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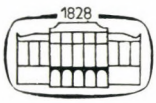

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In Memoriam

IMRE BARTA
1899–1978

On 17th November, 1978, Professor Imre Barta, one of the pioneers in clinical haematology in Hungary, passed away at Pécs.

He began his studies in medicine at Budapest University Medical School and continued them in Prague and then in Pécs, where he received his M. D. in 1924. From 1924 to 1929 he was registrar, then until 1936 lecturer at the Department of Medicine of Pécs University. In 1936 he became senior lecturer and then assistant professor. The title of his thesis was "The Pathology and Therapeutics of Blood and the Haemopoietic System". During this period he was granted a fellowship at the Charité Clinic in Berlin (1928–1930) to work under the guidance of Professor Schilling. At this time he was mainly interested in the toxic granulation of neutrophil cells. The years he worked with Professor Schilling, whose department at that time was one of the centres of haematology, the newly flourishing discipline of medicine, gave him the impetus to his research career.

From 1936 to 1960 he was head of the Department of Medicine, and later director, of the County Hospital of Mohács (Hungary).

Professor Barta was a specialist in internal medicine, but his main interest was centred upon haematology. The field of his research was the cytology of the bone marrow, spleen and the lymph nodes. He was among the first to perform bone marrow puncture in Hungary.

Although first and foremost he was a clinical investigator engaged in the research of blood cell morphology, he continuously followed with youthful ardour the new emerging disciplines of science: biochemistry, biophysics and immunology, and readily applied the new methods in haematological diagnostics.

He was an ardent teacher and educator. As a medical student in Pécs I had the privilege of attending his haematological courses.

From 1960 to 1968 he held the post of professor, and was head of the Department of Medicine of Pécs University. He soon established a school in haematology where many young, gifted students were trained under his guidance.

His scientific writings covered chiefly the field of haematological diagnostics, cytomorphology and cytomorphological diagnostics. In addition to numerous scientific papers he wrote two monographs: "Spleen in Internal Medicine" and "Clinical Cytology".

Professor Barta was the first President of the Hungarian Society of Haematology, and Honorary President until his death. He was a member of the Editorial Board of *Haematologia* since its foundation in 1967, and of *Folia Haematologica.*

His devotion to haematology and teaching together with his pleasing personality earned him the highest appreciation of everyone who had the pleasure of being his friend, student, and patient or colleague, and who will cherish his memory.

Susan R. Hollán

Haematologia 12 (1—4), pp. 7—13 (1978/1979)

New Ideas about Self-Tolerance and Auto-Immunity*

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(Received September 4, 1978)

Recent advances in our understanding of B cell ontogeny strengthen the theory that self-tolerance is generated principally by clonal deletion. B cells pass through a stage: the "baby B cell", during which they are particularly susceptible to receptor cross-linking. Further evidence of clonal deletion comes from studies on the maintenance of the liver differentiation alloantigen F. In spite of this accumulating evidence room can still be found for the concept of immunological silence: it is a prediction of the dual recognition theory of T cell receptors that differentiation macromolecules which occur only on the surface of Ia negative cells cannot be seen by T helper cells.

Ideas about self tolerance have developed in the following successive phases:

(i) Horror auto-intoxicus.
(ii) Acquired tolerance: clone elimination.
(iii) T and B cells: guidance by T cells.
(iv) Suppressor T cells.
(v) Immunological silence.

During the first phase it was realised that the immune system must distinguish between macromolecules of self and foreign origin, but methods of investigating the problem experimentally were not available. In the second phase, experiments of nature and in the laboratory showed that tolerance of foreign antigens could be acquired. It was therefore assumed that self-macromolecules would be tolerated through the same mechanism. Clone elimination was believed to provide this mechanism, not only because this fitted with the firmly established clonal selection mechanism of antibody production, but also because it fitted experimental data on the kinetics of recovery from tolerance and the effects of thymectomy on recovery. Just how clones come to be eliminated is still unclear, although the mode of antigen presentation seems to be the most important factor: direct presentation of antigen favours tolerance induction, while presentation via macrophages favours immunisation. It has also been argued that the availability of helper cell activity is crucial in making this distinction [3]; although this view is not universally accepted [5]. It has also for long been supposed that lymphocytes may pass

* Presented at the 4th European Immunology Meeting, Budapest, 12—14 April, 1978.

through a stage of ontogeny in which they are particularly sensitive to clone elimination; this view has found recent experimental support, as outlined below.

