the blood flow, and causes hypoxia. All these factors and the increased platelet count contribute to the development of thrombosis.

The tendency to bleeding may be explained by a weakening of the vascular wall and its reduced ability to react with constriction due to excessive stretching

of vessels by the increased volume of circulating blood. Another cause of bleeding in polycythaemia vera is the relative deficiency of fibrinogen as compared to the increased erythrocyte count [15, 16].

As a result of these changes the blood clot is exceptionally friable and its retraction is impaired with a large number of blood cells dropping out of the clot (Reiman's sign characteristic of polycythaemia). According to some authors, in polycythaemia vera the platelets show qualitative changes: a reduced serotonin content and a low level of platelet factor III [18]. Changes in the plasma were also described: a deficiency of prothrombin, and of factors V and VIII [15], sometimes also a deficiency of factor VII [18] suggesting the occurrence of disseminated intravascular clotting with a secondary activation of fibrinolysis [15]. Thromboelastographic investigations demonstrated pathological changes during exacerbations and their regression during remissions of the disease [9].

A striking observation was that during spontaneous or treatment-induced remission, most disorders of haemostasis decreased or disappeared.

In patients with polycythaemia vera, surgical procedures should, if possible, be avoided, in view of the frequent postoperative complications. If there is an emergency requiring prompt surgical intervention, the patient should be prepared to it by blood-letting, and if the surgical treatment is indispensable, but not an emergency procedure, it is indicated to treat the patient first with repeated blood-letting and then with cytostatics until a full remission of polycythaemia is obtained.

Patients with polycythaemia vera are usually advised to lead an active life to prevent thrombosis. Blood-letting of 500 ml may be applied every 1 – 3 days until normal values of blood tests are achieved. Long-term treatment is conducted with cytostatic agents or radioactive phosphorus. The most frequently used cytostatic drug is busulfan in doses of 2 – 6 mg daily. Excessive reduction in white blood cell count and development of thrombocytopenic purpura may prevent further treatment with this agent. Other cytostatics used in the treatment are chlorambucil, melphalan, cyclophosphamide, etc. [5, 10, 11, 20].

Radioactive phosphorus used in specialized centres is convenient in administration. According to some authors the survival of patients after such treatment is much longer than after chemotherapy; according to others there is no difference in survival. The more frequent incidence of acute leukaemia and bone marrow aplasia after treatment with radioactive phosphorus is explained by proponents of the method as due to longer survival of the patients [7]. Other authors suggest that the said complications point to the necessity of abandoning the method. Radioactive phosphorus is not used in preoperative management.

If bleeding occurs, it is advisable to transfuse fresh or frozen plasma and when anaemia develops indications may arise for transfusion of fresh whole blood. In treatment of thrombosis in patients with polycythaemia vera, anticoagulants are not recommended and heparin should be given only exceptionally.

In symptomatic polycythaemia associated with reduced arterial oxygen saturation profuse blood-lettings are contraindicated since they increase tissue hypoxia. However, in this form of disease when operation is envisaged and disor-

ders of haemostasis are present it is necessary, if possible, for improving the general condition to reduce the haematocrit value below 50%. Symptomatic erythraemia requires causative treatment, while the administration of cytostatics and radioactive phosphorus are absolutely contraindicated.

Reports on surgical operations performed in patients with polycythaemia vera are scarce. In the world literature single cases have been reported [2, 3, 12, 13], except the extensive materials of Fitts et al. [4], Wasserman and Gilbert [18] and Rigby and Leavell [14].

Management and Results

In the years 1968–1973, in our Institute 9 patients with polycythaemia vera were subjected to 11 operations because of vital indications (Table 1).

The age of the patients ranged from 51 to 68 years, with a mean of 60 years. The group under consideration included 1 man and 8 women. In all these patients the diagnosis of polycythaemia vera had been established previously and they had been treated at the outpatient clinic. The duration of follow-up from the time of diagnosis to the development of pathological changes requiring surgery was 1 to 11 years.

Table 1

Operations carried out on patients with polycythaemia vera in the years 1968–1973

No. of operation	Patient	Sex	Age	Diagnosis	Type of operation
1	E. G.	F.	65	Gallstones in gallbladder and common duct	Cholecystectomy with Kehr's drainage of common duct
2				Stenosis of Vater's papilla	Relaparotomy, transduodenal sphincterotomy with drainage of Wirsung's duct
3	M. O.	M.	59	Bleeding gastric ulcer	Partial gastrectomy modo Rydygier
4				Previous osteosynthesis with nail of left femur	Removal of nail
5	B. M.	F.	58	Chronic cholecystolithiasis	Cholecystectomy
6	S. M.	F.	51	Bleeding gastric ulcer. Hydrops of gallbladder	Hofmeister-Finsterer partial gastrectomy
7	A. G.	F.	68	Anal sarcomatous leiomyoma	Abdomino-sacral amputation of rectum
8	F. Z.	F.	62	Left renal atrophy	Left nephrectomy
9	Z. M.	F.	56	Left crural varices	Linton's operation
10	J. S.	F.	66	Bilateral crural varices	Linton's operation of right leg
11	Z. M.	F.	58	Left femoral arterial thrombosis	Thrombendarterectomy

Before the operation two main methods were used to reduce the polycythaemia: blood-letting and treatment with cytostatics, in the first place with busulfan. Sometimes both these methods were applied. In one case (operation No. 6) ^{32}P was administered prior to surgery. However, the obtained reduction of the haematocrit and erythrocyte count was explained as due to bleedings from a gastric ulcer, which was the indication for surgery, rather than to the action of phosphorus. Similar bleedings from a gastric ulcer in patient M. O. (operation No. 3) caused a sufficient reduction in blood cell mass and the patient did not require preoperative preparation. In two other operations (Nos. 2 and 4) carried out 6 and 3 weeks respectively after the first operation the red cell count continued to be close to the normal range and these patients similarly did not require preoperative blood-letting or administration of busulfan. In one case (operation No. 5) the surgical intervention was carried out at a time when the values for haematocrit, erythrocytes, haemoglobin and platelets were higher than those accepted by us as appropriate for major surgery.

The duration of preoperative reduction of the red cell mass was up to 20 weeks and the mean values achieved by this management were: haematocrit, less than 50%; erythrocyte count, less than 4,500,000; and haemoglobin, less than 15 g/100 ml.

In 4 cases (operations Nos. 1, 6, 9, 11) including 3 patients after long-term busulfan treatment a reduction in platelet count was found (to 48,400; 53,800; 81,400 and 33,000 respectively) but no excessive intraoperative blood loss was observed.

In all patients operated upon, the postoperative course was uneventful, without haemorrhagic or thrombotic complications. Mean hospital stay was 57

Table 2

Blood counts before and after preoperative

Operation No.	Patient	Haematocrit per cent	RBC	Hb g per 100 ml	WBC	Platelet count
1						—
2	E. G.	67	6,120,000	17.6	13,000	—
3						
4	M. O.	75	6,600,000	19.6	—	—
5	B. M.	69	5,680,000		15,400	357,000
6	S. M.	66	6,500,000		5,200	142,600
7	A .G	54	5,830,000	18.3	5,500	204,600
8	F. Z.	59	6,600,000	18.3	22,400	497,000
9	Z. M.	52	4,920,000	15.9	4,100	57,200
10	J. S.	52	5,400,000	17.6	3,200	98,000
11	Z. M.					
Mean		62	5,955,000	17.8	9,820	321,800

days including 26 days after the operation. In one case (operation No. 7) the patient received during the operation an autotransfusion of 1500 ml. The blood was taken from her prior to the operation.

Discussion

In the pertaining literature some views are expressed that operations on patients with polycythaemia vera are linked with a high risk of complications and a high mortality rate reaching 40%.

According to Fitts et al. [4] in their material of 28 patients operated upon, haemorrhagic complications developed in all cases and were the cause of 4 deaths.

In 1966, Wasserman and Gilbert published their experience obtained in connection with 81 major operations performed on patients with polycythaemia vera [18]. Haemorrhagic complications developed in 37 cases causing the death of 13 patients. These authors emphasized a doubtless correlation of the incidence of complications with the method of preoperative management. They isolated 4 groups of patients characterized by the length of time between the reduction of morphological blood tests during preoperative management and the day of the operation. Group I, 4 months or more; group II, from 1 week to 4 months; group III, 1 week; group IV included patients not treated preoperatively or treated unsuccessfully. In the last non-controlled group including 28 patients complications occurred in 23 cases leading to death in 11. In groups II and III, the frequency of an uneventful postoperative course was over 60% of all the patients subjected to surgery. On the other hand, in group I comprising patients treated over 4 months preoperatively, complications occurred in 5% of the cases only.

management and type of treatment

Type of preparation			Haematocrit per cent	RBC	Hb g per 100 ml	WBC	Platelet count
Busulfan	Bloodletting	^{32}P					
+	−	−	42	5,100,000	−	6,900	81,400
−	−	−	−	3,660,000	11.3	15,200	−
−	−	−	32	3,420,000	10.4	7,200	176,000
−	−	−	38	4,060,000	11.6	6,150	138,000
−	−	−	54	4,860,000	16.8	11,350	470,800
−	−	+	45	4,300,000	14.0	3,250	48,400
−	+	−	41	4,380,000	14.0	2,400	25,300
+	+	−	46	4,430,000	14.8	5,300	127,600
−	+	−	42	4,120,000	14.0	5,100	52,800
−	+	−	46	4,830,000	13.2	2,900	132,000
−	−	−	43	4,510,000	12.4	6,700	33,000
			42.9	4,333,000	13.3	6,580	153,300

In our operated cases no complications were observed which could have been ascribed to a long preoperative management (Table 2), which was shorter than 2 weeks. Accepting the grouping of Wasserman and Gilbert according to the duration of treatment, the patients observed by us could be included into groups I or II which showed the lowest percentage of complications.

Successful surgical treatment of patients with polycythaemia vera is determined by careful haematological preoperative preparation. The main principles of this management are the reduction of red cell mass to normal or close to normal, the platelet count to near normal, and the maintenance of these values for a long period prior to surgery.

In the light of the experience of other authors and our present observations it may be assumed that immediately before the operation the haematocrit value should not exceed 50% and the platelet count 500,000 – 600,000/cu.mm. The principle of treatment in outpatient clinics is to maintain the blood values at these levels, if this succeeds, the patients do not need any special preoperative management. On the other hand, in these patients a reduction in the white blood cell and platelet count is sometimes observed as a result of cytostatic treatment which may inhibit the production of these cellular elements in a higher degree than that of red blood cells. Such patients should then be treated surgically solely on some vital indication. It may be necessary to use in these cases platelet concentrates during the operation, particularly if the platelet count is below 50,000/cu.mm. If it is possible to postpone the operation, cytostatic treatment should be discontinued and the intervention performed only after the platelet count has reached levels; above 100,000/cu.mm. Until that time, polycythaemia can be controlled by blood-letting.

Operations are contraindicated on patients with uncontrolled polycythaemia vera. In Wasserman and Gilbert's 37 cases of polycythaemia vera with postoperative complications the mean blood values were: haematocrit 54%; RBC 6,900,000; haemoglobin 15.8 g/100 ml; platelet count 286,000 cu.mm. This points to a high risk of operations in uncontrolled cases. When an emergency operation must be done, immediately before the operation 1500 ml of blood should be withdrawn and the procedure should be repeated postoperatively to keep the blood counts within the normal range. In every case an attempt should be made to postpone the operation and to reduce the erythrocyte mass and platelet count by cytostatic agents such as busulfan or to withdraw blood at intervals of several days. The blood obtained may be used for autotransfusions during the operation as it was done in one of our cases. It should be checked whether there is a deficiency of fibrinogen or blood clotting factors and to correct it, if possible [18].

Conclusions

1. Clinical experience shows that operations on patients with polycythaemia vera are connected with a high risk of postoperative complications. Thus, in such patients, indication for surgery should be considered with caution.

2. The success of the operation depends on the satisfactory control of polycythaemia, i.e. a reduction of the erythrocyte mass to a haematocrit value below 50%, and of the platelet count below 500,000/cu.mm, and the maintenance of these values at the given level for a possible long time prior to surgery.

3. Operations on patients with polycythaemia vera should be performed in close cooperation with specialists in haematology and blood transfusion.

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Thrombocytokinetic Studies with ^{75}Se-Selenomethionine in Thrombocythaemias

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Thrombocytokinetics were determined with ^{75}Se-selenomethionine in three patients with primary and nine patients with symptomatic thrombocythaemia, and in six cases platelet survival time with the ^{51}Cr method. Platelet survival time was normal in all patients according to both methods. The results showed that the raised blood platelet count in thrombocythaemia depends solely on the increased thrombocytopoiesis. In untreated cases this correlation was directly proportional.

Studies on thrombocytokinetics in both primary and secondary thrombocythaemia are scarce and even these observations are based on few patients examined by using ^{51}Cr- and DF^{32}P-labelled platelets.

These methods allow not more than an indirect evaluation of thrombocytopoiesis by determination of the platelet turnover [6, 12]; directly, they only show the survival time [1, 5, 7, 19].

The introduction into thrombocytokinetic studies of a new isotopic marker, ^{75}Se-selenomethionine which is incorporated into the platelets via the megakaryocytes, allowed a simultaneous evaluation of bone marrow thrombocytopoietic activity and of platelet survival time [8, 13]. In view of the scarcity of studies on thrombocytokinetics it seemed useful to report the results obtained in 12 patients with primary or symptomatic thrombocythaemia.

Material and Methods

Three patients with primary and nine with symptomatic thrombocythaemia were included in the study (Table 1).

Secondary thrombocythaemia developed in 5 cases in the course of chronic myelocytic leukaemia and in 1 patient each with osteomyelofibrosis, polycythaemia vera, cervical tuberculous lymphadenitis, primary autoimmunohaemolytic anaemia, and after splenectomy performed 7 years earlier because of traumatic rupture of the organ. The patients showed no symptoms of haemorrhagic diathesis and received no anticoagulants. The control group consisted of 10 subjects with no changes in the thrombocytopoietic system and the platelets.

Table 1

Clinical, haematological and isotope investigations in thrombocythaemias

Diagnosis	Number of cases	Platelet count in 1 cu. mm × 10^3	Megakaryocytes in bone marrow	Survival time of autologuos ^{51}Cr platelets (days)	Survival time of ^{75}Se platelets (days)
Primary thrombocythaemia	3	from 1 200 to 2 000	numerous or very numerous, with normal thrombocytopoiesis*	from 10 to 11 (determined in 3 patients) 10.6 ± 0.4	from 9 to 13 9.6 ± 0.4
Symptomatic thrombocythaemia	9	from 487 to 18 000	fairly numerous, numerous or very numerous, with normal thrombocytopoiesis**	from 9 to 10 (determined in 3 patients) 10.8 ± 1.6	from 9.5 to 12 10.8 ± 1.3

* In 2 cases the determinations were based on megakaryograms, in 1 case on bone marrow biopsy and in 1 case on the myelogram.
** In 2 cases the determinations were based on bone marrow biopsy and the remaining cases on myelograms.

^{75}Se-selenomethionine of 1 – 4 MCi/mg (Amersham) was used. It was introduced intravenously in a single dose of 2.5 μCi/kg body weight. Blood samples of 16 ml volume were collected at 2 – 3-day intervals for 20 days. The platelets were isolated by differential centrifugation, their activity was measured, and from the results a curve was plotted. The time between the half-period of increase of platelet activity and its decrease corresponded to the mean survival time of the platelets (Fig. 1).

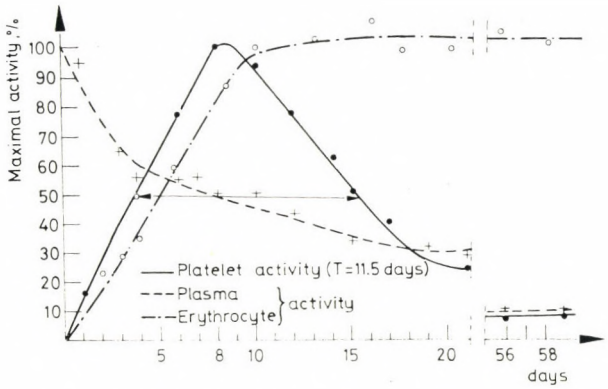

Fig. 1. Activity of ^{75}Se-selenomethionine in plasma, erythrocytes and platelets (in percentage of maximum activity)

In 6 patients, 2 with primary and 4 with symptomatic thrombocythaemia, the survival of autologous platelets was determined with the use of ^{51}Cr according to the authors' modification of the method of Aas and Gardner [11].

Results and Discussion

In the control group, after administration of ^{75}Se-selenomethionine, curves of increasing and decreasing platelet radioactivity were obtained with a peak between the 6th and the 11th day (Fig. 2). Mean survival time of platelets determined from these curves ranged from 9 to 13 days (mean, 10.07 $\pm$ 1.7 days). In 11 patients of the 12 with thrombocythaemia platelet activity curve was obtained with a much higher and steeper peak which fell between the 5th and 12th day (Fig. 3), and mean platelet survival time was 9 – 13 days. Survival of ^{51}Cr-autologous platelets was normal in all the six subjects examined (Table 1).

The maximum amount of ^{75}Se-selenomethionine incorporated into the platelets (represented by the peak of the curve) with a normal survival time reflects the intensity of thrombocytopoiesis in the bone marrow. The latter value and the peripheral platelet count should be directly proportional. This was confirmed by the results in Cases 1 to 10 shown in Table 2, concerning 3 patients with primary

Table 2

Comparison with control values of maximum radioactivity of platelets labelled with ^{75}Se-selenomethionine and platelet counts in thrombocythaemias

Case No.	Maximum radioactivity imp./min	Maximum radioactivity divided by maximum radioactivity in controls	Platelet count in 1 cu.mm × 10^3	Platelet count divided by mean platelet count in controls
1.	6203	5.5	1750	6.2
2.	6679	5.9	1200	5.0
3.	5217	4.5	1200	4.8
4.	8611	7.6	2000	8.0
5.	2916	2.6	570	2.4
6.	3090	2.8	810	3.3
7.	4414	3.9	1170	4.6
8.	4268	3.8	900	3.7
9.	6987	6.8	1800	7.5
10.	3571	3.1	600	4.0
11.	1493	1.3	663	2.65
12.	369	0.32	160	0.67

and 7 with symptomatic thrombocythaemia. The ratio of the peak values of the curves (depending on the thrombocytopoietic activity) to analogous values in the control cases corresponds to the ratio of the platelet count in the pathological cases to the control platelet counts. These values showed a high correlation ($r = 0.85$).

The obtained results indicate that the increase of the platelet count in thrombocythaemia, notwithstanding its nature, depends solely on enhanced thrombocytopoiesis and that these values are directly proportional. This correlation could not be revealed directly by any of the methods used so far for the evaluation of the intensity of thrombocytopoiesis.

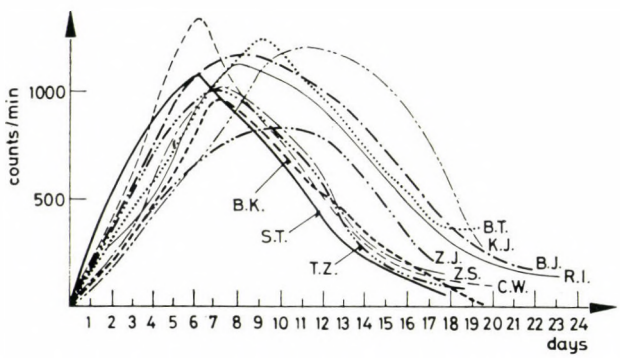

Fig. 2. Activity of ^{75}Se-selenomethionine in the platelets of 10 control subjects

Different results were noted in 2 patients (Cases 11 and 12, Table 2) with symptomatic thrombocythaemia in the course of chronic myeloid leukaemia and polycythaemia vera treated with busulfan which produced a fall in the platelet count in the period of investigation with ^{75}Se-selenomethionine from 887,000 to 663,000 and from 487,000 to 160,000 per cu.mm blood, respectively.

In studies of platelet kinetics by means of ^{75}Se-selenomethionine the obtained curves were comparatively flat. In the case of polycythaemia the curve ran even below those obtained in the control group (Fig. 4). The quotients of the peak values on the curves of these patients and the peak values in the control cases were much lower than those for the platelet counts in the diseased and healthy subjects (Table 2). The lower values obtained with ^{75}Se-selenomethionine might be explain-

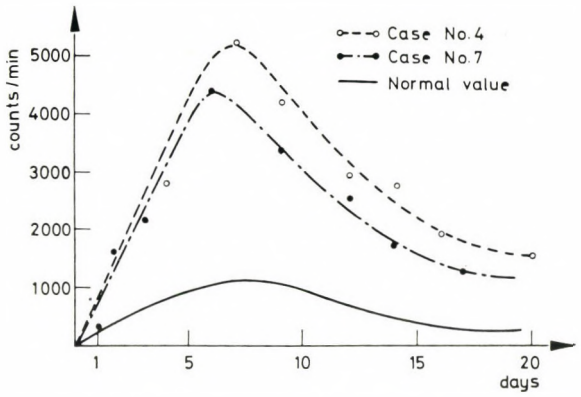

Fig. 3. ^{75}Se-selenomethionine platelet kinetics in 2 cases of thrombocythaemia

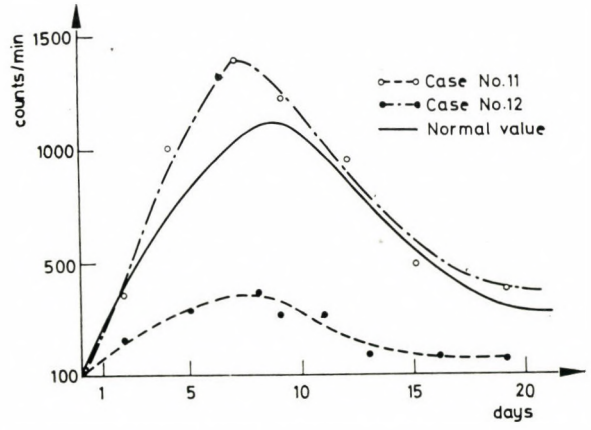

Fig. 4. ^{75}Se-selenomethionine platelet kinetics in 2 cases of thrombocythaemia in the course of busulfan treatment

ed by a considerable inhibition of thrombocytopoiesis by busulfan with a slower decrease of the platelet count in peripheral blood, dependent only on their ageing. During the investigations the supply from the bone marrow of newly formed platelets was much smaller and this explains the low radioactivity of the peripheral blood platelets.

Most of the papers published to date on thrombocytokinetics in both primary and symptomatic thrombocythaemia report on a normal [4, 5, 7, 9, 13], and some on a shortened [2, 3, 5, 7] platelet survival time. The only report on a prolonged survival time in several cases of thrombocythaemia [1] must have been due to a misinterpretation of the curve of DF^{32}P-labelled platelets, since the label may partly be incorporated *de novo* via the megakaryocytes into the platelets.

Numerous factors may lead to a reduction of platelet survival in thrombocythaemia, such as the formation of abnormal platelets, splenomegaly, thrombotic processes, haemorrhagic diathesis or immunization processes. Part of this has found confirmation in experimental studies. It has been demonstrated in several cases that platelets from patients with polycythaemia vera exhibit a shortened survival time not only in the diseased subject but also in healthy recipients [7]. This indicates that an intra-platelet defect may be the cause of the shorter survival. Another cause may be a considerable splenomegaly leading to an increased accumulation of platelets in the spleen with a shortening of their life [12]. The thrombotic processes and haemorrhagic diathesis frequently associated with thrombocythaemia, may also shorten the survival of platelets [4, 12]. In all these cases, in spite of the shortened survival thrombocythaemia occurs due to the enhanced thrombocytopoiesis.