The third phase followed the discovery of T and B cells, and was devoted mainly to investigation of the differences in tolerance susceptibility and stability in the two lymphocyte classes. Failure of response could be shown to result from tolerance in either helper or effector cells. T cells appear to be principally responsible for guiding responsiveness, because they are susceptible to induction by lower concentrations of antigen than B cells, or at least in comparisons between mature T and B cells.

The fourth phase followed the discovery of suppressor T cells. These cells can recognise antigen, idiotype, allotype, and immunoglobulin class. They can regulate the activity of other lymphocytes, although just how important they are in doing so is not clear. In descending order of confidence they are thought to control (i) chronic allotype suppression; (ii) clonal dominance; (iii) return-to-rest of the immune system after a normal response; (iv) tolerance of the foetal allograft; and (v) self-tolerance.

Discussion of the last phase is postponed until later. Documentation of this introductory survey can be found in recent reviews (7a; *Brit. Med. Bull. 32*, 99, 1976). We turn now to some recent developments.

Clonal abortion

The first immunoglobulin to be detected in the B cell lineage appears within the cytoplasm of pre-B cells. Subsequently, before these cells emerge from their site of origin in foetal liver or bone marrow, immunoglobulin can be detected on their surface at a stage defined as the baby B cell. At this stage the cells are exquisitely sensitive to cross-linking of their surface immunoglobulin receptors to which they respond by irreversible inactivation. Cross-linking can be brought about by means of antigen [13], or anti-immunoglobulin antibody [14] and probably also by anti-idiotype [4]. The effect has been termed, with special reference to the inactivation brought about by exposure to antigen, 'clonal abortion'. The lack of surface IgD, as in baby B cells, may be responsible for their particular susceptibility to clonal abortion [17]. The problem with this hypothesis is that anti-μ antibodies, which do not react with IgD, are effective in producing clonal abortion [14]. IgD must therefore act in an indirect manner, if it does so at all.

These discoveries lend new support to the old idea that lymphocytes pass through a stage of ontogeny during which they are particularly susceptible to the induction of tolerance, and that it is at this stage that self-tolerance is acquired. The suggestion is now that clonal abortion, occurring among baby B cells, may be an important mechanism of self-tolerance. Stage-specific tolerance has undoubtedly become much more attractive, but before it is accepted as an important mechanism certain reservations have to be made. One would predict that prolonged exposure of an animal to antigen, starting in early life (or after lethal irradiation and marrow reconstitution), should be relatively effective in inducing tolerance. So far as T

cells are concerned this is not the case [11], but then these cells may obey different rules from B cells. Nor does it appear that B cells obey this prediction in respect of T-independent antigens [7], but here again the rules may be special. The necessary experiments have not been performed with T-dependent antigens: they would not be easy to carry out rigorously because of the problem of ensuring continuous exposure to antigen of baby B cells in vivo.

So far as self-tolerance is concerned, work on haemoglobin has been cited in favour of the clonal abortion theory [15]. The conclusion has been reached that B cells in the rabbit are tolerant of self-haemoglobin, on the grounds that rabbits respond to human haemoglobin-A chain by producing antibodies directed only against those parts of the chain unique to humans. The conclusion is different from that reached with the liver cytoplasmic protein F, cited below, where tolerance of self-F among B cells appears not to occur. More data are needed: the discrepancy between the haemoglobin and F data may simply reflect quantitative differences in the exposure of B cells to the two antigens.