All the studies on thrombocytokinetics in thrombocythaemia have revealed an increased thrombocytopoiesis. Quantitative evaluation was mostly based on the calculation of platelet turnover and showed a more than 13-fold increase of platelet count [6].

Platelet turnover, however, is an indirect means of thrombocytopoiesis assay, accounting for the time of platelet survival and their count in peripheral blood, under the assumption that platelet production corresponds to platelet destruction if the peripheral count shows no change. The thrombopoietic system in thrombocythaemia was subjected to a more precise evaluation in the studies of Harker et al. [6] with the use of the isotope-morphological method devised by these authors. An enhanced thrombocytopoiesis was demonstrated in 7 cases of primary and 8 cases of symptomatic thrombocythaemia (5 patients with polycythaemia vera and 3 with chronic myelocytic leukaemia) as well as the absence of differences between these two groups, in agreement with our results presented above.

Harker et al. also found that the increased thrombocytopoiesis in these patients was associated with an increased number and volume of megakaryocytic nuclei. On the other hand, symptomatic thrombocythaemia accompanying inflammatory processes and neoplastic diseases occurs only with an increased megakaryocyte count. The intensity of thrombocytopoiesis is measured with Harker's method

as the total megakaryocyte mass which in cases of thrombocythaemia may be increased as much as 15-fold [6].

Results of thrombocytokinetic studies in thrombocythaemias with the use of ^{75}Se-selenomethionine have been published for 2 patients only [8]; they revealed an enhanced platelet production and a normal survival time.

The present results agree well with those of up-to-date investigations on thrombocytokinetics in thrombocythaemias in which mostly a normal platelet survival time was observed together with enhanced thrombocytopoiesis. The obtained results indicate that in untreated cases the increased platelet count is directly proportional to the enhanced thrombocytopoiesis. Besides, the value of ^{75}Se-selenomethionine for following platelet kinetics in the course of thrombocythaemia has to be emphasized.

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Hodgkin's Disease of the Spleen: Some Clinical Aspects and Problems

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Eight cases of Hodgkin's disease of the spleen are reported. In 7 cases clinical signs of the disease appeared in advanced age. Characteristics were the absence of lymph node involvement and a very poor general condition. The diagnostic importance of laparoscopy is stressed and surgical treatment, and complex chemotherapy are recommended.

Hodgkin's disease is not easy to diagnose because in the early clinical stage it causes no pains. Lymph gland enlargements, fever of the Pel—Ebstein type, sweating at night and pruritus, together with many other well-known symptoms, are indications for suspecting its onset. The diagnosis rests on histopathological evidence [5, 6, 8, 10, 19] and it is difficult to recognize the deep seated variations of the disease [3, 4, 14, 16, 21]. In such cases, diagnosis takes much time. It is therefore important to know the signs which call attention to the disease even when its picture is not yet complete.

The introduction of lymphography of abdominal and mediastinal endoscopy, of aimed biopsy and laparoscopic inspection has, however, facilitated the charting of the successive phases in the clinical development of Hodgkin's disease [1, 11, 11a, 17, 20].

The spleen has rarely been identified as the original seat of malignant lymphogranulomatosis, but a primary splenic origin has rarely been considered in such cases owing to their confusion with varieties of the disease stemming from deep lymphoglandular involvement.

For this reason it might be rewarding to report on 8 cases of Hodgkin's disease of splenic origin. We do so in the hope of defining those clinical symptoms which may help in diagnosing a suspected case.

Case 1: Carolina S., a single woman aged 60

The family history was non-contributory. She had had keratoconjunctivitis at birth with bilateral residual blindness and then a dry dextral pleurisy at 21 years. At the age of 58, after about one month of increasing malaise, herpes zoster appeared in the region around the left sixth, seventh and eighth intercostal nerves.

The disease developed unusually slowly and about 40 days after the herpetic eruption sweating at night occurred, and fever which was soon to take a pseudo-undulatory form. Prolonged treatment with maximum doses of antibiotics and sulphonamides was totally inefficacious.

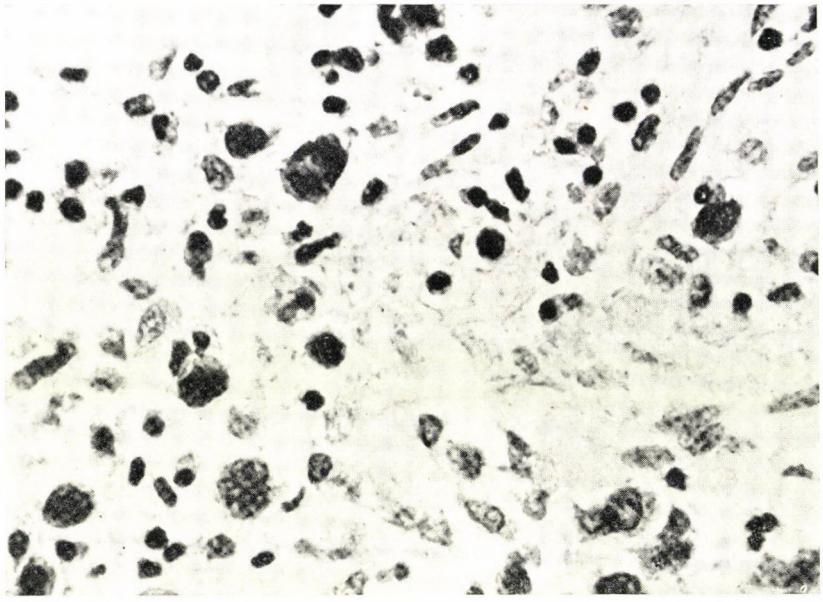

Fig. 1. Spleen biopsy: Hodgkin's granulomatous tissue with reticular cells, histiocytes, large multinuclear cells and lymphocytes; normal splenic tissue is not recognizable. Case 1 (Haematoxylin-eosin, ×40)

On entering hospital the patient presented a mild anaemia, terminal signs of bilateral keratoconjunctivitis, herpes, and a slight splenomegaly with some hardening. The overall lack of clinical symptoms was, however, in sharp contrast with the patient's severe prostration. Haematological tests were negative except for anaemia and the VES, and so were radiographs of the digestive organs, the lungs and bile ducts. Then an abdominal endoscopy revealed granulomatous lesions within the limits of the spleen, and these were confirmed by biopsy (Fig. 1). After prednisone and cyclophosphamide treatment, temperature returned to normal and prostration was much reduced. In the course of the next 6 weeks there were, however, clear signs of a granulomatous invasion of the liver. Splenectomy was therefore inadvisable and the patient was put under medical treatment. Within 10 months of diagnosis, or about one year from the start of the disease, lympho-granulomatosis had become general. Post-mortem examination showed a wide-spread granulomatous condition of the liver and the abdominal lymph glands.

Case 2: Luigi V., a monk aged 55

The family history was negative. The patient has had an operation for anal fistula at 32 years, and a second similar operation following a recurrence of the condition at 39 years. As a missionary in Libya from the age of 35 to 50, the patient suffered no illness. At 54 there had been a sudden and unexpected attack of enteral haemorrhage the cause of which was never explained in spite of a very thorough hospital examination. At 55 years, after 2 months of serious asthenia, there was a sudden onset of pain in the left hypochondrium with the temperature rising to 39°C. The condition persisted in spite of symptomatic therapy and the patient was sent to hospital. Large doses of antibiotics and cortisone were administered resulting in an immediate abatement of fever and a recovery of the general condition.

Later there was a marked increase of fever accompanied by sweating at night, and this further confirmed the suspicion of Hodgkin's disease, suggested by the patient's enlarged spleen, asthenia and weak condition. Laparoscopy showed adhesions in the perisplenic area with whitish nodules, characteristic of a spleen of the salami type. The addition of mechlorethamine to the antibiotic and corticosteroid therapy produced a most striking effect. Discharged in a period of marked improvement, the patient was successfully treated at home for the next 3 months. His general condition then grew worse and he was again brought to our hospital where laparoscopy now revealed Hodgkin's granulomas in the liver. Despite appropriate therapy, undulatory fever continued, the patient's condition deteriorated progressively, death occurring after about 4 months. It was not possible to ascertain whether the disease had spread to the lymph glands.

Case 3: Francesco T., a monk aged 58

The family history included two brothers who had died apparently with fever at 34 and 42 years of age, respectively. Apart from occasional common complaints the patient had had suffered from no particular illnesses except for gastric disturbances and, at 50 years, diffuse eczema which cleared up within a few months. When he was 57, he began to complain of pollakiuria which besides resisting normal treatment was marked by fever and a general deterioration in health. The patient was then admitted to the urology ward of a hospital where aseptic cystitis was identified by cytoscopic inspection, also a hard swollen spleen and leukopenia, for which reason he was transferred to our hospital.

Suspecting Hodgkin's disease of primary splenic origin, we applied laparoscopy and lymphography, but without obtaining any useful indications. After 2 months of ineffective therapy with antibiotics and steroids, splenectomy was performed and this confirmed the clinical evidence of Hodgkin's disease involving the spleen. Treatment with regular doses of mechlorethamine, antibiotics and corticosteroids followed, and there was a moderate improvement for about 3 months, after which a sudden enlargement of all superficial lymph nodes occurred and the general condition deteriorated gradually. Death ensued one month later.

Case 4: Maria Z., an unmarried housekeeper aged 65

The patient's father had died of aplastic anaemia the cause of which was uncertain. The history revealed no noteworthy data, while the early pathological record showed only an attack of pulmonitis with complete recovery at the age of 5. At 65, for no apparent reason, increasingly severe asthenia set in with a steady loss of weight and a deterioration of the general condition. Home treatment had proved ineffective and the patient was admitted to hospital. Here the undulatory type fever failed to respond to antibiotics, and the haematochemical, serological and cultural tests produced no satisfactory diagnostic evidence. Treatment with corticosteroid and antibiotics brought some improvement and the patient was discharged. Her health was relatively good for about 2 months, after which continuous fever with periods of remission, abundant sweating and extreme asthenia supervened and she had to return to hospital. Hardened splenomegaly was observed, and because of the associated symptoms it was decided to perform aimed splenocentesis under laparoscopic control in the vicinity of a small whitish patch and its vascular network. In the spleen, Sternberg cells were identified.

Polychemotherapy was prescribed and the symptoms diminished to some extent, although the size of the spleen remained unchanged. This improvement was maintained for about 9 months, then with the onset of high fever the general condition deteriorated sharply, icterus developed, the liver was considerably enlarged and hard superficial lymph nodes were found. No pain was felt on palpation. In spite of an intensive regime of polychemotherapy the patient died about 30 days later. Autopsy revealed a lymphogranulomatous invasion almost wholly confined to the spleen and the liver.

Case 5: Leonardo D., a naval rating aged 25

The family and the personal history were non-contributory. In February, 1971, the patient, then 24 years old, had been serving in the Red Sea when he had suffered an attack of undulatory fever. He had been hospitalized, possible tropical diseases had been excluded, and in the absence of any exact diagnosis antibiotic and antifebrile therapy had been prescribed. However, the fever persisted and the patient developed increasing asthenia amounting almost to adynamia. On discharge from the Navy he underwent further clinical and radiological check-ups which seemed to point to acute articular rheumatism. This was supported by vague forms of arthralgia and myalgia. Hospital treatment included a general cleaning-up of the dental and tonsillar areas, the suspicion being that this was due to an atypical form of rheumatic fever. Meanwhile leukopenia, and thrombocytopenia developed with an exceptionally high sedimentation rate.

Antibiotic and steroid treatment was applied, but the high fever persisted and in a later phase splenomegaly, followed 2 months later by the appearance of

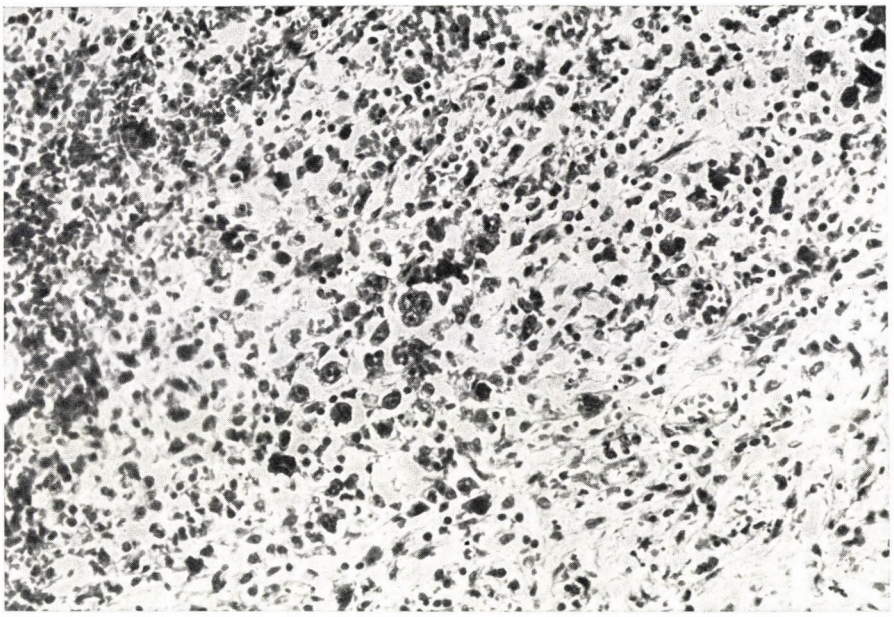

Fig. 2. Post-mortem spleen section: granulomatous invasion of splenic parenchyma with almost total destruction of the tissue structures; in centre, large multinuclear cell surrounded by numerous histoid cells. Case 5 (Haematoxylin-eosin, ×25)

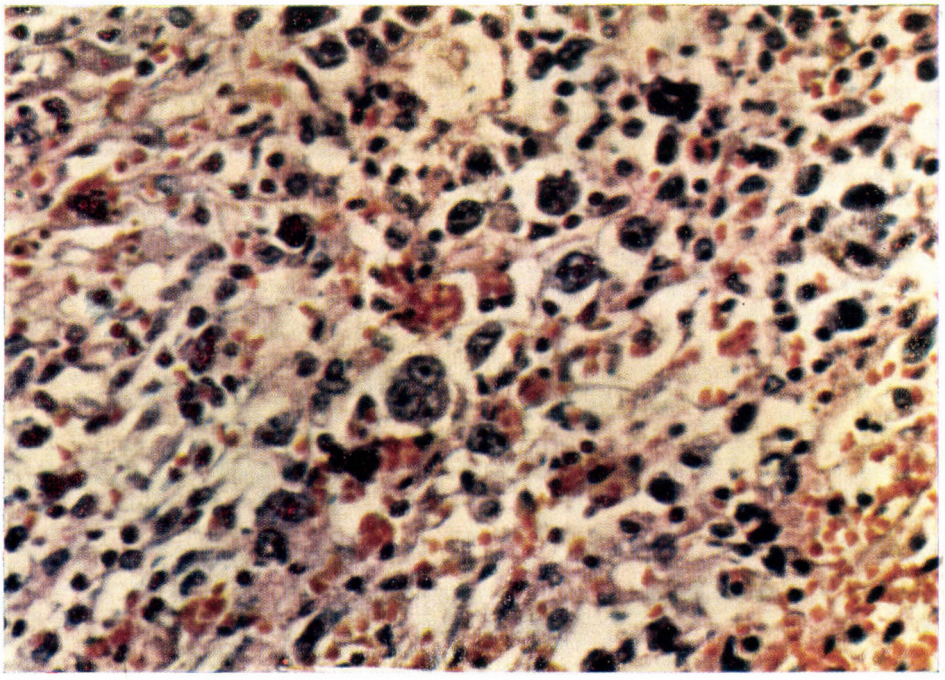

Fig. 3. Detail of Fig. 2: in centre, large multinuclear cell surrounded by smaller ones and by histoid cells; considerable vascular congestion (Haematoxylin-eosin, ×50)

jaundice of the cholestatic type. At this time the patient was admitted to our hospital. Here every attempt was made to determine the cause of the disease, but after a further month of steroid, antibiotic and intensive symptomatic treatment the patient died. Necroscopy revealed Hodgkin's disease localized in the spleen and the liver (Figs 2 and 3) without involving the lymph glands. Judging by its clinical course, the disease was probably of splenic origin.

Case 6: Lucilla C., a housewife aged 62

Family and early personal history were negative. Bouts of fever and feeling of prostration had begun in the spring season when she was 59. Symptomatic and steroid treatment had no result, and the asthenia became worse adversely affecting the general condition. The same year, in autumn, the patient was hospitalized for mild jaundice and was then found to have an enlarged spleen, slight

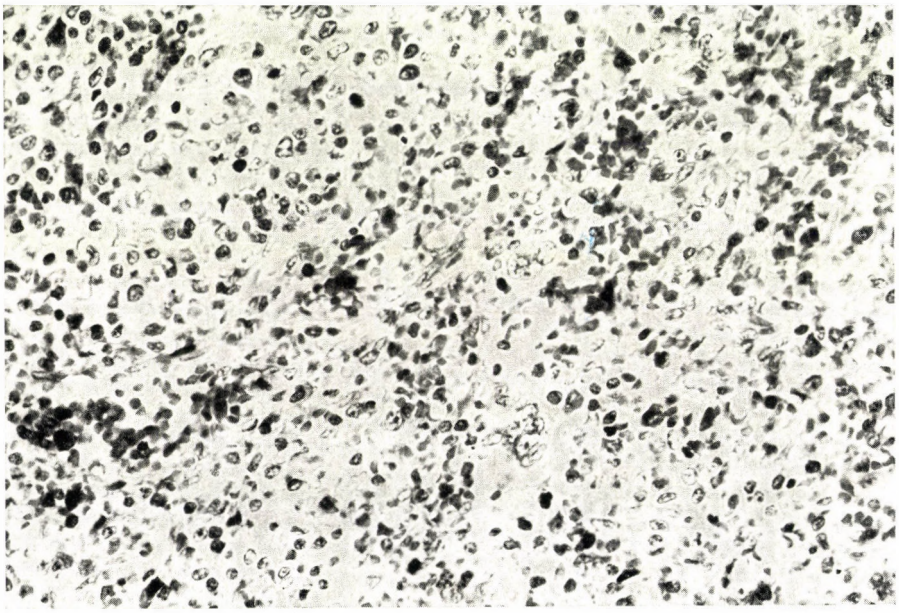

Fig. 4. Granulomatous tissue invasion of red and white splenic pulp; two large multinuclear cells in centre. Case 6 (Haematoxylin-eosin, ×40)

hepatomegaly, anaemia and leukopenia. Banti's syndrome was diagnosed; splenocentesis was performed resulting in the identification of lymphoreticulosis. Antibiotics and corticosteroid were prescribed and the subjective symptoms and fever disappeared.

The patient enjoyed moderately good health until April of the following year when there was a return of hyperpyrexia accompanied by a rapid decline of the general condition. Back in hospital a further enlargement of the spleen was noted together with severe leukopenia and persistent anaemia. Laparoscopy revealed splenomegaly with fibrosis and a slight hepatomegaly, although the organ

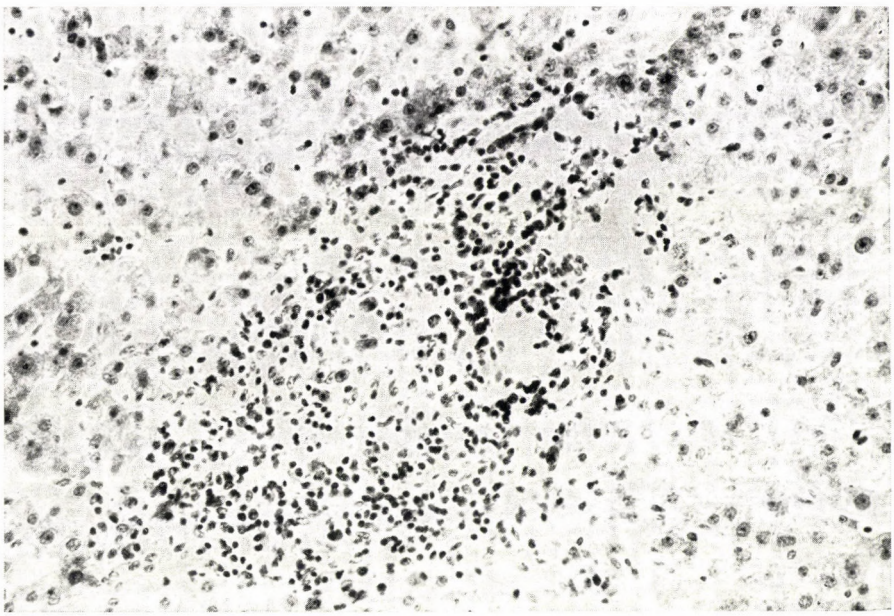

Fig. 5. Lymphogranulomatous infiltration of a portobiliary space. Large multinuclear cells in centre of the nodule, with lymphocytes and histiocytic cells around them. Case 6 (Haematoxylin-eosin, ×25)

appeared to be normal. After renewed antibiotic and cortisone treatment, the patient improved until the end of July when hyperpyrexia with severe prostration recurred without any plausible explanation other than splenomegaly and mild hepatomegaly. She was then brought to our hospital for exploratory laparotomy. A picture supporting the findings revealed by the previous laparoscopy was obtained and it was decided to perform splenectomy and hepatic biopsy. Histology showed Hodgkin's disease of the spleen and microscopic innidiations in the liver (Figs 4 and 5). All symptoms cleared up completely after the operation and the patient has since enjoyed good health.

Case 7: Filippo M., aged 58

Two maternal aunts and a brother had died of tuberculosis and the patient himself had been operated upon at the age of 18 for anal fistula which was prob-

ably of tuberculous origin. He had had typhoid fever when 27 and at 35 had tried to cure himself of intestinal amoebiasis which continued to affect him for some years. At 48, while under an orthopaedic examination for 5L and 1S discopathy, splenomegaly had been discovered, but the condition was judged to be innocuous. At 58 the patient had noticed that his spleen was rapidly increasing in size. On his own account he sought haematological and serological checks but did not succeed

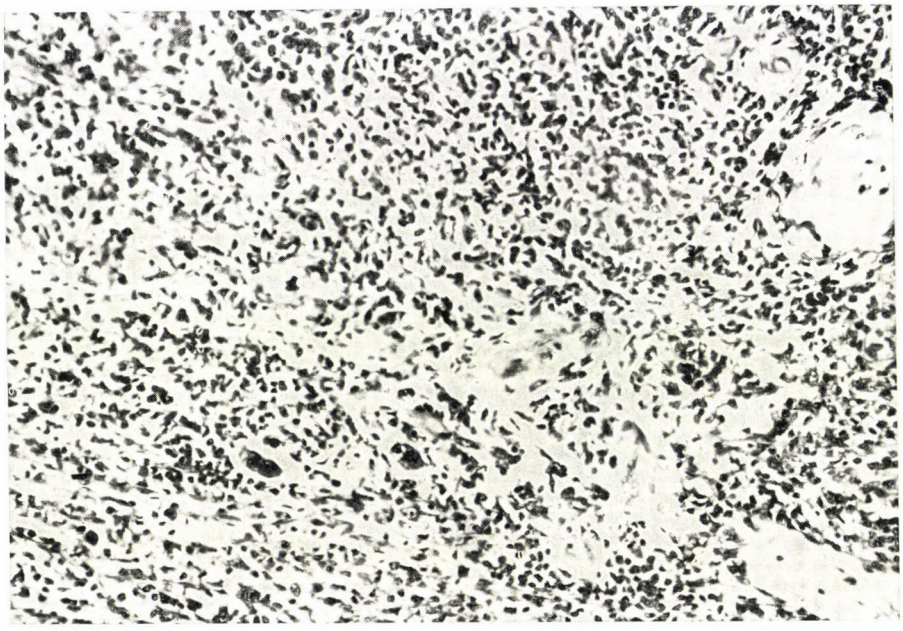

Fig. 6. Lymphogranulomatous tissue invasion into splenic pulp; large multinuclear cells and histoid cells at bottom left. Case 7 (Haematoxylin-eosin, ×25)

in obtaining relevant diagnostic indications. Ten days later, and after complaining for some days of asthenia, the patient was afflicted by severe pain in the spleen followed by high fever. The symptoms were treated and the fever cleared up within a week, but shortly thereafter he was running a high temperature accompanied by gradually increasing asthenia which did not yield to antibiotics.

In our hospital the patient was given 75 mg of prednisone, after which fever, asthenia and the other symptoms disappeared. A further increase in size and firmness of the spleen was then noted, and this gave immediate grounds for suspecting Hodgkin's disease of splenic origin. Without delay we performed a splenectomy which fully confirmed the diagnosis, while bringing to light microscopic innidiations in the liver, although a very thorough examination and palpation of the organ suggested that it was quite normal (Figs 6 and 7). Shortly after the splenectomy, jaundice appeared; symptomatic and cytostatic therapy was applied, but without effect. One month later the patient died.

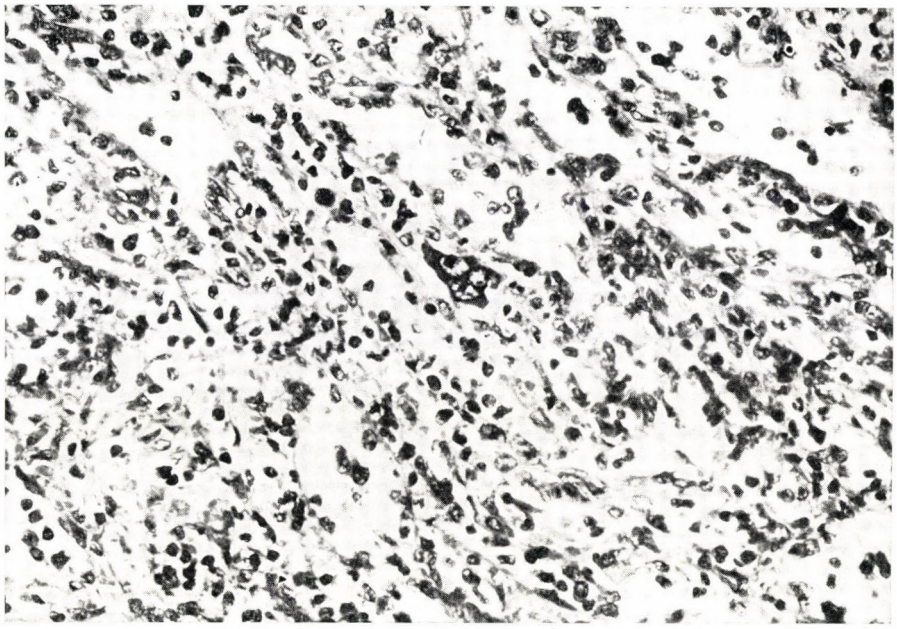

Fig. 7. Lymphogranulomatous tissue of polymorphous appearance, with large multinuclear cell in centre. Case 7 (Haematoxylin-eosin, ×40)

Case 8: Margherita G., a housewife aged 62

The mother had died of acute leukaemia and the father of diabetic coma. A sister who had been killed in an air accident at 59 had had slight splenomegaly, and a niece of 15 had died of acute leukaemia.

When the patient had been 44 years old, a neurinoma was excised from the cerebellopontile angle. Six months later she had had an icteric episode of undefined nature. Then, at 52, while being medically examined for loss of weight the patient had been found to be suffering from splenomegaly with mild pancytopenia. At first the spleen had remained unchanged, but during the 2 subsequent years it increased steadily in size to reach down to the umbilical line. Asthenia was present though fever was slight.

At this point the patient was admitted to our hospital. After check-ups telecobalt therapy was applied on the splenic area, the only condition discovered having been a non-uniform parenchymal uptake of radiocolloids. As the symptoms showed no change, the treatment was followed 2 months later by splenectomy. On examination the spleen displayed an invasion of malignant granulomatous tissue, though the liver as well as the abdominal lymph nodes proved histologically free of Hodgkin's disease. Since the splenectomy the patient has received no treatment of any kind and now 2 years after the operation she is in perfect health.

Conclusions

From the clinical point of view the finding common to all cases was the absence of any change involving either the superficial or the deep lymph nodes.

The general symptomatology gave no indications since it differed only slightly from that accompanying other grave diseases. However, the general condition was invariably affected and, judging from our findings, in a greater degree than in cases of lymphogranulomatosis of lymphoglandular origin [22].

Moreover, except in rare instances and then only in certain phases of the illness, fever took a typical Pel–Ebstein course. This could not be considered an indication, just as sweating at night and pruritus were in themselves no indications. Yet it is beyond doubt that in the context of splenomegaly a combination of some or all of these symptoms is an important guide to diagnosis.

In certain cases of malignant lymphogranulomatosis of splenic origin, suspicion is more than justified when the general condition is deteriorating. Even more must lymphogranulomatosis be suspected when to the deterioration of the general condition accompanied by marked asthenia is added a hard enlarged spleen, even if it causes little pain or none at all on palpation.

So far as our patients are concerned, splenic scintigraphy was not conclusive except in one case in which a defective parenchymal uptake was recorded. Other laboratory tests were equally uncharacteristic, with the obvious exception of biopsies.

A useful contribution to the diagnosis of suspected Hodgkin's disease of splenic site is supplied by laparoscopy which reveals a spleen of a typically porphyroid appearance. This condition, which calls for an aimed biopsy, is nearly always a decisive one in reaching the exact diagnosis. However, justification for laparoscopy with aimed biopsy, or of laparotomy with splenectomy must always have ample support in a well-founded clinical suspicion of Hodgkin's disease involving the spleen, and the suspicion should be resolved as soon as possible.

In view of the prognostic importance of the primary site of the disease, it is evident that where it is of splenic origin, the earlier the diagnosis the more favourable the prognosis. In any event, wherever the original site of Hodgkin's disease may be, liver involvement occurs in the final stage of the granulomatous invasion [10, 18]. It follows that lymphogranulomatosis of splenic origin may rapidly affect the liver to such an extent as to cause death even before the process has extended to the lymph nodes.

Removal of the spleen, thus of the elective site of the disease, is necessary on grounds of anatomy and portal physiology [18], as well as for those other reasons which today give support to splenectomy not only as a cure in certain cases of malignant granuloma but also for the valuable contribution of laparotomy to the correct staging of the disease [1, 9, 11a].

Splenectomy does not, however, necessarily result in remission even when it is performed in the early phase of the disease. This has been shown by our Cases 3 and 4 in which splenectomy was soon followed by death. In contrast, the patient

described in Case 6 is alive and in perfect health about two years after splenectomy, in spite of histologically confirmed liver metastases at the operation.

These clinical findings have been supported by the observation that, in addition to numerous other factors, not all of them well-known, in our cases there was an apparent relation between the histological type of the granulomatous tissue of the diseased spleen and the length of the remission period. Thus, in cases displaying a porphyroid spleen the patient survived longer than in those characterized by a diffuse invasion of Sternberghian tissue. And here – at least in our opinion – the prognosis of Hodgkin's disease of splenic origin might be based on a firmer footing than mere probability, though our own material is not in itself sufficient to offer a more reliable approach to this aspect.

Localization of Hodgkin's disease in the spleen raises clinical problems of no small importance. And it is into this category that belong those family and personal histories of tuberculous and parasitic lesions which may not only be the cause of protracted splenomegaly but also the responsible or co-responsible causes of a weakening of the organism's immunological resistance. In this respect, inherited tuberculosis seemed relevant in Cases 1 and 7; and leukaemia occurring in the subject's forbears and collaterals in Case 8.

In our cases, the clinical diagnosis of Hodgkin's disease of splenic origin has been established at presenile age in 7 out of 8 patients. This is in complete agreement with the observation of Clarke et al. [2] that the frequency of Hodgkin's disease in the abdominal region increases with age. In view of this, we would not reject a possible correlation between diminished immunological resistance, basic to the process of ageing [7], and the primary appearance of Hodgkin's tissue in the spleen, since this organ is known to contribute to active immunization while its senile involution normally occurs much later than in the lymph glands.

During diagnostic examinations it is impossible to establish whether the deep lymphoglandular regions have been affected by previous metastases, particularly in the upper subdiaphragmatic area, and/or in the hepatic parenchyma, even when on direct inspection and careful palpation the latter appears perfectly normal (Case 7).

To sum up, the clinical course taken by our cases was peculiar to each patient and in 6 cases out of 8 the survival period was very brief. This agrees with the hypothesis of Macmahon [13] and the observations of Clarke et al. [2] that in presenile age malignant granulomatosis displays the characteristics of other lymphomas. The peculiarities and variations displayed by our patients were, however, conditioned in each instance by the anatomico-functional reaction of the spleen attacked by granulomatous aggregations.

In conclusion, whenever a more or less serious deterioration of general health is accompanied by splenomegaly, and even more so by a sudden change in the semeiological characteristics of a pre-existing splenomegaly, it is necessary to make such inspections and check-ups as will enable the possibility of Hodgkin's disease of splenic origin to be taken into serious consideration. Diagnostic suspi-

cions of this kind leave no option but to perform abdominal endoscopy with aimed biopsy as well as other complementary tests.

Moreover, prompt surgical treatment and suitable chemotherapy are essential in all cases of Hodgkin's disease localized in the spleen, even when patients are suspect only clinically. And where subsequent difficulties are intrinsic they may be overcome by an appropriate staging of the disease.

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Haemolytic Anaemia in Malignant Lymphoreticuloendothelial Diseases

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Among 102 patients with malignant lymphoreticuloendothelial diseases, ten had autoimmune haemolytic anaemia, six haemolytic anaemia without positive direct antiglobulin test, and five had a positive direct antiglobulin test without anaemia and hyperhaemolysis. The pathogenesis of haemolytic anaemia in these diseases is discussed. It is assumed to be different in the cases with chronic lymphocytic leukaemia and in those with Hodgkin's disease.

Haemolytic anaemias (HA), especially those of autoimmune origin associated with malignancy of the lymphoreticuloendothelial system, are in the focus of interest because of the frequent coincidence of the two diseases. Autoimmune haemolytic anaemias (AHA) may serve as a model for studies on the pathogenesis of autoimmune mechanisms [1, 2, 3, 5, 11, 18]. During the last two decades, a number of papers [6, 9, 18, 21] have discussed the problem. The clinical picture and the course of AHA in these malignant diseases show considerable differences.

The present paper discusses the natural history of 21 cases with HA and malignant lymphoproliferative disease.

Material and Methods

Since 1969 we have treated 102 patients with malignant lymphoreticuloendothelial diseases. Thirty-two had chronic lymphatic leukaemia (CLL), 53 Hodgkin's disease (HD), 14 lymphosarcoma and 3 reticulosarcoma.

The diagnosis of HA was made on the basis of clinical signs of hyperhaemolysis, the peripheral blood picture, the serum bilirubin level, and the reticulocyte count. In the majority of patients, survival time of the red cells was examined by the ^{51}Cr technique. The direct and indirect antiglobulin tests were carried out at different temperatures and, when necessary, enzyme-treated erythrocytes were used. Bone marrow puncture was carried out and immunoglobulins G, A and M were determined in every case.

Results

Among a total of 48 patients with acquired HA, a positive direct antiglobulin was found in 37. In the latter group HA was associated with malignant lymphoma in 21 patients. The direct antiglobulin test was positive in 15 patients, while in 6 patients red cell survival was shortened but no anti-erythrocyte autoantibodies could be demonstrated by the antiglobulin test.

The cases presented in the Table are grouped as follows. The first group comprises patients with a positive direct antiglobulin test. These constitute 10% of all malignant diseases. The second group comprises 6 cases of HA without detectable antibodies. The third group includes 5 cases with positive direct antiglobulin test but without signs of marked anaemia and hyperhaemolysis throughout a long period of observation.

Table 1

Haemolytic anaemia in malignant lymphoma

Diagnosis	Number of cases	Haemolytic anaemia with direct antiglobulin test		Direct antiglobulin test positive without anaemia
		positive	negative	
Chronic lymphocytic leukaemia	32	4	5	5
Hodgkin's disease	53	5	1	—
Lymphosarcoma	14	1	—	—
Reticulosarcoma	3	—	—	—
Total	102	10	6	5

The main characteristics and the course of the diseases are shown in Fig. 1.

In the majority of cases, HA was associated with CLL, in 6 cases with HD, in 1 case with lymphosarcoma. Seventeen of the patients were males, 4 females. HA became manifest in 6 patients simultaneously with the malignant disease. Mean survival time of these patients was 11 months, in contrast to the 45 months of the rest.

Immunoglobulin values were different between the CLL and HD groups. Most of the patients with CLL had low values for IgG, and hardly any IgM. In 5 of the 6 patients with HD, IgG values were significantly higher than the normal upper limit. The absolute IgG values for both groups are shown in Fig. 2.

The direct antiglobulin test was positive in 15 cases, the indirect test in 8. In cases Nos 17, 18 and 21, the direct antiglobulin test was positive during the entire period of observation (in one patient for 4 years) without evidence of hyperhaemolysis and marked anaemia. Red cell survival was normal.

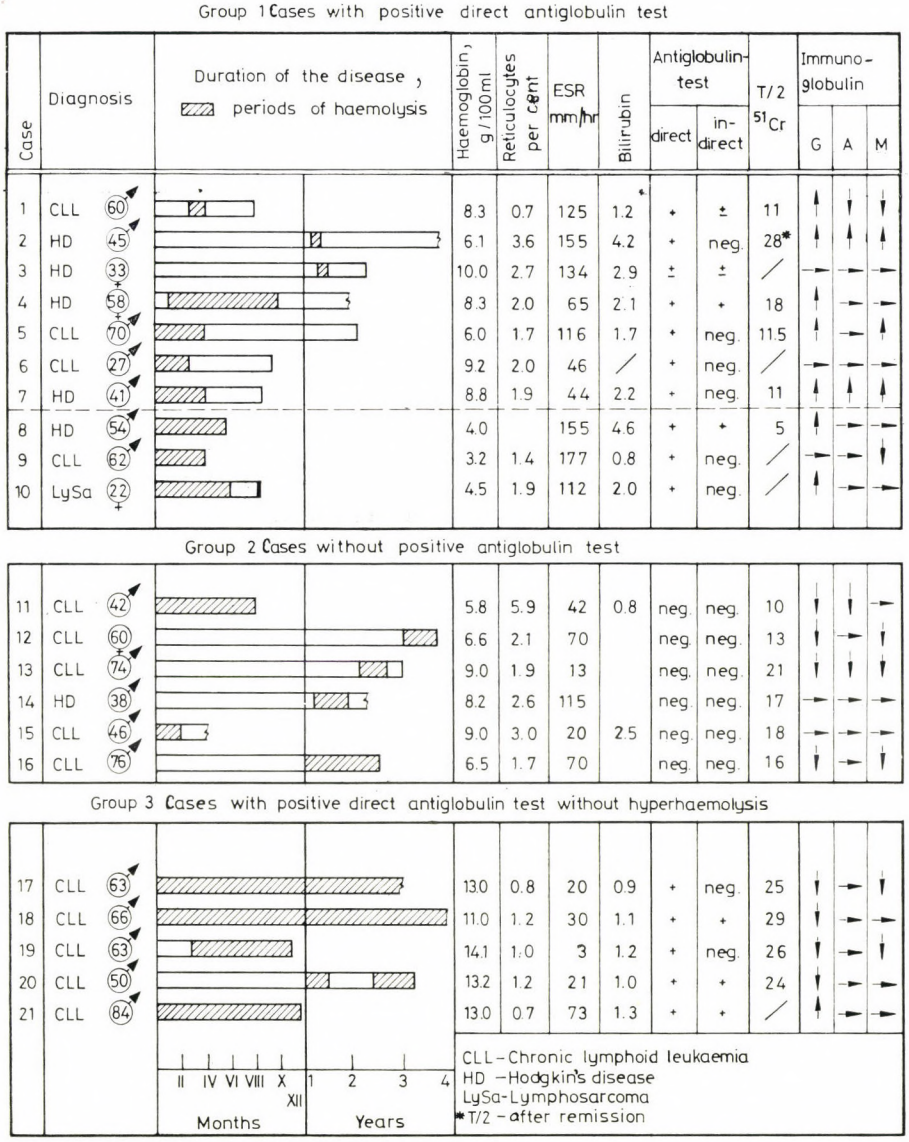

Fig. 1. Main characteristics as reflected in the antiglobulin test, erythrocyte survival time and serum immunoglobulins of cases with haemolytic anaemia in malignant lymphoma

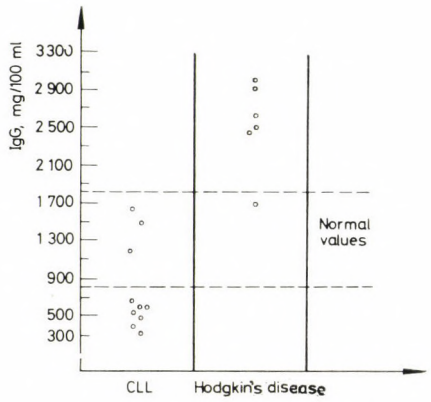

Fig. 2. IgG values of patients with chronic lymphocytic leukaemia and Hodgkin's disease, both associated with haemolytic anaemia

Report of some typical cases

Case 2 from the first group. A 45-year-old man had fallen ill in May, 1968. The presenting symptom was enlarged nodes. Hodgkin's disease-IIIA was verified by biopsy. He had not received active therapy until February, 1969, when

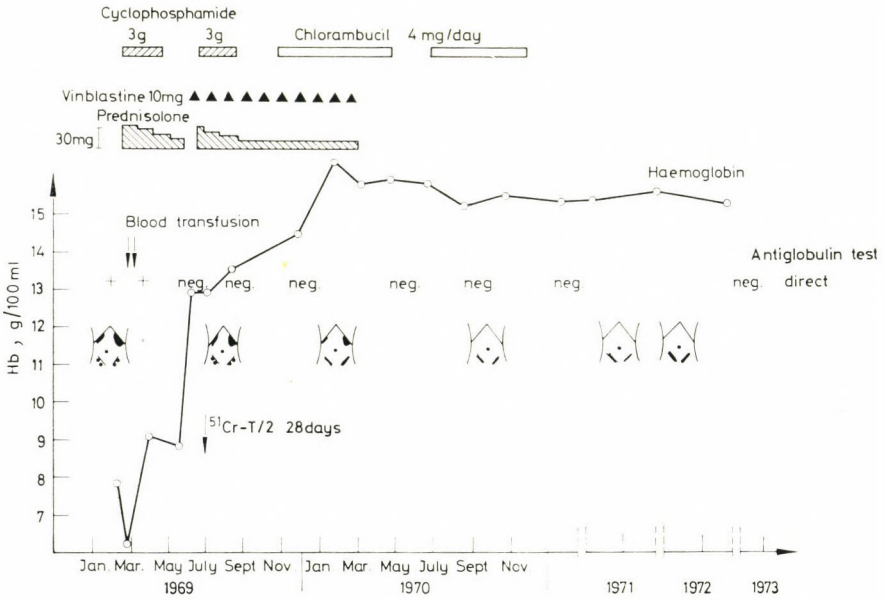

Fig. 3. A case with autoimmune haemolytic anaemia in Hodgkin's disease

he was admitted to our Department in a serious condition. Temperatures were up to 39 °C. The patient was wasted, pale, with a yellowish tinge of the skin. He had lymphadenopathy and the liver and the spleen were palpable 3 cm below the costal margin (Fig. 3). ESR, 155/160; Hb, 7.7 g per 100 ml; PCV, 28%; RBC, 2.8 million; WBC, 5900; serum bilirubin, 3.6 mg per 100 ml (indirect 2.5 mg per 100 ml). The direct antiglobulin test was strongly positive. Serum total proteins amounted to 6.4 g per 100 ml, with 45% gamma globulin fraction on electrophoresis. IgG, 2700 mg.

The patient was given prednisolone, 30 mg/day with cyclophosphamide in a total dose of 6 g, vinblastine 10 mg/week and chlorambucil 4 mg/day for one year. A full clinical and haematological remission was achieved and maintained for three years. Repeated antiglobulin tests yielded negative results. Red cell survival time was within normal limits.

Comment. This case represents a typical AHA in a patient with HD. HA disappeared as soon as an effective treatment of HD had been applied.

Case 8 from the first group. A 54-year-old man in October, 1970, developed symptoms of weakness, fatigue, pruritus, enlarged lymph nodes. He was admitted to our Department in November. Within a week he developed severe anaemia with temperatures up to 38.5 °C and lymphadenopathy. The spleen and the liver were palpable 3 cm below the costal margin. Laboratory findings at admission: ESR, 60/75 mm; Hb, 11.8 g per 100 ml; PCV, 38%; RBC, 3.9 million; WBC, 5800; reticulocytes, 0.1%; serum bilirubin, 0.8 mg per 100 ml; IgG, 2500 mg per 100 ml. Direct and indirect antiglobulin tests were strongly positive. Histological diagnosis: Hodgkin's sarcoma. Laboratory findings 2 weeks after admission: Hb, 4 g per 100 ml; PCV, 12%; RBC, 1.4 million; WBC, 6200; ESR, 155 mm.

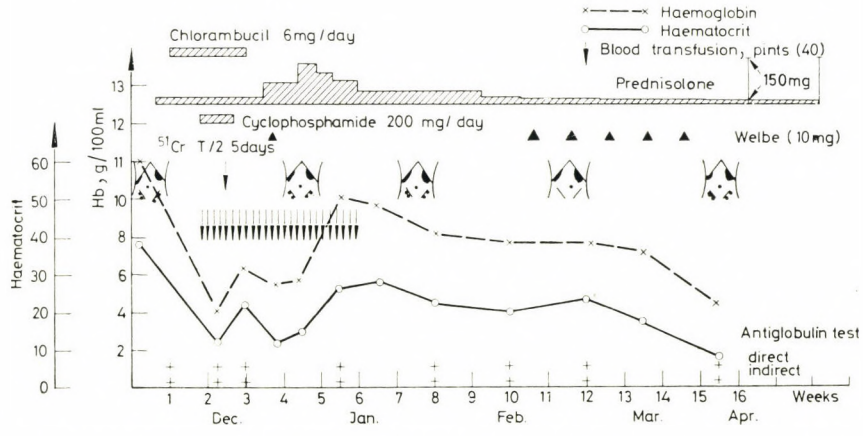

Fig. 4. Hodgkin's sarcoma and extremely severe uncontrolled autoimmune haemolytic anaemia

The bone marrow smear showed severe hypoplasia. ^{51}Cr-RBC survival time was 5 days. He was given prednisolone up to 150 mg/day and during the six weeks of the severe haemolytic episode, 40 pints of blood were given. A partial remission could be achieved in this therapeutic regimen, with the Hb level rising to 10 g per 100 ml. Treatment was continued with vinblastine 10 mg/week and prednisolone 20 mg/day, without any obvious effect. The patient died of pneumonia and HA five months after admission. The direct and indirect antiglobulin tests were strongly positive during the whole course of the disease.