Differentiation allo-antigens as models of self-tolerance

What kind of experiment can be designed to answer the question whether tolerance of self is acquired? Thus far attention has concentrated on attempts to grow lymphocytes in the absence of a macromolecule to which they would normally be exposed, and then assessing reactivity towards the macromolecule. In one form of this experiment, the source of the macromolecule is removed by extirpating an organ [16]. In the other, a source of lymphocytes is transplanted into an environment free of a given antigen. This second approach has been exploited particularly successfully with differentiation allo-antigens, in an experimental design first introduced for study of the skin allo-antigen Sk [9].

Mice are polymorphic for Sk, which occurs in common laboratory strains as two allelic types, Sk-1 and Sk-2. Chimaeras can be prepared in which irradiated mice of type Sk-1/1 are reconstituted entirely with bone marrow cells of genotype Sk-1/2. Neither bone marrow cells, nor lymphocytes or other cells of bone marrow origin, produce Sk. Lymphocytes of genotype Sk-1/2 do not normally react against antigen of type 2. In the chimaeras reactivity towards this antigen is acquired after an interval of a few weeks, presumably because lymphocytes derived from the transplanted bone marrow have matured in an environment free of Sk-2. In support of this conclusion, the presence of a large skin graft of type Sk-2 on the chimaeras can inhibit the development of reactivity [9].

The Sk molecule is located in the cell surface, and for quantitative work a soluble antigen is needed. The liver differentiation allo-antigen F fulfills this requirement. This is a phylogenetically ubiquitous 40,000 dalton soluble intracellular protein of unknown function [10]. Like Sk, F occurs in common laboratory mouse strains in two allelic forms, types I and II. The polymorphism can be typed by T cell reactivity or by immunophoresis (Lane, D.P., unpublished data) or iso-electric focussing (Anders, R.F., unpublished data). Interestingly, the anti-

body produced in allo-immunised mice appears to be directed entirely towards the common parts of the molecule. This implies, as mentioned above, that B cells are not tolerant of this molecule.

In a study similar to that carried out with Sk, mice of F type I were repopulated with bone marrow cells of F genotype I/II. They were then challenged with type II F, to which they proved able to respond. Tolerance of type II F could be maintained in these chimaeras by thrice weekly injections of an estimated 10 μg of F protein in the form of whole liver homogenate. This establishes an antigen concentration threshold for tolerance induction at least as low as that previously encountered with foreign protein antigens, and makes it unlikely that suppressor T cells are involved.

This result, it might be supposed, greatly strengthens the case for acquired tolerance of self-antigens, since the two differentiation allo-antigens so far tested have behaved in the same way. However, a doubt remains. Neither Sk nor F can be regarded as a random sample of self macromolecules, for both were selected for study on the grounds that they had already been found able to provoke a T cell response. It seems unlikely that F should be the only intracellular liver protein for which mice are polymorphic. An additional piece of evidence in the same direction is that M. C. Raff and N. A. Mitchison were unable to detect differentiation allo-antigens on the mouse neuroblastoma C1300, using an experimental design similar to that used for Sk. Our experiment, in fact, was almost a carbon copy, for C1300 happens to derive from the very strain of mouse employed in the Sk study. The modest conclusion follows that while some self-macromolecules exemplified by Sk and F do generate tolerance actively, others which may well form the majority need not to do so.

Programmed self-tolerance

Acquired tolerance is a form of regulation of the immune response which is essentially antigen-driven. An alternative is that lack of reactivity with self is pre-programmed, and does not depend on exposure of lymphocytes to self-macromolecules. The immunological system simply remains silent in relation to these molecules. Selection of V-genes, it has been proposed, might have this effect [1]. This proposal accords with recent genetic studies which have shown that the repertoire of V-genes may be quite extensive [e. g. 2]. Nevertheless, one may question whether a model of this sort can cope with the numbers required either/or of genes and/or of selective forces. An alternative form of pre-programming depends on the dual recognition theory [19]. The belief has long been held that some self-macromolecules avoid confronting the immune system by lying sequestered, and as a corollary that exposure of these normally sequestered molecules may be a cause of auto-immunity. This idea can now be reformulated in terms of the ability of T helper cells, the major class of regulatory T cell, to recognise antigens only in association with Ia antigens of the major histocompatibility complex. These antigens have a restricted distribution within the body, and they have not been

found on most differentiated cells. Macromolecules present only on Ia^- cells, presumably the majority of differentiation macromolecules, have no means of associating with Ia molecules except through reprocessing by Ia^+ cells. To the extent that they can avoid reprocessing they should, therefore, escape the attention of T helper cells, and thus need no other mechanism of self-tolerance. How much reprocessing of self-macromolecules actually occurs is unknown, but in the light of these considerations it becomes a matter of great interest.