Comment. This patient was a typical example of the simultaneous acute onset of the malignant lymphoproliferative disease and AHA. Both the malignant disease and the associated HA failed to respond to intensive corticosteroid and cytostatic treatment.

Case 11 from the second group. This was a 44-year-old man, with a family history of malignant reticuloendothelial disease; his father had died 8 years before of HD. The patient had had no symptoms until he had been revaccinated against small-pox in the spring of 1972. Throughout the summer he had had complaints of discomfort and fatigue. He had consulted his family doctor several

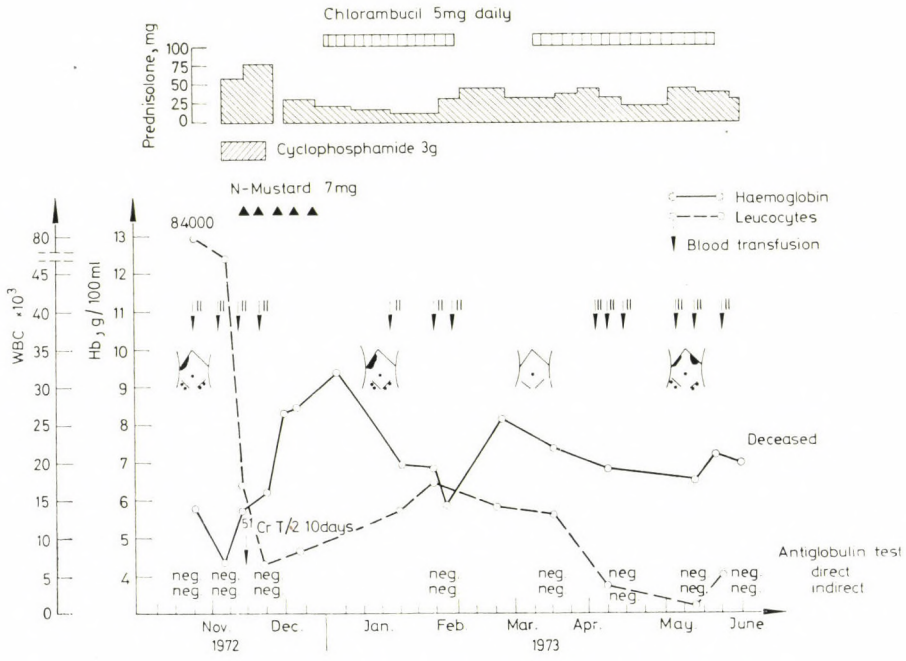

Fig. 5. A case of chronic lymphocytic leukaemia with severe haemolytic anaemia without positive antiglobulin test

times. The laboratory findings had been negative except for a mild anaemia. In November of the same year he was admitted to our Department with a severe haemolytic episode and anaemia. The skin was yellowish, the temperature, 38.5 °C. Enlarged lymph nodes. Spleen not palpable. ESR, 47/95 mm; Hb, 5.8 g per 100 ml; PCV, 16%; RBC, 1.9 million; WBC, 83,000; platelet count, 9300; reticulocyte count, 5.9%. Total serum proteins, 5.15 g per 100 ml. Serum protein electrophoresis: gamma globulins 11.7%, IgA decreased, IgG and IgM normal. Bone marrow biopsy showed hyperplasia; 70% of the cells were lymphocytes. RBC survival amounted to 10 days. The direct and indirect antiglobulin tests were negative on several occasions. The patient was given high doses of prednisolone, cyclophosphamide, chlorambucil, nitrogen mustard, and several blood transfusions. An incomplete remission of short duration followed. The patient died of sepsis and thrombocytopenic haemorrhagic complications.

Comment. This was a typical case of an obvious haemolytic process without detectable red blood cell autoantibodies. In this group of patients the course of the disease was usually short, averaging 6 to 12 months after the presenting symptoms.

Case 17 from the third group. A 63-year-old man with a negative family history. In 1968, CLL had been diagnosed for which he was treated periodically. The patient had been referred to us early in 1972. His complaints included weakness, fatigue, and enlarged lymph nodes. The spleen was palpable 4 cm below the costal margin. Laboratory findings: ESR, 46/80 mm; Hb, 13 g per 100 ml; RBC, 4.2 million; WBC, 72,000; platelet count, 94,000; differential count, 90% lymphocytes. The bone marrow smear revealed 60% lymphocytes. Total serum proteins amounted to 6.7 g per 100 ml; electrophoresis: gamma globulin 13%. IgG and IgM values were below normal during the period of observation. ^{51}Cr-

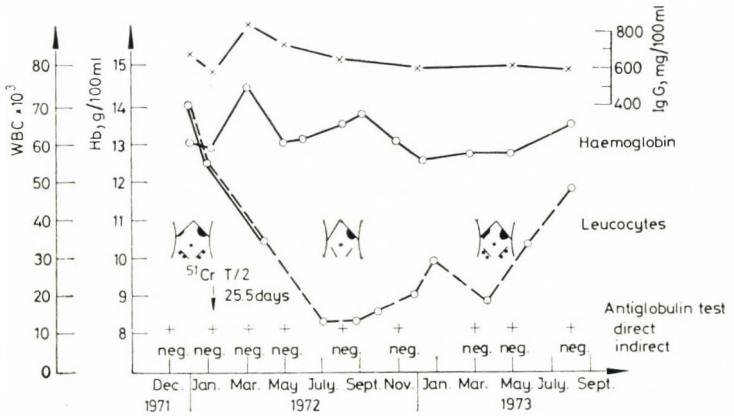

Fig. 6. A case of chronic lymphocytic leukaemia with persistent positivity of the direct antiglobulin test without anaemia or signs of increased haemolysis

RBC survival, normal (25.5 days). The direct antiglobulin test was invariably positive, the indirect test, negative. During his stay in the hospital he underwent recurrent facial erysipelas infections and pneumonitis. Blood Hb levels were near the lower normal limit.

Comment. The case is representative of a group in which in spite of the direct antiglobulin test no signs of hyperhaemolysis were observed. Red blood cell counts were normal. RBC survival time was within normal limits. There was a marked hypogammaglobulinaemia with repeated infections. Survival of similar patients was usually long, averaging about $3^1/_2$ years.

Discussion

Since the publication of the first cases of acquired haemolytic anaemia (AHA) associated with CLL and HD [13, 16], there has been a continuous interest in the subject [7, 9, 12, 18]. Dameshek and Komninos [5] reported 19 cases, Dausset and Colombani [9] 28, Dacie [4] 59, Pirofsky [18] 73 cases of AHA in malignant lymphoma of the reticuloendothelial system. According to these literary data, the frequency of AHA varied from 14.3 to 82.6%. This high frequency of AHA has not been encountered in other malignant diseases. A number of authors [2, 4, 18] has tried to explain this intriguing phenomenon.

Pirofsky [18], Videbaek [21] and others have stated that AHA occurs more frequently in association with CLL than with HD or other lymphomas. In our material the occurrence of AHA was identical in both diseases. Eisner et al. [10] found only 3% AHA in HD, whereas Matthias [14] described an incidence of 25%.

We assume that the pathogenesis of AHA is different in the cases with CLL and in those with HD. Patients suffering from CLL usually exhibit poor antibody production or develop even an immune paralysis. In contrast, immunoglobulin levels were above the normal limits in most of our patients suffering from HD. It is known that CLL is usually accompanied by anaplasia of B lymphocytes resulting in deficiency of humoral immunity. In HD, the T lymphocyte-related delayed immune response is impaired. We may assume that AHA in CLL is due to the presence of an aberrant malignant forbidden clone instrumental in causing an occult [19] or manifest form of HA with or without detectable anti-erythrocyte autoantibodies. Autoantibodies were permanently present in our third group of patients but it seemed that they did not possess the ability of producing manifest hyperhaemolysis. This was well reflected in the normal survival time of red blood cells in this group. Dresch et al. [8] made similar observations in two cases. Weiner [23] observed Coombs positivity in 21 normal subjects among 60,000 healthy blood donors. The finding was confirmed by Dacie [4] and Pirofsky et al. [17]. These authors suggest that the process of antiglobulins does not necessarily indicate hyperhaemolysis, or that a haemolytic ineffectiveness of some autoantibodies may be involved.

Schubothe [20] differentiated between therapy-resistant and therapy-responsive forms of idiopathic AHA. Our 3 patients in the first group were evidently of the therapy-resistant type.

AHA in HD is usually transitory and responds readily to therapy (our cases Nos 2, 3, 4, 7 and 14). We suppose that in a state of depressed cellular immunity, which is common in untreated active HD, the proliferation of a forbidden clone of lymphocytes may occur in a patient who is genetically disposed, and thus able to form antibodies. As soon as proliferation of the abnormal cell clones has been suppressed by adequate therapy, the AHA subsides, as in our case No. 2. The theory of Gershwin and Steinberg [11] on the thymic suppression function by which the thymus or the T lymphocytes regulate or under normal conditions suppress a continued lymphoid hyperplasia may apply to such cases. In HD where delayed immunity is impaired, the suppressive function becomes abolished providing a possibility for the proliferation of abnormal clones of B lymphocytes, which in turn may play an important role in producing AHA.

The absence of detectable autoantibodies in malignant lymphomas with HA is difficult to explain. In Pirofsky's opinion [18], autoimmune HA is common, but a positive antiglobulin test is rare. We are in full agreement with this statement, in contrast to Videbaek [21].

Wasi and Black [22] examined the half-life of erythrocytes in 20 patients with CLL, of which 6 had also anaemia. Survival time was significantly shortened in one case only. We have determined ^{51}Cr-RBC survival in 7 patients with severe anaemia whose malignant lymphoproliferative disease was under control. Our findings revealed a normal erythrocyte survival time in this group of patients, a finding in contrast to the observation of Dresch et al. [8].

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Der Einfluß von kombinierten elektrischen und magnetischen Feldern auf biologische Zellen und andere Partikel*

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Eine bestimmt konfigurierte Kombination eines niedrigen elektrischen und niedrigen magnetischen Feldes führt zu Bewegungen von Partikeln, die entgegengesetzt zu der bekannten 3-Finger-Regel gerichtet sind. Die elektrische Ladung der Partikel spielt für die unerwartete Richtung der Ablenkungskomponente keine oder eine untergeordnete Rolle. Von großer Wichtigkeit ist dagegen die Ionenkonzentration des Aufschwemmungsmediums. Es kann bisher noch keine theoretische Deutung dieses Phänomens angegeben werden, die alle Ergebnisse befriedigend erklärt. Es ist jedoch zu vermuten, daß das Phänomen eine Informationsübersetzung "magnetische Feldstärke" in "Bewegung von Partikeln" ermöglicht und daß dies auch für bestimmte biologische Phänomene von Wichtigkeit sein dürfte.

In den letzten Jahren wurden zahlreiche Arbeiten über eine biologische Wirkung magnetischer Felder veröffentlicht [3]. Offenbar kann aber eine biologische Wirkung eines magnetischen Feldes nur so zustande kommen, daß die Information des magnetischen Feldes in eine andere Information, z. B. in eine entsprechende chemische oder eine strukturelle Veränderung übersetzt wird. Bisher ist meines Wissens noch kein derartiger allgemeiner Übersetzungsmechanismus gefunden worden. Im folgenden möchte ich über Beobachtungen berichten, die möglicherweise eine Grundlage für eine derartige Informationsübersetzung liefern. Ausgangspunkt unserer Beobachtungen waren elektrophoretische Untersuchungen.

In den letzten 10 bis 15 Jahren habe ich mit zahlreichen Mitarbeitern das Muster ionisierbarer Gruppen auf Zelloberflächen untersucht und geprüft, welche funktionellen Schlüsse man daraus ziehen kann. Hierfür war es notwendig, eine Routinemethode zur Messung der elektrophoretischen Beweglichkeit von Zellen oder anderen Partikeln zu haben, denn die Messung der ionisierbaren Gruppen an Zelloberflächen läßt sich am einfachsten elektrophoretisch durchführen. Hierfür werden die Partikel in einer wäßrigen Salzlösung aufgeschwemmt. An die Aufschwemmung wird ein elektrisches Feld gelegt und mikroskopisch die Wanderungsgeschwindigkeit der Partikel gemessen. Von der Firma Zeiss, Oberkochen, wurde nach unseren Angaben für Routineuntersuchungen das Zytopherometer entwickelt (Abb. 1). Der Hauptteil des Apparats besteht

* Nach einem Vortrag auf der Jahrestagung der Physiologen der DDR in Magdeburg am 26. 9. 1972.

aus einer geschlossenen rektangulären Meßkammer mit den Abmessungen von $20 \times 10 \times 0.7$ mm, in der die Partikelsuspension aufgeschwemmt ist. An den beiden Enden der Kammer liegen die Einfüll- oder Auslaufstutzen und die Elektroden. Die Meßkammer ist von einer Kühlkammer umgeben, die die Joule'sche Wärme ableitet, die beim Stromdurchtritt durch die Partikelsuspension entsteht. Das Ganze ist auf ein Spezialmikroskop montiert, das die Beobachtung der Zellgeschwindigkeit mittels eines Gitters und einer Stoppuhr bestimmen läßt.

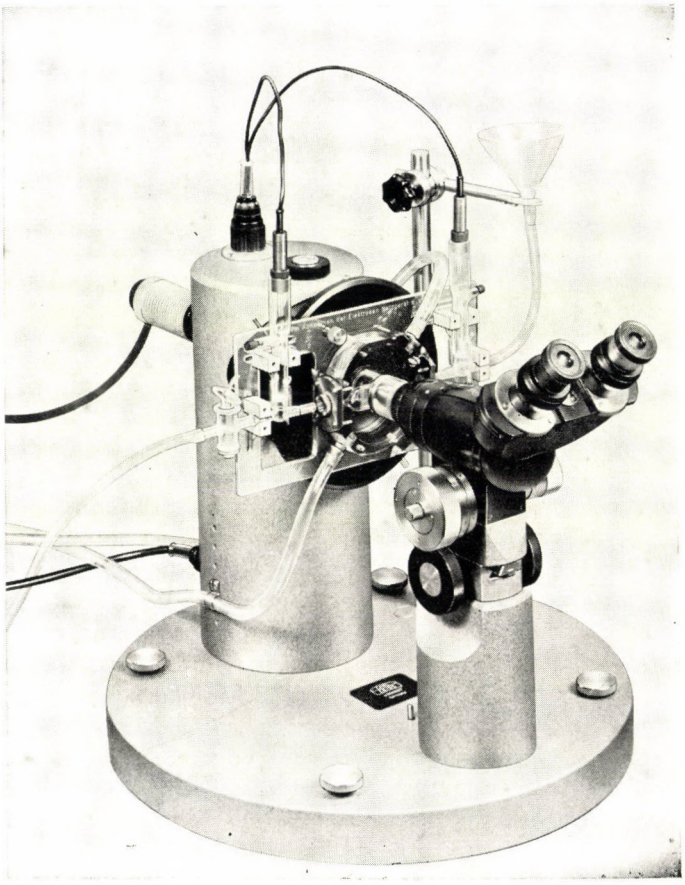

Abb. 1. Zytopherometer

Im vorigen Jahr hatte ich den Besuch eines amerikanischen Gastprofessors, Roy C. Gunter. Unter anderem untersuchten wir folgende Frage: Was geschieht, wenn man Zellen oder andere Partikel nicht nur einem elektrischen Feld aussetzt, sondern zusätzlich noch im rechten Winkel ein magnetisches Feld anlegt. Ausgangspunkt der Überlegung war die bekannte Beobachtung, daß elektrisch geladene Partikel, die sich in zwei so gelagerten Feldern befinden, eine Ablenkung

erfahren, die durch die bekannte 3-Finger-Regel wiedergegeben wird: Die Ablenkung bildet sowohl mit dem elektrischen als auch dem magnetischen Feld einen rechten Winkel. Dieser Vorgang kann an den lila gefärbten Permanganationen gezeigt werden: ein elektrisches Feld bewegt die Ionen in einer bestimmten Richtung; wird ein magnetisches Feld normal zu dieser Richtung angelegt, so kommt es zu einer seitlichen Anlenkung der Ionen.

Aufgrund dieser Überlegungen haben wir an unser Zytopherometer ein magnetisches Feld in der Richtung des Mikroskoptubus angelegt. Die Stärke des magnetischen Feldes in der Meßkammer betrug etwa 30 Gauss, war also relativ niedrig. Es wurde dadurch erzeugt, daß wir einen elektrischen Strom durch eine koaxiale Spule um den Mikroskoptubus führten. Auf diese Weise konnten wir die Meßkammer des Zytopherometers unbeeinträchtigt beobachten. Die elektrische Feldstärke variierte etwa von 0.1 bis 1.0 V/cm.

Mittels dieser Anordnung haben wir nun folgende Beobachtungen gemacht [4]. Bei alleiniger Einschaltung des elektrischen Feldes bewegen sich die negativ geladenen Erythrozyten zur Anode. Wird das elektrische Feld durch ein magnetisches überlagert, so kommt es zu einer Ablenkung, jedoch entgegengesetzt zu der Richtung, die die 3-Finger-Regel erwarten läßt. Eine solche unerwartete Ablenkungsrichtung konnte bei Erythrozyten, Thrombozyten, Gold- und Silberpartikeln, Polyvinyltoluol- und Polystyrenpartikeln beobachtet werden. Darunter sind sowohl positiv wie negativ geladene Partikel enthalten. In Tab. 1 ist zu sehen, daß

Tabelle 1

Partikel	Suspensionsmedium	Ablenkungswinkel
Plättchen	Plättchenreiches Plasma	43
Plättchen	0.9 % NaCl + 0.1 % EDTA	42
Erythrozyten	Plättchenreiches Plasma	30
Erythrozyten	0.9 % NaCl + 0.1 % EDTA	36
Fe_2O_3	0.9 % NaCl	15
Silber-kolloidal	0.9 % NaCl	40
Quarz	0.9 % NaCl	45
Polystyrol (⌀ 0.79–9.5 μ)	0.83 % NaCl	27°—37°

Ablenkung in Winkelgraden (± 10 %) von verschiedenartigen Partikeln in einem elektrischen Feld (~4 V/cm) und einem rechtwinklig überlagerten magnetischen Feld (~30 Gauss).

das Ausmaß der Ablenkung unter sonst identischen Versuchsbedingungen von der Art der Partikel abhängig ist. Der Diameter der Partikel spielt nur eine geringe Rolle für das Ausmaß der Ablenkung. Dies geht aus Versuchen mit Polystyrenpartikeln hervor, deren Diameter von 0.79 bis 9.5 μ variierte; der Ablenkungswinkel fiel hierbei nur von 37° auf 27°.

Auf der anderen Seite ist der Ablenkungswinkel von der Stärke des Magnetfeldes abhängig. Er steigt z. B. bei Erythrozyten bis zum stärkst angelegten Feld

von etwa 50 Gauss dauernd an. Anders war dies bei der Variation des elektrischen Feldes: Auch hier stieg die Ablenkung bis zu einer Feldstärke von 4 V/cm an, eine höhere Spannung ergab jedoch keine Änderung.

Eine besondere Bedeutung für das beobachtete Phänomen hat die Ionenkonzentration des Aufschwemmungsmedium: Be ieiner Konzentration von 10% Natriumchlorid werden Latexpartikel beinahe um 90° abgelenkt.

Dieser Befund spricht dafür, daß die Salzkonzentration des Aufschwemmungsmediums eine besondere Bedeutung für das Ablenkungsphänomen hat und daß die elektrische Ladung der Partikel für die unerwartete Richtung der Ablenkungskomponente keine Bedeutung zu haben scheint. Dies wird durch folgenden Befund noch unterstrichen:

Erythrozyten wurden in einer physiologischen Kochsalzlösung aufgeschwemmt und an die Aufschwemmung ein synchron wechselndes elektrisches und magnetisches Feld von 5 Hz gelegt. Der Wechsel des elektrischen Feldes verhinderte die durch die elektrische Komponente bedingte Bewegung der Erythrozyten. Bei diesem Ansatz betrug die entgegen der 3-Finger-Regel gerichtete Ablenkung wie erwartet 90°. Auch folgender Befund zeigt, daß die elektrische Ladung der Partikel als solche keine oder nur eine untergeordnete Rolle für die unerwartete Richtung der Ablenkungskomponente spielt: Werden Erythrozyten mit Neuraminidase inkubiert, so verlieren die Zellen etwa 4/5 ihrer elektrophoretischen Beweglichkeit. Werden solche Zellen nun dem kombinierten elektrischen und magnetischen Feld ausgesetzt, so kommt es ebenfalls zu der gegen die 3-Finger-Regel gerichteten Ablenkung, die jedoch wesentlich stärker ist als jene der unbehandelten Erythrozyten. Schließlich sollte noch untersucht werden, ob das Phänomen mit einer möglichen Inhomogenität des elektrischen Feldes in der Meßkammer zusammenhängt, die auf dem quadratischen Querschnitt der Meßkammer beruhen könnte. Die Versuche wurden zu diesem Zwecke in einer Meßkammer wiederholt, die eine zylindrische Form hat und bei der die Elektroden flächenförmig an den beiden Enden des Zylinders angesetzt sind. Auf diese Weise kommt ein streng homogenes elektrisches Feld in der Meßkammer zustande. Auch hier kam es zu der entgegen der 3-Finger-Regel gerichteten Ablenkung der Partikel nach Anlegung der kombinierten Felder.

Vor längerer Zeit wurden von Kolin Untersuchungen über den Einfluß kombinierter elektrischer und magnetischer Felder auf die Bewegung von suspendierten Partikeln veröffentlicht [1]. Mit hohen magnetischen Feldern erhielt er Abweichungen, deren Richtung abhängig war von dem Verhältnis der Leitfähigkeiten des Mediums und der Partikel. Bei unseren Experimenten war jedoch die Richtung der Ablenkung immer gleich und schien nicht von der Leitfähigkeit der Partikel abzuhängen. Dies geht daraus hervor, daß elektrisch sehr schlecht leitende Erythrozyten dieselbe Ablenkungsrichtung aufweisen wie die vermutlich sehr viel besser leitenden Silberkolloide. Außerdem war die Stärke unserer Felder um Größenordnungen niedriger als bei den Kolinschen Versuchen.

Kolin u. Mitarb. [2] haben auch mit niedrigeren magnetischen Feldern gearbeitet und konnten zeigen, daß eine Flüssigkeitsströmung durch exzentrische,

heterogene magnetische oder elektrische Felder zustande kommt. Wir haben auch diesen Möglichkeit geprüft, indem wir die Achse des magnetischen Feldes parallel aus der optischen Achse um etwa 1 cm herausrückten. Damit war die Heterogenität des magnetischen Feldes künstlich geändert, ohne daß es zu einer stärkeren Änderung der Ablenkung gekommen wäre. Diese Versuche zeigen, daß unser Phänomen vermutlich neuartig ist und nichts mit den Kolinschen Versuchen zu tun haben dürfte.