Antigen reprocessing by macrophages

In a general way the processing and presentation of antigen is a principle function of macrophages, and is one which they perform extraordinarily efficiently in respect of foreign proteins in general [11]. Any mechanisms restricting reprocessing are therefore likely to apply only to cell surface antigens. There is another possibility, namely that Ia molecules discriminate in their associations between self and non-self, but this raises all sorts of difficulties: in effect it shifts the burden of discrimination from lymphocytes to macrophages, without diminishing it. Indeed, this second possibility is not worth discussing further until we know more about the rules of physical association between Ia and other molecules.

What we know about the reprocessing of relevant antigens has largely been learned with allo-antigens. With these antigens it has been possible to obtain the following evidence for limited reprocessing.

1. The anti-minor cytotoxic response can be donor-restricted. This occurs, for example, when $H\text{-}2^k$, $H\text{-}Y^+$ cells are transplanted into $H\text{-}2^{k/b}$, $H\text{-}Y^-$ hosts. In contrast, the response of $H\text{-}2^b$, $H\text{-}Y^-$ hosts to these cells is host-restricted [6].

2. Latent help. For example, Thy-1 alone (AKR. Thy-1.1 AKR. Thy-1.2) generates little 1° but can boost the 2° response. In contrast, Thy-1.1 + helpers (CBA AKR. Thy-1.1) generates a 1° response [12].

3. Antigens show marked variation in the speed with which they activate help. For example, H-2I reaches a maximum after one injection, whereas H-2K requires five.

4. Functioning helper antigens increase with increasing immunisation. For example, for Thy-1, only 2 helpers in 1° (segregating in AKR × CBA cross), 25 in 2° (B6 × BALB).

Some but not all of the evidence bearing on reprocessing of helper antigens has been discussed before [8, 12], and the rest is our unpublished work.

None of these findings need necessarily be interpreted in terms of macrophage reprocessing, but it is tempting to do so. Taken together, they suggest that cell surface antigens can follow alternative routes into the immune system, one a direct one, and the other indirect via reprocessing onto the surface of host macrophages. The second route is relatively slow, and is favoured by immunological attack on the foreign cell via other antigens.

A cautionary note concerning cytotoxic T cells

We have been concentrating on regulation of the response of helper T cells. These cells certainly in turn regulate the responses of B cells and the activity of macrophages, but are they equally important for cytotoxic T cells, the other main effector branch of the immune system? Until recently, there was little evidence to indicate that this might be so, apart from in vitro effects probably mediated by nonspecific factors. Very recently, evidence of a more specific regulatory effect has been obtained, in which helper T cells appear to recognise the Ia antigens on cytotoxic T cells in the same manner as they do on B cells [18]. So far the evidence of antigen specific cooperation is of an indirect character, and will need to be confirmed and extended.

A new technique for analysing the response to allo-antigens

A useful tool for studying the nature of antigen processing and the regulation of the immune response to cell surface allo-antigens is monoclonal antibody of high titre. Such sera could be used to elucidate the number of antigenic sites on allo-antigens as well as for routine procedures such as depletion of cells expressing the relevant antigenic phenotype.

As a prototype for this approach, we have studied the parameters of production of hybrid cell lines secreting cytotoxic Thy-1 antibodies. A rapid solid phase cytotoxic assay has been developed which enables screening of a large number of samples, and thereby allows us to detect hybridoma clones of low frequency. The frequency of positive clones is proportional to the levels of plaque forming cells in the spleen cell population; fusions using spleen cells from xenogenic immunisations, therefore, have had a higher frequency of clones than those from alio-immunisations A number of anti-Thy-1.1 antibody producers have been isolated and, in addition, two anti-Thy-1.2 producers (F7F9 and F7D5). These cells grow in vivo and sera from tumour-bearing mice have often had cytotoxic titres exceeding 10^{-6}.