*

An den Untersuchungen waren in erster Linie Dr. Roy C. Gunter, sowie, Dr. R. A. Dimich, Dr. H. Jaeger und Dr. J. N. Mehrishi wesentlich beteiligt.

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Influence of Combined Electric and Magnetic Fields on Biological Cells and other Particles

It has been shown that the special combination of low electrical and low magnetic fields causes trajectories of suspended particles the directions of which are opposite to the so-called 3-finger rule. The electrical charge of the particles is of little or no significance for this unexpected phenomenon, the ion concentration of the suspension is, however, of great importance. There is no satisfactory theoretical explanation of the phenomenon which is assumed to facilitate magnetic field strength information to be translated into particle movements, which may be of a certain biological importance.

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GOT, GPT and LDH Levels and Blood Viscosity Factors after Recent Myocardial Infarction
Effect of ABO Blood Groups*

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A study was carried out on correlations between biochemical factors (GOT, GPT, LDH) and blood viscosity factors (blood and plasma viscosity, aggregation of red cells, formation of artificial red/white and white thrombi) in patients after recent myocardial infarction. GOT levels showed a significant correlation with the viscosity of blood at high shear rate ($P < 0.005$) in all patients, in patients with complications or concurrent diseases ($P < 0.05$), or in patients of A or O blood groups ($P < 0.02$). GOT levels correlated with the rate of degradation of artificial white thrombus ($P < 0.02$) and of the red/white thrombus ($P < 0.05$, blood group O only). GPT levels correlated with the apparent viscosity of red/white thrombus ($P < 0.05$). Significant differences were found in the levels of GOT and GPT between patients of A and O blood groups. Correlations of GOT or GPT and of aggregation of red cells showed significant differences between patients of A and O blood groups ($P < 0.01$). It is suggested that elucidation of the molecular mechanics of infarction might require subgrouping of patients according to their ABO blood groups.

Introduction

While it was shown [1, 2] that blood viscosity may be elevated in patients who suffered a myocardial infarction, these results could have been influenced by several factors which may not necessarily be related directly to the myocardial damage. For example, blood viscosity could have been affected by an increase in the blood catecholamine level, a possible increase in free fatty acids, the hypoxia that sometimes accompanies acute myocardial infarction, or even the absolute bed rest that these patients are usually subjected to.

Consequently, we have made a study of the correlation between blood viscosity factors and the biochemical evidence of myocardial damage as expressed by the levels of aspartate transaminase (GOT), alanine transaminase (GPT) and lactate dehydrogenase (LDH).

* The experimental part of this study was carried out in the Haemorheology Laboratory established by L. D. at Glasgow Royal Infirmary during a Commonwealth Visiting Professorship at the University of Strathclyde, Glasgow.

Significant correlations were found during this study between the blood viscosity factors [3] (blood viscosity, plasma viscosity, aggregation of red cells, apparent viscosity and formation of artificial red/white and white thrombi) and the enzyme levels. Some of the correlations became apparent only after the data recorded were divided according to the patients' ABO blood group. Division of patients according to their blood groups proved more fruitful than a division into a group with complications and/or concurrent diseases, and a group without any complications and/or any concurrent disease.

Materials and Methods

The patients studied were admitted to the Glasgow Royal Infirmary. They were selected for this study when the diagnosis of recent myocardial infarction was supported by enzyme levels and by sequential changes in the electrocardiogram. Blood viscosity factors were determined between two days and two weeks after the episode. A single determination of each of the blood viscosity factors (blood and plasma viscosity, aggregation of red cells, and apparent viscosity and formation of artificial thrombi) was carried out on each of the patients.

All enzymes were assayed by initial reaction rate procedure at 37°C. Transaminase and lactate dehydrogenase assays were carried out on the LKB 8600 reaction rate analyzer using Boehringer test combination reagents. Enzyme activities were expressed in International Units [4, 5]. Normal ranges of values established by truncated probit analysis [6] were as follows: Aspartate transaminase (GOT): 9–35 I. U./litre; alanine transaminase (GPT): 5–26 I. U./litre; lactate dehydrogenase (LDH): 115–465 I. U./litre. Higher normal ranges (98% confidence limits) have been obtained with the same assay procedures in a normal healthy non-hospital population [7]; namely, 10–37 I. U./litre for GOT, 6–37 I. U./litre for GPT, and 0–130 I. U./litre for creatinine kinase.

The viscosity factors studied included blood viscosity, plasma viscosity, aggregation of red cells, and formation and apparent viscosity of artificial thrombi. The latter were determined in the variable-frequency thromboviscometer [8] at 37°C, using freshly shed venous blood. Artificial red/white and white thrombi [9, 11] were formed at 60 and 180 cycles/min, at total amplitude (arc) of 110 degrees of the circle, the corresponding mean shear rates being 26 and 80 sec^{-1}, respectively. Blood viscosity was determined at 37°C in the rotational rhombospheroid viscometer [12]. Plasma viscosity was measured in the British Standards M3 micro-U-tube capillary viscometer at 20° and 37°C. Erythrocyte sedimentation rates were followed in Westergreen tubes. Aggregation of red cells was estimated [13, 14] from the ESR corrected for plasma viscosity and adjusted to a haematocrit of 30%.

The above tests were carried out on blood anticoagulated with 7.5 mg of solid EDTA (sequestrene) per 5 ml of blood.

Statistical results were calculated using Sharp and Monroe desk calculators and computer PDP8I (using "Focal" language). The values computed included means, standard deviations, linear regressions, error of estimate (Sy.x). Significance of differences between slopes ($= \Sigma xy/\Sigma x^2$) was computed using F values. Significance of differences between means was calculated using Student's *t*-test.

Results

Age, sex, haematocrit, ABO blood groups, enzyme levels and diagnosis of patients are contained in Table 1. Distribution of patients according to their blood group, sex, and presence or absence of complications or concurrent disease, or anticoagulation, is shown in Table 2.

Table 1

Laboratory data and diagnosis for patients with recent myocardial infarction

Code	Age	Sex	Blood group	Hct	GOT	GPT	LDH	Comments
C1	62	M	A+	48	144	74	1440	diabetes
C2	47	M	O−	40	60	55	390	no complication
C3	50	M	O+	44	76	18	760	no complication
C4	46	M	O+	47	79	27	1580	pulmonary embolism, warfarin
C5	40	M		47	79	23	580	no complication
C6	51	M	A+	51	160	44	1680	pulmonary embolism
C7	56	M	A+	46	196	50	1400	no complication
C8	63	F	O−	53	160	26	1200	warfarin
C9	50	M	O+	41	70	70	850	no complication
C10	49	F	O+	44	232	24	1760	pulmonary embolism, warfarin
C11	62	M	O+	39	40	19	330	bronchial cancer
C12		M		48	—	—	—	cardiac arrest
C14	65	M	A+	57	300	60	3000	no complication
C15	51	F	O+	28	90	35	608	pneumonia and multiple myeloma
C16	41	M	O+	46	14	7	200	epilepsy
C17	49	M	A−	52	120	33	1160	no complication
C18	64	M	O+	42	65	20	800	no complication
C19	60	M	A+	47	144	56	840	no complication
C20	62	F	AB+	47	55	20	520	no complication
C21	70	M	A+	49	122	60	1120	no complication
C22	69	M	A+	45	140	44	840	no complication
C23	52	M	A+	48	85	56	2400	alcoholic
C24	71	M	O+	44	—	—	—	cardiac arrest
C25	59	M	A−	45	264	36	1320	warfarin
C26	59	F	B+	40	—	—	—	hypertension
C27	50	M	O+	52	100	20	1200	no complication
C28	69	F	A+	43	40	17	285	thyrotoxic

Results of rheological studies are summarized in a form of linear regressions; also, only those regressions are included which show a statistical significance or throw some additional light on the interaction between enzymes and the viscosity factors.

Table 2

Distribution of patients according to ABO blood groups, sex and complication or treatment

Blood group	Sex	No complication and no anticoagulation	Complications and/or anticoagulation
A	male	6	4
	female	0	1
O	male	4	4
	female	0	3
AB	male	0	0
	female	1	0
B	male	0	0
	female	0	1

Table 3

Significance and correlations between enzyme levels and blood viscosity factors in patients with recent myocardial infarction

Enzyme (X) vs. viscosity factor (Y)	n	Linear regression		P
		angle*	r	
GOT vs. blood viscosity:				
at 180 sec^{-1}	22	0.0150	0.6880	<0.005
at 108 sec^{-1}	24	0.0149	0.5900	<0.005
at 36 sec^{-1}	24	0.0181	0.4559	<0.05
GOT vs. plasma viscosity at 37°C	24	0.0011	0.5246	<0.01
GOT vs. rate of degradation of white thrombus	22	0.0070	-0.5273	<0.02
GPT vs. rate of formation of the red/white thrombus	24	0.1431	0.5165	<0.01
GPT vs. viscosity of red/white thrombus	24	0.1299	0.4171	<0.05

* Angle, m, of the linear regression equation; r is a correlation coefficient; ($y = mx + b$);

Note that functions of GPT vs. blood viscosity or vs. plasma viscosity show correlation coefficients of 0.0 order, while the same functions of GOT are consistently significant.

Table 3 shows significant correlations between GOT and blood viscosity measured at high shear rate; between GOT and the rate of degradation of artificial white thrombi; between GPT and the rate of formation and apparent viscosity of the artificial red/white thrombi. As an appreciation of the various parameters of artificial thrombus formation would otherwise be difficult, the variable-

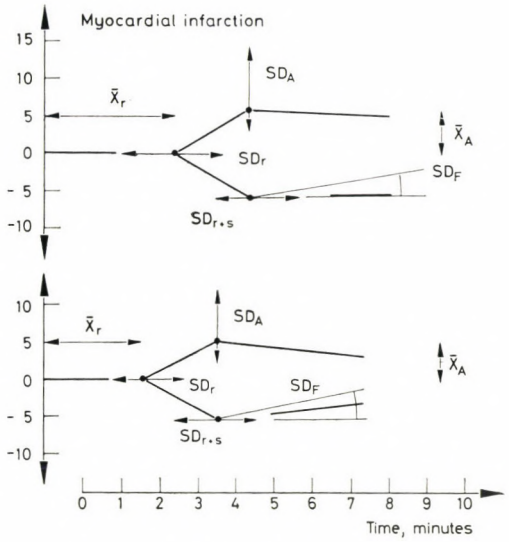

Fig. 1. Mean VFTV dynamic coagulation/thrombus formation curves for the series of patients with recent myocardial infarction. The upper curve corresponds to the mean values for artificial red/white thrombi formed at 60 cycles/min; the lower curve corresponds to the mean values for artificial white thrombi formed at 180 cycles/min, at 37°C. Means and standard deviations were obtained from log-normal distribution and reconverted into numerical values. $\bar{x}$: arithmetic mean; SD: standard deviation; subscripts r, r + s, A and F refer to the latent clotting times, total thrombus formation time, apparent viscosity of the thrombus, and the rate of degradation of the thrombus, respectively. Curves are plotted in the deflection units of the scale (1 cm of the scale equals 2.15 degrees of the circle); a Newtonian fluid of viscosity of 1 P will cause a deflection of 0.83 cm at 60 cycles/min, and one of 2.49 cm at 180 cycles/min

frequency thromboviscometer (VFTV) curves are given for the red/white and for the white thrombus. Curves in Fig. 1 are based on means and standard deviations of the VFTV parameters obtained for the series of patients studied.

The first series of results show that blood viscosity increases as the level of GOT increases. Increases in GPT run parallel with the increases in the rate of formation and in the apparent viscosity of artificial red/white thrombi. Increases of GOT correlated negatively with the rate of degradation of artificial thrombi.

The patients were divided into two groups. The first group contained patients without complications, without any concurrent disease, and without anticoagula-

tion. The second group contained patients exhibiting complications or suffering from some concurrent disease, or treated by anticoagulants. Table 4 indicates that while the second group showed significant correlations between the levels of GOT and plasma or blood viscosity, the first group behaved differently. The group of patients in which the myocardial infarction was associated with no complication, and in which no concurrent disease was present and no anticoagulation deemed necessary, showed no significant correlation between the factors described above.

Table 4

Correlations between GOT and blood and plasma viscosity in patients with recent myocardial infarction. Group 1 contains patients without complication or any concurrent disease; Group 2 contains patients showing complications, concurrent diseases, or being on anticoagulants

Group	n	X	Y	Sy. x	Angle	r	P
Blood viscosity (Y) vs. GOT (X)							
1	13	117	5.02	1.146	0.00391	0.1926	n.s.
2	11	118	5.61	1.429	0.02181	0.7608	<0.01
Plasma viscosity (Y) vs. GOT (X)							
1	13	117	1.249	0.0962	0.000553	0.2350	n.s.
2	11	118	1.319	0.1013	0.002029	0.8383	<0.005

Note: plasma viscosity, cP, measured at 37°C;
blood viscosity, cP, measured at 37°C and at the shear rate of 216 sec^{-1};
GOT level is given in international units per litre;
r: correlation coefficient;
P: significance limit of the correlation coefficient.

It appeared that other factors must have played a role and that patients should be divided according to other criteria.

In a number of correlations which showed no statistical significance when all patients were grouped together, some significant results emerged when the patients had been divided according to their ABO blood group.

Table 5 shows that correlation coefficients might differ between A and O blood group patients. In some cases, such as correlations between GOT and viscosity of artificial white thrombus, or GPT and viscosity of artificial white thrombus, the signs of correlation coefficients were opposite for A and O blood group patients. In other cases, such as correlations between GOT and aggregation of red cells, or GPT and aggregation of red cells, or LDH and the rate of degradation of white thrombus, the angles of linear regressions were different, and the correlation coefficient for the sum of both groups was lower than for either of these groups.

That the difference between A and O blood groups is real and statistically significant, at least in some of the correlations, was shown by computations of differences between the slopes of linear regressions (Table 5). Thus, the difference between A and O blood groups was significant in the correlation between GOT and aggregation of red cells, and GPT and aggregation of red cells.

Table 5

Correlations between enzyme levels and blood viscosity factors in patients with recent myocardial infarction

Enzyme vs.	Viscosity factor	Blood group	n	Angle*	r	P	$P_{A/O}$
GOT vs.	blood viscosity**	A	11	0.0159	0.6332	<0.02	n.s.
		O	11	0.0140	0.7434	<0.01	
GOT vs.	aggregation of red cells	A	11	0.6783	0.3833	n.s.	<0.01
		O	12	0.0372	0.0291	n.s.	
GOT vs.	viscosity of white thrombus	A	11	0.0427	0.6273	<0.05	n.s.
		O	11	−0.0048	−0.1054	n.s.	
GOT vs.	rate of degradation of red/white thrombus	A	11	−0.0002	−0.0722	n.s.	n.s.
		O	11	0.0057	0.5414	<0.05	
GPT vs.	aggregation of red cells	A	11	2.6773	0.2421	n.s.	<0.01
		O	12	0.5746	0.1367	n.s.	
GPT vs.	rate of formation of red/white thrombus	A	11	0.1861	0.6348	<0.05	n.s.
		O	10	0.2144	0.6042	<0.05	
GPT vs.	viscosity of white thrombus	A	11	0.1471	0.4480	n.s.	n.s.
		O	11	−0.0812	−0.4855	n.s.	
LDH vs.	rate of degradation of white thrombus	A	11	0.0003	0.1942	n.s.	n.s.
		O	11	0.0009	0.7380	<0.05	

* Angle, m, of the linear regression equation; r is a correlation coefficient.

** Blood viscosity was measured at 180 sec^{-1}.

Note: calculation of the significance limits for the difference between slopes of linear regressions for A and O blood groups was carried out using computer PDP81.

Significant differences (Table 6) were also obtained between the levels of GOT, or GPT, in patients of A and O blood groups. Significant differences were noted between the apparent viscosities of artificial white thrombi, and in the rates of their formation, in patients of A and O blood groups. Nearly as significant was the difference in the levels of LDH between A and O blood group patients.

Table 6

Means and standard deviations for GOT, GPT and LDH levels for some blood viscosity factors in patients with recent myocardial infarction

Enzymes or factors	Blood group	n	$\bar{x}$	SD	$P_{A/O}$
Enzymes:					
GOT	A	11	155.9	71.0	<0.05
	O	11	89.6	56.9	
GPT	A	11	48.3	14.7	<0.02
	O	11	29.1	17.3	
LDH	A	11	1407.7	716.8	ca. 0.07
	O	11	879.8	483.3	
Blood viscosity factors: blood viscosity, cp*	A	11	5.48	1.79	n.s.
	O	12	4.81	1.03	
apparent viscosity of white thrombus, d.u.	A	11	8.59	4.83	<0.05
	O	11	4.84	2.76	
rate of formation of white thrombus, d.u./min	A	11	4.580	2.597	<0.02
	O	11	2.429	1.265	
aggregation of red cells,** mm/hr	A	10	205.0	152.0	n.s.
	O	12	145.0	115.0	

* Blood viscosity measured at 180 sec^{-1}.

** Aggregation of red cells was estimated from the erythrocyte sedimentation rate corrected for plasma viscosity and adjusted to a haematocrit of 30%.

Calculation of significance limits for the differences between A and O blood groups was based on Student's *t*-test.

Discussion

The GOT level is used as an indicator of the myocardial damage, while the other enzymes indicate the state of hepatic function. Thus, the levels of GOT correlated with the high shear-rate blood viscosity at $P < 0.005$, and with the apparent viscosity of artificial thrombi (although for the blood group A only), and with the rate of degradation of white thrombi at $P < 0.02$.

As an increased blood viscosity and an increased viscosity of artificial thrombi (which are morphologically analogous to thrombi *in vivo*) are associated with, and indicative of, ischaemic conditions [3], the question therefore arises whether an increase of the GOT level was associated with a poorer blood flow and a less efficient tissue perfusion. The enzyme may directly affect the blood viscosity factors, but it is more likely that it serves as a marker for other intracellular pro-

teins released by the destroyed muscle fibres, and that these intracellular proteins might be responsible for the blood viscosity elevation and the enhanced thrombus formation. As a poor blood flow may by itself lead to tissue destruction, a vicious circle may be formed.

The GPT levels correlated well ($P < 0.01$) with the rate of formation of red/white thrombi and with their apparent viscosity ($P < 0.05$).

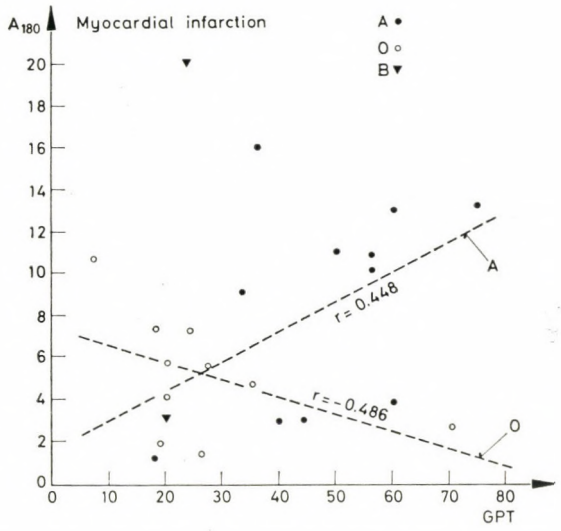

Fig. 2. Apparent viscosity of the artificial white thrombus as a function of GPT level in patients after recent myocardial infarction. Thrombus viscosity is given in deflection units of the scale; GPT level in I.U./litre. Linear regressions given for patients of A and O blood groups. This figure serves as an illustration only and not as a proof of differences between A and O blood groups

It is peculiar that the few statistically significant correlations between GOT and blood or plasma viscosity were all observed in the group with complications and concurrent diseases and none in the patients whose recent myocardial infarction was not accompanied by a complication or some concurrent disease. No definite explanation can be supplied, but the clue might reside in the effect of blood groups.

While a group of patients might show no significant correlation between biochemical and viscosity factors, the same group subdivided according to their ABO blood groups may show such a significance. It is not obvious why one of the major blood groups should display statistically significant correlations while the other major blood group fails to do so; or why a correlation between GPT level and the apparent viscosity of white thrombus should show nearly equal but opposite correlation coefficients for patients of blood group A and O (Fig. 2).

The reality of differences between the molecular rheologies of blood groups A and O has been proven by the significant ($P < 0.01$) difference between the slopes of linear regressions for red cell aggregation as a function of GOT or GPT levels; and by the higher levels of GPT and GOT in the patients of blood group A than of blood group O ($P < 0.02$, and $P < 0.05$, respectively). This result cannot be explained solely by the severity of the infarcts as the percentage of patients with complications was higher in blood group O than in blood group A (see Table 2). Furthermore, blood group A patients showed a higher viscosity of artificial thrombi, higher blood viscosity, and higher aggregation of red cells than patients of blood group O (Table 6).

Bronte-Stewart [15] and Allan and Dawson [16] showed a significant difference between A and O blood groups in the incidence of ischaemic heart disease. It has been shown that antihaemophilic factor (factor VIII) is significantly higher in persons of blood group A [17].

Some significant differences between ABO blood groups have been found in *in vitro* studies of thrombus formation in patients with renal failure [8] and hypertension [10].

No conclusion can yet be reached whether the above results are related to different, if any, pathways of myocardial infarction in patients of different blood groups. Nevertheless, some microrheological differences have been revealed by objective and instrumental methods.

Finally, although there is no evidence that would permit to conclude whether the elevation of blood viscosity factors was not a by-product of the disease rather than its cause, the very fact that there was a significant correlation between the GOT level and blood viscosity, and the GPT level and viscosity and rate of formation, of artificial thrombi, might open a new line of inquiry. The key to significant results might reside in subgrouping of patients according to their ABO blood groups.

*

We wish to thank Professor G. Morgan, Department of Biochemistry, Glasgow Royal Infirmary, for enzyme assays. Support for this study came from the Association of Commonwealth Universities, the National Health and Medical Research Council of Australia, the Bioengineering Unit of the University of Strathclyde, and the University Department of Medicine, Glasgow Royal Infirmary.

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Some Obvious Problems of a Developing Country

G. DISCOMBE

Ahmadu Bello University, Zaria, Nigeria

(Received February 16, 1974)

Zaria is the educational centre of the Northern States of Nigeria. The University, which is the only one in the Northern part of Nigeria, has to serve the needs of some 30,000,000 people. It has incorporated the older College of Arts, Science and Technology, the Institute of Administration, the Institute of Agricultural Research, the Schools of Agriculture and Pharmacy, and the Abdullahi Bayero College at Kano, a centre of Islamic Studies, and has over 4000 undergraduate students, to be raised rapidly to 10,000. There are in Zaria two teacher's training colleges, a Civil Aviation training centre and six secondary schools, as well as craft schools and religious training centres. Nearly all these are outside the old walled town.

The area is in the Northern Guinea Savanna zone, and has a rainfall of between 85 and 100 cm annually, most of which falls between June and September. The land surface is based on a Pre-Cambrian or Lower Palaeozoic metamorphic shield on which layers of alluvial and aeolian deposits laid down and later had eroded by streams, which often cut 6 metres down into the alluvium. There are plentiful stores of water in the lower alluvium and weathered basement so that isolated trees flourish and comparatively shallow wells provide water; early settlements have been on the residual granite outcrops called inselbergs partly for protection, partly because the water table there is nearer the surface; but apart from these it is a gently undulating countryside, dun during the dry, green during the wet season, lightly wooded with many evergreen trees, full of birds and many other animals. There are large areas of lateritic soil, useless for farming but good for road foundation.

Animal husbandry is mainly nomadic, but agriculture, formerly shifting, is now settled, and a variable proportion of farmers also work off the farm. With a population density of about 50 per sq.km and about 30% fallow land it seems possible that the population may soon be pressing against the maximum the area can support. The limiting factor is water, and in one area where the population is dense, the rapidly developed village of Samaru near the University Campus, 5 of 12 public wells are constantly contaminated with *Escherichia coli* (P. A. Bello: Thesis for Diploma in Microbiology, A. B. U., 1971); little is known of well pollution in remote areas. Few rivers flow throughout the year, though in many

dry beds there is a considerable subsurface flow which can be tapped. Most lakes and permanent ponds are contaminated with bilharzia.

Dams are being built everywhere; the largest, Kainji, on the River Niger, some 200 miles away, supplies electric power, water for irrigation and up to 10,000 tons of fish a year; another for electricity generation is being considered on a tributary of the River Benue; but smaller impounding dams for the storage of water during the dry season are being built everywhere – near the University on the Kubanni and Galna rivers to provide 45×10^3 cu.m daily; one on the Kano River for irrigation, and others for fish farms. That bilharzia will spread is obvious; not so obvious is what has to be done when the basins are choked by the sand brought down by the turbulent flows of the rainy season.

The religion of the majority is Islam, which grew steadily in influence since its introduction probably in the 13th century and became the majority religion after the Jihad (Holy War) of Usman dan Fodio in 1804 – 1810. Islam is held firmly, but with tolerance. Until about 30 years ago Koranic schools provided the only education, and even now secular primary education is available for only about 15% of that age group. Various Christian sects number about 12% of the total, but are more common among the immigrants from the South and West.

It is too soon to comment on the scars left by the Civil War, but the above geographical, social and religious factors provide the background to the present attempt to establish higher education suitable for the needs of the Northern States of Nigeria.

Medical education

An unusual arrangement is that the University controls most of the local hospital services. In order to obtain beds for clinical instruction of students the University had to take over two general hospitals, one in Zaria and one in Kaduna, which had previously been run by the Northern Regional Government and are now in theory owned by, and will be returned to, the Interim Common Services Agency for the Northern States. In doing so, it was found necessary to establish schools of nursing, medical laboratory technology, and for various other kinds of medical auxiliaries. In order to separate the problems of providing a service to patients from those of providing academic instruction to medical students, the University established an Institute of Health to deal with the service problems; all members of the Faculty of Medicine are members of the Institute of Health, but the reverse is not true. This rather surprising arrangement seems to work quite well, and the load on the two hospitals is increasing rapidly.

The first 28 students of the Medical School graduated in 1972; in 1973 another 38 graduated, and by 1980 an annual 200 is planned. Great stress is being laid on community medicine, which means such hygienic procedures as are practicable in a largely illiterate rural community, together with the development of various auxiliary personnel to form a team under the direction of the medical

practitioner and in association with the agricultural adviser. Instruction about practice in a small town is given at Malumfashi, about 70 miles away.

However, in some ways this resembles the blind leading the blind, for there are innumerable uncertainties. The population contains about 25% of the sickle cell trait and 5% of glucose-6-phosphate dehydrogenase deficiency, the latter being a much commoner cause of haemolytic disease of the newborn than is Rhesus incompatibility. These are, of course, conditions which are likely to yield only to family planning over many generations and, indeed, may be worth retaining lest malaria ceases to be controlled. The incidence of trace metal deficiency diseases on these heavily leached soils is completely unknown though in plants boron deficiency is common.

Among the preventible, or curable, disorders the first is falciparum malaria, which is holoendemic in the whole region, but with a very high rate (45% or more) of parasitaemia at all ages throughout the year at least in the Sahel zone north of Kano, for no systematic search has been carried out in villages nearer Zaria. This high incidence of malaria contributes significantly to the high frequency of folic acid deficiency and haemolytic megaloblastic anaemia of pregnancy. The problem of reducing the incidence of malaria among the dispersed villages is enormous, but there are signs that a campaign of regular spraying combined with drug treatment at quite long intervals may be effective – but this must wait for the training of health personnel for each village or group of villages.

The second is the control of schistosomiasis. In many villages *Schistosoma mansoni* can be recovered from 15–20% of the inhabitants, and it undoubtedly contributes greatly to the morbidity in that area. Nowhere in the world, except perhaps in China, has effective control been achieved, but there are hopes that in this area, with few constant flowing streams and little continuous irrigation, control may be possible.

The third is the control of *Aspergillus flavus*, a mould which is particularly common on badly stored ground-nuts and which produces the liver poison aflatoxin. Cirrhosis of the liver in Africa is usually macronodular, with multiple nodular hyperplasia, suggesting recovery from multiple focal necrosis (the lesion produced by aflatoxin) rather than the micronodular cirrhosis of Laennec, more common in Europe. This macronodular cirrhosis produces few subjective effects; indeed, it is compatible with good athletic performance, but frequently changes into primary hepatoma, and even if it does not, may suddenly induce hepatic failure. The cause of the cirrhosis is uncertain, but it seems likely that aflatoxin is an important factor, and if this is so, the incidence of the disease will be reduced only by improved methods of harvesting and storing ground-nuts, and, for that matter, all kinds of grain and pulse, for *A. flavus* is an ubiquitous fungus which grows rapidly on damp nuts, grain or pulse. Again, this is a problem for the agricultural adviser rather than for the medical practitioner. Fortunately, onchocerciasis is not a major problem of the Nigerian savanna, and that other feared disease, leprosy, is now being brought under control.

However, precise figures on the incidence of disease are difficult to obtain because of the almost universal ban on examination of the body after death. Without autopsy, knowledge of the lethal conditions of the population is very difficult to obtain. With care, moderately precise information on the incidence of meningococcal meningitis, which appears in epidemic form; and on poliomyelitis, which is not rare, can be obtained; but for disease of the liver, kidney and heart we shall have to wait until laboratory facilities spread from the laboratories of the larger cities. But this is a slow business which has to depend on the training of skilled technicians, who are as yet few. Even though the Institute of Health has established a school which hopes to train 24 high-grade and as many middle-grade laboratory technicians each year, it will be a decade or more before each city has a laboratory which can stand comparison with those of Europe. Fortunately the need has been recognized, the money has been made available, and the students are coming forward – and there is nothing wrong with the ability of the students. With teams of health personnel in the villages, and medical and scientific workers heading the teams, they will transform the picture of tropical Africa. Meanwhile the University Hospital has to aim at a more complete diagnostic cover during life, even if it requires very sophisticated apparatus.

After working here a year, I feel privileged to have an opportunity of helping to lay foundations on which Nigerians, trained in Nigeria for Nigerian needs, will build.

*

I am greatly indebted to Professor Umaru Shehu, Director of the Institute of Health, Ahmadu University, for valuable suggestions and for reading the manuscript.

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Don de Sang — don Sacré

R. ARNAUD

Centre Régionale de Transfusion Sanguine, 37000 Tours, France

Reçu le 11 Mars 1974

La Transfusion Sanguine est la forme la plus complète de succès thérapeutique grâce à des résultats incontestables et souvent spectaculaires aussi bien en réanimation médicale qu'en réanimation chirurgicale.

Ceux qui ont vécu cette courte période où s'est imposée l'utilisation du sang humain et de ses fractions, ont assisté et participé avec foi et enthousiasme: ils ont eu la joie profonde de la satisfaction de l'efficacité de leur action.

La Transfusion a été de succès en succès:

— l'hémorragie cataclysmique est compensée,
— le déficit de l'hémophile n'est plus un problème,
— l'aventure Rhésus a été un émerveillement: de Landsteiner à la prémunition, cette catastrophe est maintenant condamnée,
— hier, la découverte de l'Antigène Australie a été l'éveil de recherches passionnantes.

Mais ce qui ne doit jamais être oublié, c'est l'homme généreux qui a permis et qui permet tous les jours ces résultats. Aucun d'entre nous, même après 25 ans, n'est encore blasé de ce geste exceptionnel qu'est le Don du Sang.

— 3 500 000 dons ont été faits en France en 1973,
— des millions d'hommes de toute race, de toute couleur, de toute religion ont donné, en cette même année, de par le monde.

Comment ne pas être frappé par cette croisade, par cette foule participant dans l'univers entier à cette lutte contre la mort?

On ne peut s'empêcher d'attacher à ce don du sang, une sorte de marque sacrée. De tout âge, le sang a été l'un des éléments du sacrifice en offrande aux Dieux: le sang purificateur, le sang de l'ennemi vaincu, le sang de l'être qui donnera la force et surtout la vie.

Tous les mythes anciens se retrouvent, qu'il s'agisse des Indiens de l'Amérique ou des peuplades anciennes de l'Europe, de l'Afrique ou de l'Asie. Le sang représente la vie d'aujourd'hui et de l'au-delà. Notre religion chrétienne, dans ses rites les plus sacrés, célèbre le sang du rachat, le sang de la vie éternelle, le sang de l'alliance nouvelle.

Or, en transfusion, l'appel se fait aussi pour la vie; nous utilisons sensiblement les mêmes mots: "donnez votre sang et vous sauverez une vie humaine". C'est le leitmotiv de base de tous nos appels répétés par tout le monde sous des formes plus ou moins différentes.

La Ligue des Sociétés de la Croix Rouge s'est réunie le 8 mai 1974 à Genève pour une journée mondiale consacrée au Don du Sang et le slogan a été encore repris: "Sang donné, Vie sauvée."

Ce slogan est de matière à être compris de tous les publics: il est vrai et il n'y a rien à ajouter, la lutte est pour la vie.

Toutefois, si le côté sacré du sang n'est qu'une image, tout de même le don du sang relève d'idéaux supérieurs: charité, abnégation, solidarité, fraternité humaine, altruisme, don de soi, devoir civique. Tous ces mots traduisent les sentiments élevés, nobles de l'homme qui accompagnent le don. On ne croit plus à l'égoisme de cet homme mais à sa générosité quand on a assisté à ces gestes collectifs du don du sang pour "sauver" un malade ou à des gestes anonymes de l'isolé qui vient donner et qui effectue ce geste gratuitement, pour rien. En effet, une des plus belles acquisitions de la transfusion a été le bénévolat.

L'idéal qui s'est créé autour de la Transfusion ne pouvait être compris sans le bénévolat, et puisque bénévolat, il en découle la gratuité du sang. Gratuité, bénévolat, le don du sang prenait toute sa valeur.

Dès 1936, le conseil des gouverneurs de la Ligue de la Croix Rouge avait compris que la Transfusion Sanguine était parfaitement une mission dans l'esprit de ses activités.

En 1948 à Stockholm, la XVII^{e} conférence internationale de la Croix Rouge précisant ses objectifs dans le développement de la Transfusion, établissait les grandes règles directrices et en particulier le bénévolat, souhaitant que "le principe de la gratuité du sang donné et reçu soit universellement appliqué".

La Croix Rouge a fait œuvre de pionnier. Les premiers centres ont été créés en 1925 par les sociétés de Croix Rouge en Angleterre, en Hollande et en Australie.

En France, Tzanck créait en 1923 l'œuvre de la Transfusion. En 1943, sous l'impulsion du Médecin Général Julliard au Maroc, du Professeur Benhamou à Alger, apparaissent les premières véritables structures transfusionnelles françaises, mais la marque spécifique de la Transfusion en France se précisa dans cette magnifique loi du 21 juillet 1952 établie par le Docteur Aujaleu, Directeur Général du Ministère de la Santé.

Cette loi, depuis 25 ans, a marqué brillamment toute l'organisation transfusionnelle française. Elle a servi de modèle à de nombreux autres pays.

Cette loi établit des centres de Transfusion nationaux et la politique de ces centres est basée sur le don du sang *anonyme*, *inconditionnel*, *volontaire* et *bénévole*.

C'était donner à la Transfusion son caractère propre; c'était faire du sang un produit noble et non une marchandise. Le donneur de sang bénévole et anonyme accomplit un geste désintéressé, efficace, dont il peut être fier. D'autre part il fait confiance à ceux qui lui demandent, et cette confiance qu'il fait, établit entre lui et le Centre un climat d'amitié, de respect vis-à-vis l'un de l'autre: le donneur

est sûr qu'il participe, ce n'est pas pour lui un devoir, c'est une foi et un enthousiasme qu'il partage avec ceux de la Transfusion.

Le don est absolument gratuit, le donneur sait que personne n'osera tirer un bénéfice de ce qu'il a donné. "Devant une vie à sauver, le prix du sang est de ne pas en avoir", a dit le Docteur Tzanck, le grand précurseur, et l'expérience montre que tous les pays où le don de sang est bénévole, collectent plus de sang par rapport à la population, que les pays où le don est rémunéré.

Le bénévolat est maintenant en vigueur dans la plus grande majorité des pays: 100% dans l'Europe de l'Ouest, 40 à 50% dans l'Europe de l'Est, dans les autres continents le pourcentage est plus variable, mais en Australie et au Canada le bénévolat est généralisé. Progressivement de nombreux pays et de grandes organisations comprennent et évoluent, dans leur politique, pour se rallier à cette œuvre magnifique.

Il est, en effet, pour le moins désagréable de parler d'achat du sang humain. Ce commerce soulève une certaine réprobation et fausse l'idéal de la transfusion: c'est un peu les trente deniers de Judas, pour le prix du sang du Christ, que les siècles ont condamnés.

Le légiste réprouve également: "l'homme ne peut rien vendre de ce qui compose essentiellement son corps, pas même le sang" a écrit le Professeur Savatier, Professeur de Droit à la Faculté de Poitiers.

L'existence d'une rétribution conduit bien souvent à l'exploitation des classes les plus pauvres, surtout en Inde et en Asie, exploitation d'autant plus scandaleuse que les dons sont fréquents et mettent en danger la vie du donneur. "Quel voyage que celui des hippies drogués allant à Katmandou", décrit dans le roman, et qui n'est pas qu'un roman, "Flash ou le Grand Voyage" de Charles Duchaussois. Tous les gars du voyage savent les jalons où l'on peut vendre son sang. C'est au Kouweït que le sang se vend le plus cher. On le paie jusqu'à 28 ou 30 dollars le flacon! Quel scandale aussi que ces trafiquants d'esclaves à Haïti! Les journaux du monde entier les ont accusé et flétri.

Certes, tous les dons rémunérés n'ont pas ce caractère affreux et ils ne sont souvent que l'indemnisation de frais, et un simple encouragement. Mais ces dons n'ont tout de même pas l'élégance du geste gratuit.

"Le point de départ de la 'Chaîne' du sang ne peut être qu'un don" a également écrit le Professeur Savatier.

Malgré les difficultés rencontrées, aussi bien les obstacles psychologiques que matériels, il est du devoir des Centres de Transfusion de conserver au don du sang son caractère bénévole et inconditionnel.

Les Centres de Transfusion doivent expliquer et instruire. Ils doivent légitimer leur action, vis-à-vis du donneur, par les résultats obtenus. Ils doivent gagner la confiance du donneur, et pas un homme ne refusera le sang de la vie de l'autre s'il sait ce qu'est la transfusion sanguine.

Le Professeur Jacques Ruffie, professeur du Collège de France, dans une allocution s'adressant aux donneurs à l'occasion du 25e anniversaire de la Fédération Française des Donneurs de Sang, a parfaitement exprimé ce qu'est

la transfusion sanguine et ce qu'elle doit continuer à être: "Au cours de ces 25 dernières années, nous venons de vivre ensemble une aventure exaltante qui restera sans doute pour l'humanité l'un des épisodes les plus étonnants de sa longue histoire, car la Transfusion constitue non seulement un acquis fondamental de la biologie et de la médecine moderne, mais aussi la première vague de solidarité consciente qui doit lier les hommes à l'échelle planétaire."

La Transfusion Sanguine constitue l'un des grands évènements sociologiques du monde contemporain.

Correspondance: Prof. R. Arnaud, Centre Régionale de Transfusion Sanguine, 40, rue Jules Simon, 37000 Tours, France

News Items

International Symposium on Acute Leukaemia

The Hungarian Study Group for Leukaemia will organize under the auspices of the Medical Section of the Hungarian Academy of Sciences, the Hungarian Paediatric Association, the Hungarian Society of Haematology and the Hungarian Cancer Society, a Symposium on the

Treatment of Acute Leukaemias

to be held in Budapest, on the 10th-11th of April, 1975.

Outstanding specialists of the topic have been invited to give lectures, and the active participation of scientists interested in the problem is welcome.

The registration fee is $ 50.–

Address: Hungarian Peadiatric Association
Bókay J. u. 53.
H-1083 Budapest, Hungary

International Society on Thrombosis and Haemostasis

The Vth Congress of the International Society on Thrombosis and Haemostasis will be held in Paris, July 21–26, 1975, at the Faculté de Droit, 92 rue d'Assas.

President: Prof. J. P. SOULIER

Vice-President, Secretary General: Prof. J. P. CAEN

The main topics of the plenary sessions are as follows: platelet-vessel wall interaction, blood coagulation, thrombosis.

Simultaneous sessions and symposia are scheduled every day in the morning and afternoon.

The participants wishing to present a paper are invited to send us abstracts on the proposed topics: platelet-vessel wall, coagulation, thrombosis (clinical and

therapeutical aspects), fibrinolysis, immunohaemostasis, artificial surfaces, experimental thrombosis.

Unpublished works will be presented, whether in simultaneous sessions or as Posters. Speaking time in simultaneous sessions will be limited to ten minutes. A discussion of one hour with the authors of Posters will be held every afternoon. The Posters will be changed every day.

The abstracts should not exceed 200 words. Deadline for the registration of papers and for submission of abstracts is March 1, 1975. English will be the official language of the Congress.

All scientific correspondence regarding the Congress should be sent to:

Prof. J. P. CAEN
Service Central d'Hématologie
Hôpital Lariboisière
2, rue Ambroise Paré
75475 Paris Cedex 10

All administrative correspondence should be sent to:

CONGRESS-SERVICES
1 rue Jules-Lefèbvre
75009 Paris

5th Jean Julliard Prize

The 5th Jean Julliard Prize, which was established by the International Society of Blood Transfusion in memory of its first Secretary General, will be awarded during the XIVth International Congress of Blood Transfusion to be held in Helsinki (Finland) from July 27 to August 1, 1975.

The prize is reserved for investigators under 40 years of age in recognition of recently completed scientific work related to blood transfusion.

In order to qualify, candidates must forward five copies of an unpublished manuscript or a recently published paper to the Secretary General, Dr. F. Josso, 6, rue Alexandre Cabanel, 75739 Paris Cedex 15, France, *before the 1st of April 1975.*

The Jury for selecting the recipient of the Juillard Prize was designated during the last meeting of the Executive Committee of the International Society of Blood Transfusion in Amsterdam on August 25th 1974 and consists of the following members:

Dr. F. Kissmeyer-Nielsen (Denmark)
Dr. B. P. L. Moore (Canada)
Dr. H. R. Nevanlinna (Finland)
Dr. S. Seidl (West-Germany)

The value of the prize is 3000 Swiss francs.

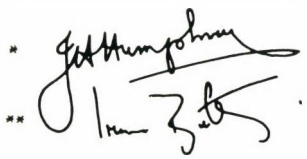
International Reference Preparation for Human Serum IgG, IgA, IgM

International Reference Preparations for serum IgG, IgA and IgM have been available for the past few years; their contents are indicated in International Units (I. U.). Estimates of the weight of the immunoglobulin corresponding to one I. U. have shown considerable variation when carried out in different laboratories. This is why it has previously been recommended that serum immunoglobulin concentrations estimated by comparison with the International Reference Preparation be expressed in International Units per ml (Rowe, Grab and Anderson, 1970). However, International Units have not gained general acceptance for clinical use. Therefore, we now recommend that human serum immunoglobulin concentrations continue to be estimated relative to the International Reference Preparation but that the values may be calculated in terms of weight based on the best available estimates (Rowe, Grab and Anderson, 1972).

On this basis, the following are the weights of immunoglobulins corresponding to one I. U., and the concentrations of immunoglobulins in a solution prepared by dissolving the contents of one ampoule of the International Reference Preparation by the addition of one ml of distilled water.

	μg/I. U.	mg/ml
IgG	80.4	7.58
IgA	14.2	1.34
IgM	8.47	0.80

Publications giving values for concentrations of serum IgG, IgA and IgM should state that these were obtained by comparison with the International Reference Preparation.



Rowe, D. S., Grab, B., Anderson, S. G. Bull. Wld. Hlth. Org. *42*, 535 (1970).
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* Chairman of the Joint Meeting of the World Health Organization and the International Union of Immunological Societies. Meeting of Experts for the programme on the Standardisation of Immunological Reagents held in Geneva, November 5–8, 1973.

** Secretary to the above.

Acknowledgement to Reviewers

The Editors of Haematologia gratefully acknowledge the valuable help of many referees who have reviewed the papers submitted for publication in Haematologia during the past years.

Author Index

Subject Index

VINBLASTINE — Richter

Vials

Composition: Vincaleukoblastine sulfate,

Lyophilized	5 mg	in vials
Normal saline	5 ml	in solvent ampoules.

Action: Vincaleukoblastine is an alkaloid of Vinca rosea. By the inhibition of cell division it exerts a cytostatic action directed mainly to the leukocytes and tumour cells. Applied in therapeutic doses, it does not influence significantly red blood cell and platelet count, and the haemoglobin content in erythrocytes.

Indications: Generalized forms oly lymphogranulomatosis (Hodgkin's disease), lympho- and reticulosarcoma, chronic myelosis, inoperable tumours resistant to other chemotherapeutics or irradiation.

Side effects: Agranulocytosis, leukopenia, alopecia, anorexia, nausea, vomiting, subicterus, diarrhoea, albuminuria, stomatitis, urticaria, paraesthesia, headaches, epistaxis, depression. On discontinuing temporarily the treatment, all these side effects disappear. Generally, no permanent damage is caused.

Contraindications: Leukopenia, bacterial infections.

Application and Dosage: The water of the solvent ampoule is transferred into the powder ampoule, and the freshly prepared solution is injected at a slow rate intravenously or administered as intravenous drop infusion (250 to 500 ml). The dose must be adjusted individually with due consideration to the patient's need and the change in the leukocyte count. For the dosage of VINBLASTINE the following schedule is presented: After a starting dose of 0.025 to 0.1 mg/kg of bodyweight, the leukocyte count is daily checked. If this count does not drop below 3000 after the initial dose, a second dose of 0.15 mg/kg can be administered one week after the first injection, provided the leukocyte count is not less than 4000. In absence of oncolytic or leukopenic effects, the single dose can be increased in the further course of treatment to 0.2 mg/kg. A seven-day interval is to be kept between the single injections, and the leukocyte count has to be carefully checked. When a favourable effect has been achieved, dosage can be reduced to a maintenance level of 0.15 mg/kg every seven to fourteen days under continuous checking of the leukocyte count until symptoms improve. The daily checking of the leukocyte count is recommended. If leukopenia occurs (leukocyte count below 3000), the administration should be discontinued and it is advisable to give prophylactically antibiotics.