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Combining Site and Antigenic Determinants of the V Regions*

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Preparation of Fv fragment and its peptide chains V_L and V_H is described. The renaturation of completely unfolded V_L and V_H to generate an active Fv indicates that the variable domain, which is only a part of the peptide chains (L or H) contains all the information for its folding and generation of antibody combining site. Model building studies (together with Drs. Davies and Padlan, NIH) were attempted on the basis of known coordinates of other immunoglobulin V regions. The model was found to be compatible with the affinity labeling of protein 315 and with other studies on mapping the combining site. This mapping indicates that Trp 93L is the major residue interaction with the DNP hapten. Indeed V_L was found to contain the subsite for the DNP ring and its affinity to DNPOH is similar to that of Fv ($V_L + V_H$). On the other hand DNP haptens with longer side chains bind much better to Fv suggesting that the side chain of the hapten interacts predominantly with V_H. V_L exists as a dimer which can undergo conformational transition near pH 7. One conformer binds two haptens per dimer whereas the other binds only one hapten. Thus the combining site made up of two V_L chains is more flexible than that made up of $V_L + V_H$.

Antibodies were prepared against V_L and V_H. These antibodies react with many different V regions on L chains (anti V_L) or H chains (anti V_H). Anti V_H reacts with isolated H chains much better than with intact Ig. These antibodies were used to detect V regions on T cells by employing inhibition of antigen binding T cells. It was found that the inhibition by anti V_H and anti V_L affects different T cell populations. Anti-V_H inhibit the binding to Lyt-1^+cells known also to be T helper cells whereas anti-V_L inhibit antigen binding to Ly-2^+3^+ cells known to be suppressor cells. These results suggest that the expression of V regions on T cells is different from that on B cells. Probably in T cells there is unlinked expression of V_L and V_H and they are selectively expressed on different T cell subpopulations.

Introduction

The variable domains of immunoglobulins (Ig) contain all the structural elements of the antibody-combining site [12] and of the idiotypic determinants of the Ig molecule [17]. The availability of Fv fragment, derived from mouse myeloma protein 315 and composed solely of V domains ($V_L + V_H$), permits

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a more detailed analysis of the structure of the combining site as well as the antigenic determinants of V regions. Several experiments along these lines will be described.

I. The V domain contains the entire information for its folding

In an 8 M urea Fv dissociated into V_L and V_H that can be separated on DEAE cellulose in urea. When a 1 : 1 mixture of the isolated chains in urea was diluted into PBS, binding activity was fully regained [11].

The kinetics of the association process between V_L and V_H to form an active Fv was followed by monitoring their fluorescence. There is a marked increase in the fluorescence intensity of Fv in 8 M urea, along with a red shift of 11 nm. Fv reconstituted from urea by dilution into PBS has the same emission spectrum as native Fv. Thus, the renaturation of Fv (or a mixture of V_L and V_H) from 8 M urea can be followed by the decrease in protein fluorescence.

The kinetics of regain of binding activity by Fv was analyzed in these reactions by a rapid titration with equivalent amounts of DNP-lysine during the renaturation, and the fluorescence quenching due to the binding of DNP-lysine to Fv was measured. At all protein concentrations, the kinetics of regain of native fluorescence was identical with that of regain of binding activity. In addition, we found that the presence of the hapten during the renaturation process did not increase the rate of renaturation, indicating that the formation of the combining site is not the rate-limiting step in the folding and association process. The data also imply that the association constant of V_L and V_H is greater than 10^8 M^{-1} since full regain of active Fv was obtained at 10^{-8} M concentrations of V_L and V_H.