Packing: 25 vials of 5 mg.
25 solvent ampoules of 5 ml.

CHEMICAL WORKS OF GEDEON RICHTER LTD.,
Budapest – HUNGARY

VINCRISTIN

AMPOULES

Each ampoule (I) contains vincristinum sulphuricum 0.5 mg. Each solvent ampoule (II) contains sterile isotonic saline. Vincristin is an alkaloid of plant origin with powerful cytostatic action causing an arrest of mitosis at the stage of metaphase.

Indications: Acute leukaemia of childhood and adult age. Lymphogranulomatosis. Acute blast crisis in chronic myeloid leukaemia. Lymphosarcoma, Ewing's sarcoma. Wilms' tumour in the preoperative stage and in the case of metastases.
Dosage: The dose should be established with due regards to the patient's body-weight and individual tolerance.

For the treatment of acute leukaemia *adults* may be given 0.05 mg — 0.10 mg per kg body-weight weekly.

With *children* it is preferable to administer 1.5 mg per square meter of body surface weekly. As maintenance therapy 1 mg per square meter both in acute leukaemia and other forms of malignancy.

CHEMICAL WORKS OF GEDEON RICHTER Ltd.,
Budapest — HUNGARY

DEPERSOLON

AMPOULES

Composition: 21-(Desoxy-N-methyl-N'-piperazinyl)-prednisolone hydrochloride 30 mg in ampoules (1 ml).

Action: 21-(Desoxy-N-methyl-N'-piperazinyl)-prednisolone hydrochloride is a water-soluble derivative of prednisolone having the same activity as the parent compound. Duetto the water-solubility of the active principle and stability of the solution DEPERSOLON could be presented in a stable solution ready for immediate parenteral use.

Indication: DEPERSOLON is expecially indicated in emergencies and other cases where an immediate high blood level of prednisolone is required.

M e d i c a l: Allergic emergencies, attacks of asthma, status asthmaticus, Addison crisis shock due to myocardial infarction or hepatic coma. In severe systemic infections with concomitant specific antibiotic therapy.

S u r g i c a l: Shock, particularly postoperative shock, shock following burns, emergency surgery, traumatic injuries.

Contraindications: Infections where specific antibiotic theraphy is impracticable, peptic ulcer, osteoporosis, tendency to psychosis.

Dosage: DEPERSOLON ampoules may be injected intravenously or given by intravenous drip. Dosage depends on the individual case. The average single dose is 15 to 30 mg (1/2 to 1 ampuole), which can be repeated according to need.

Packing: 3 or 50 ampoules of 30 mg (1 ml).

CHEMICAL WORKS OF GEDEON RICHTER Ltd.,
Budapest — HUNGARY

LYCURIM

AMPOULES

Each dry ampoule contains 30 mg freeze-dried 1,4-di-(2-methylsulphonyloxyaethyl-amino)-1,4-didesoxy-erythrite-dimethylsuphonate.

Each solvent ampoule contains 5 ml 0.9 per cent saline.

EFFECT

LYCURIM is an alkylizing, sugaralcohol-type cytostatic whose haematopoietic effect is characterized by low doses acting exclusively on the lymphoid system; myelo- and thrombogenesis are damaged only by massive doses.

In inoperable solid tumours significant clinical remission is brought about. In processes associated with pleural or peritoneal metastases optimum effect is exerted by intracavital use. Long-term administration leads to immune suppression. LYCURIM acts on the cellular immune system, chiefly in the inductive phase of antigen production.

INDICATIONS

Malignant lymphoblastoses: lymphoid leukaemia, lymphogranulomatosis (Hodgkin's disease), reticulo-lymphosarcoma.

Solid tumours: Mammary and lung tumours, furthermore tumours which develop intracavital metastases.

Immunologic diseases: Autoimmune myositis (polymyositis, dermatomyositis, myositis associated with SLE), myasthenia gravis, recurrent endogenous uveitis.

USE AND DOSAGE

The content of the dry ampoule is dissolved in the solvent. The route and dosage are determined by the disease.

In malignant lymphoblastoses 30—60 mg should be administered daily or every other day in one dose by slow intravenous injection, for ten to twenty days.

The upper limit of the total dose depends on the response elicited and on tolerability of the drug. If necessary, 600—1200 mg may be administered in a course of treatment without side-effects or toxic manifestations.

For the treatment of pleural and peritoneal oedema in solid tumour centesis should be followed by daily intravenous doses of 30—60 mg for ten to twenty days. Intracavitally (intrapleurally, intraperitoneally) 30—60 mg combined with puncture.

In immunologic diseases, 30—60 mg should be injected daily intravenously for ten to twenty days as immune suppressive treatment.

Children should be given intravenous doses of 30 mg per m^2 body surface daily or every other day. The total dose per m^2 body surface is 360 mg.

SIDE-EFFECTS

Temporary thrombopenia, leukopenia are normalized by steroid treatment or discontinuance of the drug. Gastrointestinal discomfort is rare (nausea, vomiting).

PACKING

25 dry ampoules (1 ml) 30 mg +
25 ampoules (5 ml) solvent.

CHEMICAL WORKS OF GEDEON RICHTER, Ltd.,
Budapest HUNGARY

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

GORDOX

AMPOULES
100,000 U.

Each ampoule (10 ml.) contains
100,000 U. kallikrein-inactivator.

Pathologically activated trypsin, fibrinolysin, plasmin, chymotrypsin, and kallikrein are inactivated by Gordox which also neutralizes pathological enzyme activity of the pancreas.

INDICATIONS

Severe conditions of shock.
Pencreatitis, pre- and postoperative medication of the pancreas.
Postoperative haemorrhage, hyperfibrinolytic bleedings, pulmonary embolism, prophylaxis of unsatisfactory wound healing.
At the present state of knowledge there is no contraindication to the use of Gordox.

Packing

GORDOX is presented in packs of 25 ampoules of 10 ml.

CHEMICAL WORKS OF GEDEON RICHTER, Ltd.,
Budapest — HUNGARY

Card Indexes

Jean Bernard

Very long complete remissions in acute leukaemia. Haematologia *8*, 15 (1974).

A report of 216 own cases of acute leukaemia, all showing remissions of more than 4 years is presented. The influence of age, sex, cellular variety, the beginning of treatment and its duration are discussed.

O. M. Garson, G. C. de Gruchy

Chromosome changes in the blastic transformation stage of chronic granulocytic leukaemia, Haematologia *8*, 21 (1974).

A study of bone marrow chromosomes of patients in the blastic transformation stage of chronic granulocytic leukaemia is reported, with particular reference to the appearance of chromosome abnormalities additional to the Philadelphia chromosome (Ph^1). Thirty patients were studied, of whom 28 yielded satisfactory chromosome preparations. In all 28 patients, chromosome abnormalities in addition to the Ph^1 were demonstrated, the most common being an increase in the number of chromosomes, usually due to either an extra C or extra Ph^1. It is considered that the appearance of additional abnormalities is a reliable indication of transformation. Clinical and haematological remission was induced in 11 patients and was associated with the disappearance of the additional chromosome abnormalities.

F. Fey, A. Niezabitowski, A. Graffi

Virus cell change relationships in viral leukaemogenesis. Haematologia *8*, 29 (1974).

The previously observed *in vivo* cytotropism of Gr.-leukaemia virus to specific target cells has been demonstrated *in vitro*. Spleen colonies of spleen or thymus cells were cultivated *in vitro* and incubated with virus from myeloid or lymphoid Gr.-leukaemia. The culture media were administered to newborn syngeneic mice. The culture media of the combination of spleen cells + virus from myeloid leukaemia, and thymus cells + virus from lymphoid leukaemia were found to induce leukaemia with high frequency, whereas with the crossed combinations the incidence of leukaemia was low.

E. Deutsch, P. Höcker, E. Pittermann, A. Stacher, H. Rainer, K. Moser

Recent biochemical findings in leukaemia. Haematologia *8*, 35 (1974).

A DNA-polymerase with the template characteristics of a reverse transcriptase has been demonstrated in leukaemia cells. It can use the information from a heteropolymeric natural RNA and transform it into a DNA sequence. It could be separated from the DNA-dependent DNA-polymerase of the leukaemia cells. The reverse transcriptases in myeloid and lymphoid leukaemia differ in molecular sizes. They are inhibited by rifamycin derivatives.

H. Theml, H. Begemann, R. Issels, W. Kaboth, P. Schick, M. Winnewisser

Influence of different kinds of therapy on T-cell activity in chronic lymphadenoses. Haematologia *8*, 43 (1974).

The changes of T-cell activity in PHA culture of 16 patients before, during and after 21 therapeutic series of extracorporeal irradiation of blood (ECIB) were significantly advantageous in cases of slow progress and without definite organomegaly. After spleen irradiation and after cystostatic therapy the PHA reaction was enhanced in all cases. During the therapeutic series, PHA depression caused by ECIB, cytostasis and ACTH was considerably enhanced; the weakest effect was recorded from spleen irradiation. The positive effect on T-cell activity is ascribed to the significant improvement of circulation and the regeneration of the T-cell fraction. The simultaneous follow-up of CLL kinetics allowed some therapeutical conclusions. Depletion therapy (ECIB or leucophoresis) should be applied in slow accumulative cases, while spleen irradiation is indicated in the presence of splenomegaly, and cytostatics should be applied when clinical signs of rapid cell proliferation manifest themselves [47, 48]. Alkylating agents and ACTH administered for compensation should be applied in the frame of an effective short therapy to stimulate cellular immunity. The immune defect of CLL should not be enhanced by prolonged therapy.

E. de la Torre, P. Hernandez, G. Martinez, E. Svarch, B. Colombo

Beta-thalassaemia in Cuba. Haematologia *8*, 71 (1974).

The β-thalassaemia trait was found in 7 out of 961 secondary school students of Havana. β-thalassaemia heterozygotes were also found among patients admitted to the haematological departments of two hospitals. Haematological and clinical data of these subjects, as well as of cases of thalassaemia major and sickle-cell thalassaemia treated in the hospitals are reported. The findings are discussed and compared with data from other countries.

Z. Vodražka, Z. Hrkal

Conformation of human globin. The significance of haem for the structure of the haemoglobin molecule. Haematologia *8*, 81 (1974).

By comparing the properties of human globin with the atomic model of haemoglobin constructed by Perutz, conformation of the globin molecule is suggested and the importance of haem groups for the structure and stability of haemoglobin tetramer is shown.

D. Labie, H. Wajcman

Functional disorders of some haemoglobins mutated in the haem pocket. Haematologia *8*, 87 (1974).

Ten different abnormal heamoglobins mutated in the haem pocket have been studied systematically and the results have been compared with data in the literature. A clear structural identification has been performed together with functional studies. A stereochemical classification is tentatively proposed, based on the three-dimensional position of the abnormality.

M. Cohen-Solal, Y. Blouquit, J. Rosa

Use of the sequenator for the study of abnormal haemoglobins. Application to haemoglobins Saint Louis [β28 (B10) Leu → Gln] and Lyon [β17—18 (A14—15) Lys → Val deleted]. Haematologia *8*, 97 (1974).

During the description of haemoglobin Saint Louis (unstable, and always oxidized haemoglobin) and of haemoglobin Lyon (with deletion of two amino acids), classical methods such as fingerprints and ion-exchange chromatography failed to give fully satisfactory results. Attempts to use automatic Edman degradation with a sequenator have solved the problems. The importance and interest of this technique in such cases is discussed.

G. Amiconi, D. L. Currell, L. Zolla, C. Ioppolo, G. Maffei, E. Antonini

Physiological activity of fresh and preserved erythrocytes. Haematologia 8, 111 (1974).

The rate of reduction of ferrihaemoglobin within the erythrocytes and the oxygen affinity of red cells have been measured as a function of storage time of the blood; moreover the effect on oxygen binding properties of suspending fresh erythrocytes in different isotonic media has been reported.

1. The efficiency of the ferrihaemoglobin reducing systems decreases with time of storage as measured in the presence of glucose or lactate as substrate.
2. The well-known increase in oxygen affinity of stored blood is observed when it is determined in Tris-HCl but not in phosphate buffer; this is explained as being due to the replacement of the originally haemoglobin bound 2,3-diphosphoglycerate by phosphate ions entering the erythrocytes.
3. The oxygen affinity of fresh erythrocytes does not change significantly in the presence of different passively penetrating ions, whereas the addition of a non-diffusible polyanion, such as citrate, to the surrounding medium affects the pH; this phenomenon is only in part due to the classical Bohr effect.

S. K. Srivastava, E. Beutler

Formation and cleavage of mixed disulfide of hemoglobin-glutathione in intact erythrocytes. Haematologia *8*, 121 (1974).

A marked decrease in the level of GSH was observed in human erythrocytes incubated with glucose in the presence of acetyl phenylhydrazine. All the GSH lost could not be accounted for as GSSG. After washing and reincubation of the erythrocytes with phosphate-saline containing glucose, almost 80% of the GSH could be recovered. The rate of appearance of GSH was much faster when inosine and adenine were also incorporated in the incubation medium. The results of these studies, taken together with our previous studies of GSH-Hb mixed disulfide, suggest that in the presence of acetyl phenylhydrazine, a portion of GSH oxidized becomes bound to haemoglobin or other proteins in the form of mixed disulfide. GSH from the mixed disulfide is released in the presence of NADPH and glutathione reducatse.

S. Rapoport, M. Müller, W. Siems, M. Grieger

Protective effect of formate on GSH concentration and Heinz body formation: a preliminary model study. Haematologia *8*, 127 (1974).

The effects of H_2O_2 and of formate on the concentration of GSH and on the formation of Heinz bodies were studied in 1. glucose-free human erythrocytes; 2. sheep blood; and 3. blood of patients with a G6PD defect. In glucose-free normal human red cells, formate protected the erythrocytes both from the H_2O_2-induced decrease of GSH and of Heinz body formation. There was a definite effect on osmotic resistance. Similar results were obtained with sheep blood which is characterized by extremely low G6PD and hexokinase activities. In preliminary studies on patients with G6PD defect there was an indication of less Heinz body formation. The mechanism of the beneficial effect of formate is unclarified.

H. Arnold, K. G. Blume, G. W. Löhr

Genetic abnormalities in glutathione metabolism as a cause of drug-induced haemolytic anaemia. Haematologia *8*, 135 (1974).

In some individuals, drugs may induce haemolytic anaemia. The sensitivity to drug- induced haemolysis is caused mostly by a disorder in the glutathione metabolism of the erythrocyte. These disorders are inherited and linked to the deficiency of six enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase, glutathione synthetase, gamma-glutamylcysteine synthetase and glutathione peroxidase. The drug-induced haemolytic anaemias are impressive models of the so-called pharmacogenetic diseases.

I. Szász, B. Sarkadi, G. Gárdos

Erythrocyte parameters during induced Ca^{2+}-dependent rapid K^+-efflux: optimum conditions for kinetic analysis. Haematologia 8, 143 (1974).

Increased Ca^{2+}-dependent K^+-permeability of human erythrocytes is accompanied by changes in shape such as echinocytosis or stomatocytosis, a reduction in volume, adhesiveness, sedimentation and intracellular ion activity and an increase in intracellular haemoglobin concentration and osmotic resistance. These changes depend on the applied metabolic inhibitors and drugs, ionic composition of the medium, and incubation times. Standard conditions and procedures have been suggested suitable for kinetic analysis of the selectively enhanced K^+-transport.

I. Bernát, I. Cornides

Life span of homogeneous red cell population formed after thermal injury. Haematologia 8, 153 (1974).

1. The elimination curve of red blood cells (RBC) labelled with ^{15}N-glycine was examined. A 120—123-day mean life span was found for the RBC of healthy individuals, in good agreement with data in the literature. Most of the RBC died between the 104th and 140th day; elimination started approximately on the 70th day and was complete at 150 days.

2. Mean life span of RBC formed on the 8th day after thermal injury is 102—103 days. In this respect the individual cells differed significantly. Most of them were destroyed between the 75th and 152nd day, elimination started on the 40th day.

3. Mean life span of RBC formed on the 30th and 31st day after thermal injury amounted to 111 days. Their elimination curve was similar to the curve in the earlier stage of the illness.

4. The life span of RBC is only slightly shortened subsequent to thermal injury. This factor, as compared to the severe disorder of haemoglobin synthesis, plays a minor role in the pathogenesis of thermal anaemia.

G. A. Alexeev

Permanent intravasal haemolysis (PIH-syndrome) in hypoplastic conditions of haemopoiesis. Haematologia 8, 163 (1974).

The appearance against the background of depression of bone marrow haemopoiesis of a syndrome of permanent intravasal haemolysis (haemoglobinaemia, haemosiderinuria with positive acid and saccharose test) is regarded as a second phase of a "biphasic" disease of the Strübing—Marchiafava type. Development of PIH-syndrome up to paroxysms of nocturnal haemoglobinuria was caused by the appearance of an anomalous population of PIH-erythrocytes producing complement-sensitive erythrocytes subject to intravascular haemolysis. The morphological expression of this process is a dynamic transformation of the hypoplastic bone marrow into a partially hyperplastic one with a prevalence of cells of the erythroid series imitating the picture of erythromyelosis. The characteristic peculiarity of the PIH-syndrome is the presence in the bone marrow of "depletion zones" with fat reduction. Development against the background of a prolonged permanent intravasal haemolysis with considerable splenomegaly on account of a hyperplasia of reticular elements and myeloid metaplasia of the spleen is regarded as the third, terminal phase of Strübing—Marchiafava's disease with a potential transformation into the systemic myeloproliferative syndrome.

I. Barta

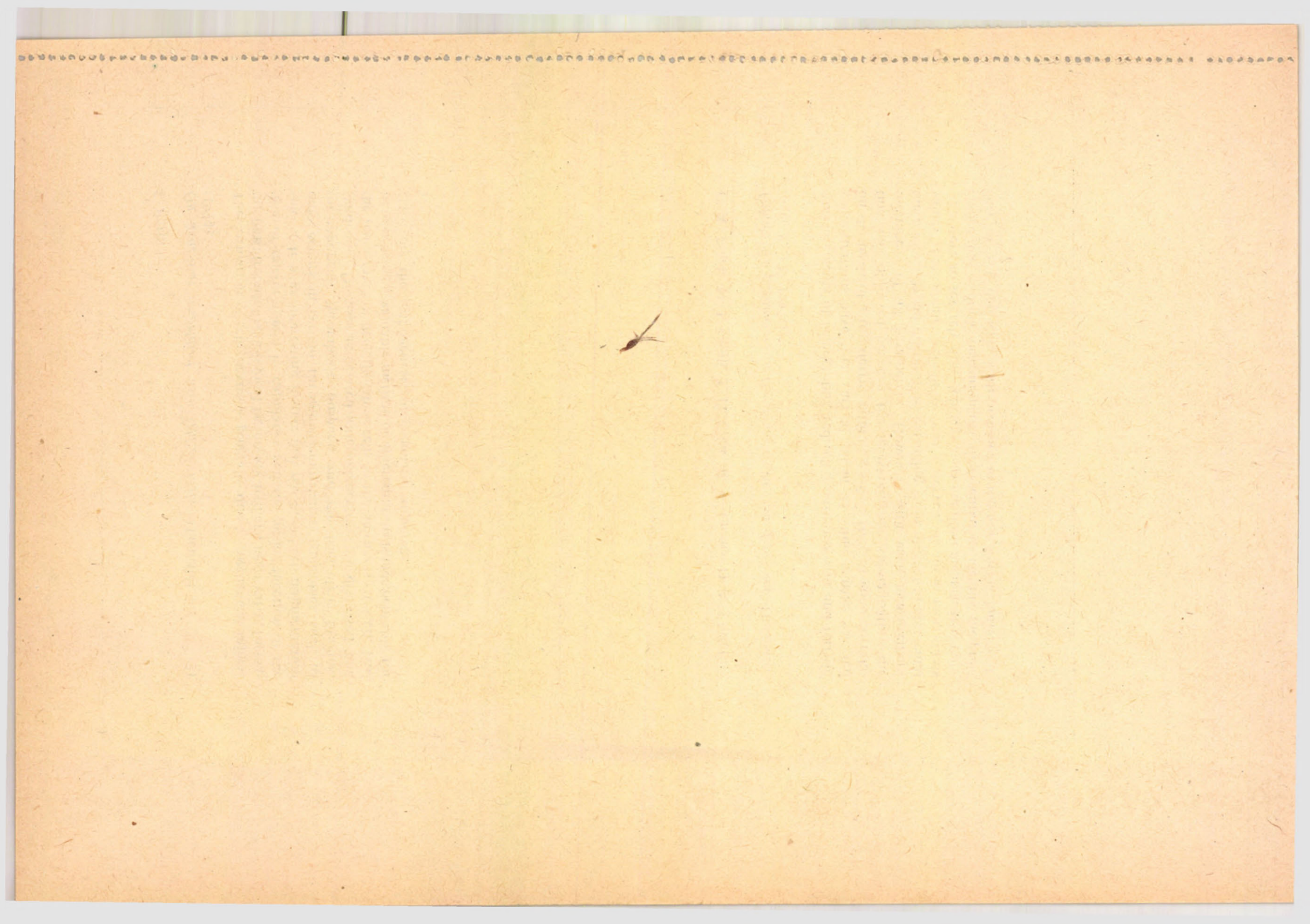
On anaemias associated with schizocytosis. Haematologia *8*, 171 (1974).

The formation of schizocytes is generally due to mechanical injury, the most frequent aetiological factors are artificial heart valve, formation of microthrombi in capillaries and the pitting function of the spleen. It is an interesting feature that red blood cell fragmentation only occurs in certain thrombohaemorrhagic syndromes such as thrombopenic thrombotic purpuras and the haemolytic uraemia syndrome. Diseases associated with schizocytosis are discussed and the importance of detecting these cells in the smears is stressed. This besides being a valuable method of differentation is indispensable for clarifying the pathogenesis of enigmatic anaemias.

C. A. Finch, N. J. Smith, J. D. Cook, R. F. Labbe, D. A. Lipschitz

Laboratory parameters in the diagnosis of iron deficiency. Haematologia *8*, 177 (1974).

Measurements of hemoglobin, red cell protoporphyrin and ferritin concentrations have been carried out in 95 adult females selected on the basis of a transferrin saturation of $<16\%$. A good correlation was observed between the measurements, substantiating the usefulness of all parameters. However, with each measurement there was a broad distribution extending from normal to abnormal values. Far greater reliability was observed in those individuals who were so severely iron deficient as to manifest anemia. It is concluded that multiple measurements are essential to identify the presence of iron deficiency with precision in the non-ânemic individual.



A. E. Mourant

Blood groups and diseases. Haematologia *8*, 183 (1974).

The ABO blood group system, and most other systems of hereditary blood factors, show wide differences in gene frequency between one population and another. Much of the variation found is probably due to random effects, but much also is almost certainly due to natural selection, certain phenotypes being more liable than others to suffer from certain diseases, with resulting loss of fertility. Most investigations have been based on determining the frequency of certain factors in those suffering from particular diseases. Thus, for instance, patients with carcinoma of the stomach have a raised frequency of blood group A, almost certainly because people of this group have a raised susceptibility to the disease. Several other established cases and many other probable cases are known of such associations. Numerous examples have recently been discovered of associations of diseases with particular histocompatibility antigens. It is suggested that the gene frequencies in many if not most genetic polymorphisms are the result of an equilibrium, varying with locality between the selective effects of different diseases and other environmental stresses on the various phenotypes of any given system.

A. S. Wiener, W. W. Socha, J. Moor-Jankowski

Homologues of the human A-B-O blood groups in apes and monkeys. Haematologia 8, 195 (1974).

Red cells of all apes except the gorilla closely resemble human red cells in their reactions with anti-**A** and anti-**B**. In chimpanzees only groups A and O occur, while in gibbons and orangutans groups A, B, and AB have been found but not group O. All gorillas tested have proved to be group B, but the agglutinogen B on gorilla red cells is extremely weak and demonstrable only after proteolytic-enzyme treatment. Monkey red cells do not react with anti-**A**, anti-**B** or anti-**H**, but their A-B-O groups are demonstrable by testing their saliva for the group-specific substances A, B and H, and their serum for anti-**A** and anti-**B**. Certain species of Old World monkeys have all four A-B-O groups, e.g., baboons and crab-eating macaques, though group O is rare in these species; in some species only group A occurs, while in other species only group B has been found. The situation in New World monkeys is complicated by the presence on the red cells of a B-like agglutinogen, irrespective of their A-B-O groups determined by saliva tests. All but one (an orangutan) of the hundreds of apes and monkeys tested by us to date have proved to be A-B-H secretors. The relationship of H to the A-B-O groups in apes and in monkeys is different from that in man.

J. Hors, J. Dausset, A. Gerbal, Ch. Salmon, C. Ropartz, S. Lanset

HL-A phenotype and anti-Rh(D) immunization. Haematologia *8*, 217 (1974).

Ninety-three Rh negative subjects were submitted to a planned schedule of anti-D immunization. The 62 immunologically responding and 31 non-responding individuals were tested for 25 HL-A antigens. No significant difference in phenotype frequency was observed between the groups.

J. J. van Rood

The HL-A system. Genetics (patho)physiology and clinical relevance. Haematologia 8, 223 (1974).

The HL-A system remains one of the most fascinating and complex immunogenetic systems in man. Its role in determining the outcome of allograft survival is certain, but we have only begun to understand how to match between unrelated donor-recipient pairs. New findings have made this issue more realistic, as it appears that not only the serologically defined antigens play an important role in graft survival, but also the area which codes for the MLC determinants. Major emphasis in future histocompatibility studies will certainly concern the loci on the same chromosome which determine whether or not an individual is predisposed to a disease. Again, only a start has been made in unravelling the factors which determine this process.

J. P. Soulier, O. Prou-Wartelle, J. Y. Muller

Paternity research using the HL-A system. Haematologia *8*, 249 (1974).

Forty-seven cases of paternity research are reported. Paternity was excluded in 11 of these cases by the HL-A system (9 of the 11 were excluded also, on the basis of blood group systems). The technique of exclusion is discussed. In 36 cases, the paternity could not be excluded. We have presented a method for estimating the fraction of the population compatible with possible paternity so as to give approximative evaluation of presumption of paternity. The limitation of such presumptive appraisals is discussed, as well as the genetic and serologic problems which have been encountered. Keeping these limitations in mind, the HL-A system appears to be a very precious tool in paternity research.

C. P. Engelfriet, J. J. van Loghem

HL-A in connection with blood transfusion. Haematologia *8*, 267 (1974).

The following aspects of HL-A, leucocyte and platelet serology that may be of importance in blood transfusion practice are discussed: the preparation of leucocyte-free blood, in particular the new method by Diepenhorst et al. using filtration over cotton wool. Heteroimmunization in rabbits showed that in blood filtered immediately after withdrawal less leucocyte antigens are present than in leucocyte-poor blood prepared in any other way; the detection of non-agglutinating, non-cytotoxic antibodies; the full expression on the red cells of a normal donor of all the HL-A antigens of his genotype.

E. Ivašková, H. Macurová, H. Bavorová, M. Micková, P. Iványi

Cross-reactions of mouse anti-H-2 sera with human HL-A antigens. Haematologia *8*, 273 (1974).

Alloimmune pure anti-H-2 (anti-H-2.8, 3, 7, 9) serum has been found to exert a strong cytotoxic reaction on human lymphocytes bearing the HL-A haplotypes in which the HL-A2 gene is involved.

I. Simonovits, G. Bajtai, R. Kellner, M. Kerényi, L. Rucz, R. Szilvás, S. Takács

Immunization of $Rh_0(D)$-negative secundigravidae whose first pregnancy was terminated by induced abortion. Haematologia *8*, 291 (1974).

The occurrence of rhesus immunization was investigated in secundigravidae whose first pregnancy had been medically terminated during the first trimester.

a) Out of 301 *non-protected* women whose pregnancy had been terminated medically within the first trimester, the second pregnancy was terminated in 121 again by induced abortion within the first trimester. One of these was found to be immunized. She had received i.m. blood injection in childhood. Twenty-four women had spontaneous abortion. One of these became immunized. Immunization was detected in the fourth month of the second gravidity. The frequency of immunization was found to be about 1.0%. To evaluate the exact rate of immunization was not possible due to the low number of cases. Among the 156 secundigravidae who had delivered $Rh_0(D)$ positive newborns at term, antibodies could be detected in 6, about 4.0% (= 3.84%).

b) Among 96 women who were protected by 50 μg anti-D IgG when their first pregnancy was interrupted, immunization occurred in one case.

c) An attempt was made to determine secondary response in women whose first pregnancy was terminated by induced abortion. A frequency of about 1.0% immunization was found after spontaneous abortion.

D. R. Stanworth

The role of the antibody in immunological cell triggering processes. Haematologia *8*, 299 (1974).

The idea is developed that the role of the antibody in immunological cell triggering processes is that of a transducer of the stimulus afforded by specific antigen. In another sense it can be considered to be a "pro-hormone", which is capable of recognizing and "priming" (i.e. sensitizing) the appropriate target cell so that subsequent interaction with antigen brings about the mobilization of specific effector groups which trigger the cell into action. Although the emphasis has been placed on the mode of action of anaphylactic antibodies in the initiation of vasoactive amine release, it is suggested that other types of cytophilic antibodies such as those engaged in the mediation of phagocytosis and lymphocyte stimulation could be fulfilling an essentially similar role. Taking this analogy further, it is tentatively suggested that IgG mediator antibody molecules possess cell-binding sites with differing specificity located within a similar region of their C_H3 domains and that the effector sites responsible for triggering different target cells are probably located within a different part of the antibody Fc region.

R. Cahill, J. B. Hay, H. Frost, Z. Trnka

Changes in lymphocyte circulation after administration of antigen. Haematologia *8*, 321 (1974).

The recirculation of lymphocytes has been studied using the model of Bede Morris whereby vessels afferent to and efferent from single lymph nodes are cannulated (in sheep), enabling the collection of *all* efferent lymph over long periods of time in conscious animals when antigen is introduced *via* a cannulated afferent lymph vessel. Antigen and specific antigen-reactive cells are confined to the lymph node or diverted from the animal by the chronic efferent lymphatic fistula. The sequential changes in cell traffic, the appearance of blast cells and specific antibody forming cells, and the appearance of biologically active materials such as MIF and a mitogenic factor are described. Evidence is presented that when allogeneic lymphocytes are confined to a single lymph node, that node is responsible for removing all lymphocytes specifically reactive to those allogeneic lymphocytes from the recirculating pool of lymphocytes of the whole animal. The potential of this finding and of the model is discussed.

T. J. Greenwalt, A. E. Steane, C. R. McConnell

The usefulness of Scatchard plots in the analysis of antigen-antibody interactions at the surface of the red cell membrane. Haematologia *8*, 335 (1974).

The Scatchard conversion of the law of mass action has been used extensively for quantitating the reactions of antibodies with red cells. We have found that it is not completely satisfactory for these measurements and suggest that use of the red cell concentration at which 50% of the absorbable antibody is bound is more suitable. The required value is obtained by plotting the percentage of antibody absorbed using a probit scale on the ordinate against the concentration of red cells on a logarithmic scale on the abscissa.

H. Braunsteiner, F. Schmalzl, R. Rindler-Ludwig

The function of neutrophil granulocytes and monocytes in the frame of cellular defence. Haematologia *8*, 341 (1974).

Among the mechanisms protecting the integrity of the human body the neutrophil granulocytes and the monocytes form a peculiar phagocytic system whose most important features include ubiquitous distribution guaranteed by the blood circulation and, at least under normal conditions, large pools of the disposable cells. The functions include phagocytosis, bactericide property, degradation or segregation of organic and inorganic materials as well as various functions within the humoral and cell mediated immunologic mechanism. As the performance of these tasks is concerned, neutrophile granulocytes and monocyte-macrophages distinctly differ complementing each other in their functions. The variations in the composition of the cellular exudates and the granulomatous process reflect the different functional peculiarities of the cells involved.

Mature granulocytes are highly differentiated and specialized non-proliferating cells. They are highly phagocytic and are characterized by conspicuous cytoplasmic granules which depending on the time of production during the neutrophile development, differ in their content of very active enzymes. These include a large set of catabolic lysosomal enzymes as well as some enzymes, especially proteases, acting preferentially at a neutral pH; various bactericidal enzymes and proteins complete the equipment. The metabolic and cytological features predispose the neutrophile PMN to a highly specialized but only short-termed function.

In contrast, the monocytes in peripheral blood display poor differentiation and reach their full functional abilities as they transform into macrophages, or histiocytes or epitheloid or giant cells. In an extravasal environment under adequate stimulation and enzyme induction a transformation occurs into cytochemically different forms of macrophages, depending upon the environment and the substances to be degraded.

The transformation into macrophages includes activation of the RNA metabolism with increase in cell size and enlargement and activation of the Golgi apparatus, endoplasmic reticulum and lysosomes, as well as marked changes of the oxidative metabolism. In contrast to the short-lived neutrophil granulocytes, the macrophages and epitheloid cells may survive for weeks and months and under special conditions they are capable of proliferating at the site of their action. The bactericidal mechanisms are essentially similar in monocytes or macrophages and in neutrophil granulocytes; in the latter they are, however, more potent and manifold but rapidly exhausted. Congenital or acquired disorders of the intracellular functions among these phagocytes are the causes of various diseases; some of the most interesting syndromes are briefly reported.

B. Fekete, Gy. Szegedi, P. Gergely, G. Szabó, Gy. Petrányi

Technical problems of spontaneous rosette formation. A suggestion for standardization of the method. Haematologia *8*, 353 (1974).

A high percentage of human lymphocytes forms rosettes with sheep red blood cells. The percentage of rosette forming cells (E-RFC) varies considerably according to the technique of the test. In the present work some technical conditions influencing the number of E-RFC have been investigated. The criteria of an optimum rosette technique capable of detecting a maximum number of E-RFC is described. It is important to keep the rosettes at 0°C, because resuspension at higher temperatures destroys them and a smaller percentage is counted.

Gy. G. Petrányi

Functional properties of T and B lymphocytes in cell-mediated immunity. Haematologia 8, 361 (1974).

Recent data on the characteristics and functional behaviour of T and B lymphocytes are summarized. The importance of surface markers (antigens, receptors, immunoglobulins) in the distinction of these two lymphocyte populations is stressed. Some evidence is offered to show that the equilibrium between T and B lymphocytes is one of the basic rules of normal immune reactivity. The functional characteristics of T and B lymphocytes, important in cell-mediated immunity are discussed together with the different sensitivity to mitogens, the specific and aspecific cytotoxic activity and the "soluble factor" release of T and B lymphocytes. Adherence, mobility and motility, and the subpopulations of the lymphocytes and some results of studies on PHA sensitivity, spontaneous cytotoxicity and moving properties of human lymphocytes are reviewed.

M. Hrubiško, M. Steriušká

Lymphocyte transformation induced by autologous platelets in thrombocytopenic patients. Haematologia *8*, 383 (1974).

Lymphocyte stimulation by autologous platelets in short-term cultures was examined in patients with idiopathic and secondary thrombocytopenia. Stimulation of lymphocytes by autologous platelets was statistically significantly higher ($P = 0{,}01 - 0.001$) in the patients than in healthy subjects. Differences among single groups of thrombocytopenias were not significant statistically. No correlation was found in the results of lymphocyte stimulation by autologous platelets and the presence of anti-platelet antibodies, immunoglobulin levels, platelet survival, i.e. between humoral and cellular immune mechanisms. The results suggested that autoimmune cellular mechanisms were involved in the pathogenesis of thrombocytopenia of diverse aetiology.

A. Hässig

The clinical use of plasma fractions. Haematologia *8*, 393 (1974).

On the basis of experience at the Blood Transfusion Service of the Swiss Red Cross, the following problems are discussed. 1. The clinical use of albumin solutions in comparison with plasma substitutes and electrolyte solutions. 2. The available and intravenously tolerated gamma globulin preparations are compared and critically evaluated. 3. The value of the coagulation factor concentrates is discussed.

O. K. Gavrilov

Subject and tasks of transfusiology as a scientific subject and its place among other medical sciences. Haematologia 8, 401 (1974).

Transfusiology is a science of the control of functions of the organism by directed action on the morphological composition and physiological properties of the blood system and extracellular fluid by parenteral injection of organic and inorganic transfusion media. Organic and inorganic transfusion media and their action on the human organism are the subjects of study of modern transfusiology. Tasks of transfusiology, contents, methods of investigation and their inter-relationship with other medical sciences are presented in this paper.

B. P. L. Moore

Prevalence of HB Ag and HB Ab in the Canadian blood-donor population. Haematologia *8*, 409 (1974).

It is an unfortunate fact that, having tested almost two million blood donations for HB Ag, we have no proof of the effectiveness of all this work in reducing the incidence of post-transfusion hepatitis. Whether the use of methods capable of detecting lower concentrations of HB Ag will materially decrease the infectivity of our blood products appears debatable (e.g. [6]). The most direct approach is still greater discrimination on the prescription of blood components and products, a point with which I feel sure that my good friend Dr. Susan Hollán, to whom this paper is dedicated, will agree.

P. A. Miescher, B. Portmann, J.-J. Farquet

Pathophysiological implications of the hepatitis associated antigen (HAA). Haematologia *8*, 415 (1974).

With the discovery of an antigen associated to the hepatitis B virus (HAA), a new tool has become available for the investigation of the relationship between this antigen and a number of pathological conditions. Depending on whether one is focussing on a given change in the liver or directly on the presence of HAA (Australia antigen), different patterns of association appear. Both approaches seem to be necessary to obtain a better insight into the actual connection between the presence of HAA, eventual antibodies to it, and a given liver change. In this paper, we are summarizing the present knowledge in an attempt to clarify some pathophysiological implications of the Australia antigen.

J. Hořejší, S. Höglund, J. Kořínek, M. Kselíková, Z. Malaska, J. Novák

Preparation of specific immunoglobulin for the prevention of hepatitis B. Haematologia 8, 427 (1974).

Isolation of IgG from the blood plasma with a high content of HBAg was made and the final product was tested by sensitive methods both immunochemical, radioimmunological and also by electron microscopy. HBAg was not detected in the final product. These findings suggest the possibility of preparing hyperimmune IgG containing a high titre of HBAb for preventive use in persons with a high risk of HB infection.

J. Roskam

Some general remarks about research in spontaneous arrest of bleeding. Haematologia *8*, 439 (1974).

In a noteworthy monograph published a few years ago [1], Sir Harold Himsworth, former Secretary of the British Medical Council, pointed out that it was essential for the growth of scientific knowledge to promote steadfast exchange between specialized and basic research. This is well shown by the part played by the sympathetic nervous system, the catecholamines and some of their metabolites in the spontaneous arrest of bleeding, in other words, spontaneous haemostasis.

P. A. Owren

Standardization of thromboplastin reagents and control plasmas. Haematologia, 441 (1974).

Different thromboplastins give different results. The standardization of reagents and the development of means for comparing results is an urgent problem, because of the clinical problems involved. In order to assess their acceptability in clinical laboratories, different reagents have been compared concerning stability, specificity and sensitivity to separate clotting factors and group of factors and to the clotting defect caused by oral anticoagulants. Certain preparations have a too low sensitivity to factors they are supposed to measure. Control plasmas have recently been introduced as reference materials for the quality control of thromboplastin preparations and for establishing the therapeutic range in oral anticoagulation. It is found that most of the commercial plasmas are highly activated, containing activated factor VII, which produces abnormally short clotting times and false high percentage values, leading to an all too low and ineffective intensity of anticoagulation.

L. Á. Pálos

The progressive antithrombin (antithrombin-III, heparin-cofactor). Haematologia 8, 455 (1974).

Problems connected with the theoretical and practical aspects of progressive antithrombin are discussed and the characteristics of the functionally defective molecule antithrombin-$III_{Budapest}$ are presented.

G. Astaldi, B. Yalçin

Haemo-lymphocytopoiesis. A review. Haematologia *8*, 461 (1974).

The old controversy by the polyphyletic and monophyletic theories concerning lymphocytopoiesis and haemopoiesis, based mostly on poor morphological criteria, has found its resolution by results of new and more functional approaches to the problem. Even the position or potentiality of the lymphocyte(s) in the stem cell puzzle is now clearer. In fact, the spleen colony assay, the polycythaemic mouse assay, the granulocyte-macrophage colony assay, and the focus assay for antigen-sensitive cells, first provided evidence for the existence of multipotent stem cells capable of undergoing either self-replication, or multiple differentiation in such a way, that they may repopulate both the lympho- and haemopoietic tissues of an irradiated animal. It appeared that a single pluripotent stem cell (colony-forming unit) may undergo qualification either for a lymphoid committed stem cell, or for a haemic committed stem cell. Then, these specifically committed stem cells may differentiate into several unipotent progenitor cells, which are: 1. the antibody-forming cell progenitors, and the antigen reactive cell progenitors, for the lymphocyte series; and 2. for the haemic series, the erythropoietin-sensitive cells, the colony- and then cluster-forming cells (granulocyte-macrophage colonies), the eventual megakaryocyte progenitor cells. In their turn, the above-mentioned unipotent progenitors give rise to progenies of morphologically identifiable blast cells, which ripe to the different mature cell stages.

E. Kelemen, E. Puskás, Cs. Kiss, Gy. Petrányi jr.

Haemopoietic precursor cells in early human embryos: light microscopic pictures. Haematologia *8*, 467 (1974).

Liver and yolk sac smears and/or histological preparations obtained from 6.5 to 22 mm crown-rump length (second month of intrauterine life) intact human embryos are presented. The morphological inhomogeneity of early, unprocessed, free haemopoietic progenitor cells of the liver is emphasized and the peculiar location of apparently similar cells in the yolk sac entoderm is mentioned.

S. Stefanović, M. Radotić, M. Dukić, M. Ristić, B. Banićević, M. S. Jančić, S. Maslovarić

Treatment of polycythaemia vera with reinfusion of autologous blood plasma obtained by phlebotomy. Haematologia 8, 473 (1974).

In 37 out of 41 patients with polycythaemia vera, complete haematological remission has been achieved by phlebotomy and reinfusion of the autologous plasma. Improvement lasted from 7 to 48 months. Repeated application of the same procedure in 18 patients led to a new remission in 2 patients lasting more than 25 months. In the blood plasma a factor inhibiting erythropoiesis has been detected in normally fed or previously stimulated rats by testing the plasma for ^{59}Fe uptake. The nature of this inhibiting factor is discussed.

W. Rudowski, Z. Klawe, M. Woźniewska, J. M. Ziemski

Major surgery in patients with polycythaemia vera. Haematologia *8*, 477 (1974).

In the period 1968 to 1973, 9 patients with polycythaemia vera were subjected to 11 surgical interventions at the Department of Surgery, Institute of Haematology in Warsaw. No postoperative complications, haemorrhagic or thrombotic, were observed. Successful surgical treatment of patients with polycythaemia vera depend on temporary control of the disease, i.e. reduction of the erythrocyte mass to haematocrit values below 50% and of the platelet count to below 500,000/cu.mm with maintenance of these reduced values for a possible long time prior to surgery.

K. Rechowicz, S. Pawelski

Thrombokinetic studies with ^{75}Se-selenomethionine in thrombocythaemias. Haematologia *8*, 485 (1974).

Thrombocytokinetics were determined with ^{75}Se-selenomethionine in three patients with primary and nine patients with symptomatic thrombocythaemia, and in six cases platelet survival time with the ^{51}Cr method. Platelet survival time was normal in all patients according to both methods. The results showed that the raised blood platelet count in thrombocythaemia depends solely on the increased thrombocytopoiesis. In untreated cases this correlation was directly proportional.

E. Storti, G. Casirola, G. Marini, G. Ippoliti, E. Ascari, E. Ferrari

Hodgkin's disease of the spleen. Haematologia 8, 493 (1974).

Eight cases of Hodgkin's disease of the spleen are reported. In 7 cases clinical signs of the disease appeared in advanced age. Characteristics were the absence of lymph-node involvement and a very poor general condition. The diagnostic importance of laparoscopy is stressed and surgical treatment and complex chemotherapy are recommended.

T. Stojčevski

Haemolytic anaemia in malignant lymphoreticuloendothelial diseases. Haematologia *8*, 507 (1974).

Among 102 patients with malignant lymphoreticuloendothelial diseases, ten had autoimmune haemolytic anaemia, six haemolytic anaemia without positive direct antiglobulin test, and five had a positive direct antiglobulin test without anaemia and hyperhaemolysis. The pathogenesis of haemolytic anaemia in these diseases is discussed. It is assumed to be different in the cases with chronic lymphocytic leukaemia and in those with Hodgkin's disease.

G. Ruhenstroth-Bauer

Influence of combined electric and magnetic fields on biological cells and other particles. Haematologia *8*, 517 (1974).

It has been shown that the special combination of low electrical and low magnetic fields causes trajectories of suspended particles the directions of which are opposite to the so-called 3-finger rule. The electrical charge of the particles is of little or no significance for this unexpected phenomenon, the ion concentration of the suspension is, however, of great importance. There is no satisfactory theoretical explanation of the phenomenon which is assumed to facilitate magnetic field strength information to be translated into particle movements, which may be of a certain biological importance.

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L. Dintenfass, C. D. Forbes

GOT, GPT and LDH levels and blood viscosity factors after recent myocardial infarction. Effect of ABO blood groups. Haematologia *8*, 523 (1974).

A study was carried out on correlations between biochemical factors (GOT, GPT, LDH) and blood viscosity factors (blood and plasma viscosity, aggregation of red cells, formation of artificial red/white and white thrombi) in patients after recent myocardial infarction. GOT levels showed a significant correlation with the viscosity of blood at high shear rate ($P < 0.005$) in all patients, in patients with complications or concurrent diseases ($P < 0.05$), or in patients of A or O blood groups ($P < 0.02$). GOT levels correlated with the rate of degradation of artificial white thrombus ($P < 0.02$) and of the red/white thrombus ($P < 0.05$, blood group O only). GPT levels correlated with the apparent viscosity of red/white thrombus ($P < < 0.05$). Significant differences were found in the levels of GOT and GPT between patients of A and O blood groups. Correlations of GOT or GPT and of aggregation of red cells showed significant differences between patients of A and O blood groups ($P < 0.01$). It is suggested that elucidation of the molecular mechanism of infarction might require subgrouping of patients according to their ABO blood groups.

MAGYAR
TUDOMÁNYOS AKADÉMIA
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