V_L and V_H contain one intrachain disulphide bond and the regain of binding activity was analyzed after complete reduction. Fv was reduced in 9 M urea, 0.1 M Tris-Cl pH 8.2 and 0.1 M β-mercaptoethanol at 37°C for one hour. Alkylation of a portion of this solution with iodoacetamide, followed by amino acid analysis, showed four carboxymethylcysteines per mole of Fv. Since either V_L or V_H each contain only one disulphide bond, we anticipated that if oxidation of the reduced Fv were to be performed in 8 M urea, no "wrong" disulphide bonds could be generated, and only the single intrachain disulphide bond would be formed. Reduced Fv (3 mg/ml) was dialyzed against 8 M urea to remove 8-mercaptoethanol and during this time it was also reoxidized. Subsequent dilution of this Fv into 0.15 M NaCl – 0.01 M phosphate buffer pH 7.4 (PBS) resulted in a complete regain of binding activity by the renatured Fv. Similar experiments were also performed with reduced V_L which was reoxidized in urea and combined with V_H in 8 M urea. Dilution of this mixture into PBS yielded completely active Fv [11].

These results reinforce the previously published evidence that the folding and specificity of the antibody-combining site are entirely dependent on the amino acid sequence [8] and are in line with the thermodynamic hypothesis of protein-folding [2]. In our case, 100% of the binding activity was regained by reoxidation

of completely reduced V domains of a homogeneous antibody. The complete renaturation of fully reduced Fv also provides strong support to the domain hypothesis of immunoglobulin structure. This study demonstrates that the V domains not only exist in immunoglobulin structure but can also fold independently of the rest of the peptide chain to form the native structure with full binding activity. Moreover, the foldings of V_L and V_H are completely independent of each other.

II. Model-building study of the antibody-combining site

X-ray structures of various Ig fragments demonstrated that the V domain can be considered as a three-dimensional framework of β sheets to which the hypervariable regions are attached. Replacements of residues at the hypervariable regions will not disturb the "immunoglobulin fold" of the framework. Hence, it should be possible to form any antibody-combining site by inserting sequences of the appropriate hypervariable regions into a known three-dimensional structure. Such an attempt was made by inserting protein 315 V region sequence onto protein 603 structure [14]. A model built in this way immediately suggested the likely site for binding DNP. This was based on H-bonding of the NO_2 group of DNP to protein residues (Asn 36L or Asn 36H), the stacking with a tryptophan residue from V_L (Trp 93L) and further adjustment to ensure the feasibility of affinity-labelling of Tyr 33L and Lys 52H by BADE and BADL [9], respectively. The possibility for cross-linking by the bifunctional reagent previously described [7] is obvious. The model was compatible with many of the binding data of the various ligands to protein 315 [10]. The next important step was to use the model as a basis for structural analysis by magnetic resonance and electron spin resonance. On the basis of the model it was possible by these methods to determine the general dimension of the site, its polarity and asymmetry, to assign contact residues with the ligand, and to determine their correct orientation with respect to the hapten [5]. The positioning of Trp 93L, Phe 34H, Tyr 34L, Asn 36L and Arg 95L and other residues allows understanding of the specificity of binding DNP ligand to protein 315.

Several interesting points emerge from the comparison of the combining site of protein 315 with those of protein New and 603. In Fab' New vitamin K_1OH complex, the naphthoquinone ring of vitamin K_1OH is in close contact with Tyr 90L [1]. Sequence homology shows that Trp 93L in protein 315 which interacts with the DNP is homologous to Tyr 90L in New, and therefore this provides an interesting example for a change in specificity by replacing one residue in the site. Note that protein 315 also binds menadione in lower affinity than DNP but protein New does not bind DNP ligands. Charge interactions play a dominant part in the binding of protein 603 to the hapten phosphoryl choline [16]. On the other hand, another model-building study on rabbit anti-type III polysaccharide [4] suggests clustering of the hydroxyamino acids Tyr, Ser and Thr and also Asn in the combining site which can form H bonds with hydroxyls of the sugar ligand.

It is clearly seen that some pattern of changes in the antibody-binding site can be deduced from such comparative studies, and these can help understanding the structural basis for antibody specificity.

In conclusion, two types of structural parameters will influence antibody specificity: 1. Size differences of the hypervariable loops. This is particularly pronounced in L1 and H3 and will affect the dimension of the site and its depth; 2. replacement in the hypervariable loops which will provide different contact residues with the ligand.

III. Location of subsites on different V domains

Since V_L and V_H can be isolated from Fv [12] it is possible to compare the binding properties of Fv ($V_L + V_H$) to that of V_L or V_H. It is shown (Table 1) that V_L but not V_H contains the binding site to DNP ligands [6]. This is in line with the model-building study which demonstrated that Trp 93L is a major contact residue for the DNP ring.

Isolated V_L which exists as a dimer, binds two molecules of DNP-lysine with approximately 1000-fold K_A than that of Fv. However, when a homologous series of DNP ligands was used to map the site of V_L dimer it appeared that the

Table 1

Comparison of some properties of the subunits of Fv 315

	V_L	V_H	Fv
Mw ($NaDodSo_4$)	12,000	13,500	12,000 + 13,500
Mw (PBS)	24,000	Aggregate	25,000
$S_{20,w}$	2.18	Aggregate	2.6
DNP binding sites	2	0	1
$K_A \times 10^{-3}$	2.3	—	2,000
Binding to DNP-Lys-Sepharose	+ (90%)	− (6%)	+ (90%)

Table 2

Binding of various ligands to protein 315 IgA and its V_L subunits

Ligand	K_I ($M^{-1} \times 10^{-3}$)		K_I (IgA)
	V_L	IgA	K_I (V_L)
DNP-OH	0.46	3	6.5
DNP-$NHCH_2COOH$	1.60	50	31
DNP-$NHCH_2CH_2CH_2COOH$	1.40	5200	3714
DNP-$NHCH_2CH_2CH_2NH_2$	1.20	870	725
DNP-$NHCH_2CH_2CH_2CH(NH_2)COOH$	2.0	3400	1700
DNP-$NHCH_2CH_2CH_2CH_2CH_2COOH$	2.2	3600	1636
DNP-$NHCH_2CH_2CH_2CH_2CH(NH_2)COOH$	2.4	1700	708

combining site of V_L dimer binds the DNP ring *per se* (e.g. DNPOH) almost as strong as Fv (Table 2).

Some significant features of the combining site of protein 315 are absent in that of V_L dimer. The increase in affinity with the increase in length of ligand side chain disappears almost completely. A remarkable feature of the results presented in Table 2 is the small difference between the association constants for DNPOH of $(V_L)_2$ and protein 315. For example, the affinity of protein 315 for DNP-lysine or DNP-aminocaproate is respectively 708 and 1636 greater than that of $(V_L)_2$, whereas the affinity of protein 315 for DNPOH is only 6.5-fold greater than that of $(V_L)_2$. This indicates that the predominant binding energy of the DNP ring is provided by V_L. It is also noteworthy that in $(V_L)_2$ there is almost no increase in K_I between DNPOH and other ligands with longer side chains, whereas, in protein 315-combining site which is composed of both V_L and V_H, the affinity for DNP-lysine is 567-fold greater than for DNPOH (Table 2). Moreover, different side chains of the ligand can sense special features of protein 315-combining site. Thus, DNP-aminobutyric acid binds about 6-fold better than DNP-aminopropylamine due to a "positive subsite" in protein 315. This feature is also absent in $(V_L)_2$. It is suggested that a significant part of the interactions with side chains of DNP ligands is due to contributions from the heavy chain, whereas the DNP ring is bound predominantly to the light chain. It is therefore a clear-cut illustration of the subdivision of the antibody-combining site into defined subsites which can in this particular case be identified with one of the chains.

IV. V_L dimer exists in two conformations

Hapten-binding measurements by equilibrium dialysis were performed with V_L that was previously dialyzed against 0.1 M buffer solution of different pH values. Fig. 1 shows the results of equilibrium dialysis at pH 6.0 and pH 8.0.

It is shown that the binding properties of V_L dimer are different at these two pH values. At pH 6.0 V_L dimer binds only one mole of ligand with higher

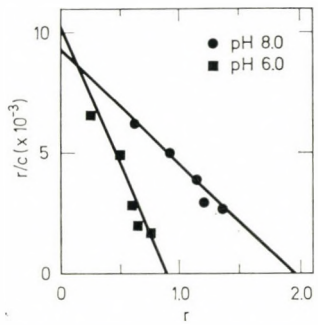

Fig. 1. Scatchard's plot for the binding of [^{3}H]DNP-Lys by $(V_L)_2$ at pH 6.0 and pH 8.0. Concentration of $(V_L)_2$ was 10^{-4} M and initial hapten concentrations were between 10^{-3} M and 10^{-4} M. *r*, moles hapten-bound per V_L dimer (MW = 24,000)

affinity ($K_A = 1.4 \times 10^4$), whereas at pH 8.0 two moles of ligand are bound with approximately 4-fold lower affinity ($K_A = 3.4 \times 10^3$). These results, in spite of measuring relatively small differences in K_A, were repeatedly obtained and indicate that V_L dimer is present in two different, pH-dependent, conformations. The transition between the two conformations was found to be at pH 6.9. In Fv no such pH dependent changes of binding were observed. We have previously demonstrated that the fluorescence of V_L is markedly enhanced in urea solutions [11]. The fluorescence spectra of V_L between pH 6 and 8 are given in Fig. 2 as compared with that of Fv under the same conditions. It is shown that a marked enhancement of V_L fluorescence occurs upon changing pH from 6 to 8, whereas the changes in Fv fluorescence are very small. The fluorescence of V_L at pH 8 is enhanced more than 2-fold compared to that at pH 6 and this presumably indicates the opening of the molecule, as in urea solution [11]. This also provides further support to the proposed change in the size of the combining site from one capable of binding one ligand (pH 6) to one that can accommodate two ligands (pH 8).

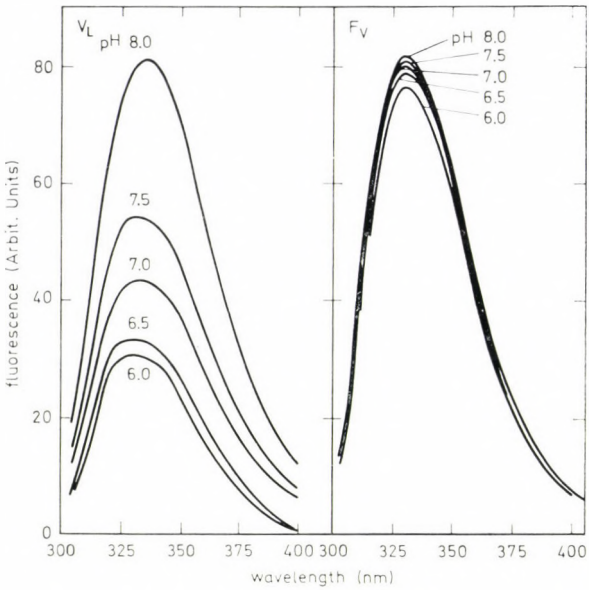

Fig. 2. pH-dependent fluorescence spectra of V_L and Fv

V. Anti-V_{L315} and anti-V_{H315} react with many different V regions

Rabbit antisera raised against V_{L315} were analyzed with V_L, L, Fv and the intact protein 315. A line of identity was formed between V_L and L indicating that the antiserum does not recognize C_L determinants. Similarly Fv ($V_L + V_H$), but not H, gives a line of identity with V_L.

Inhibition of the binding of V_L to anti-V_L is shown in Fig. 3. It is shown that L_{104} is a good inhibitor and the maximum inhibition reached is 70%. Hence, some of the V_L antigenic determinants are exclusive to V_{L315}. Intact M104 ($\lambda_1\mu$) or Hl ($\lambda_1\gamma$) did not precipitate with anti-V_L but inhibited to some extent the binding of V_L by anti-V_L (Fig. 4). Unlike the case of the homologous protein, the intact protein M104, or $HOPC_{-1}$ are poorer inhibitors than the isolated L chains.

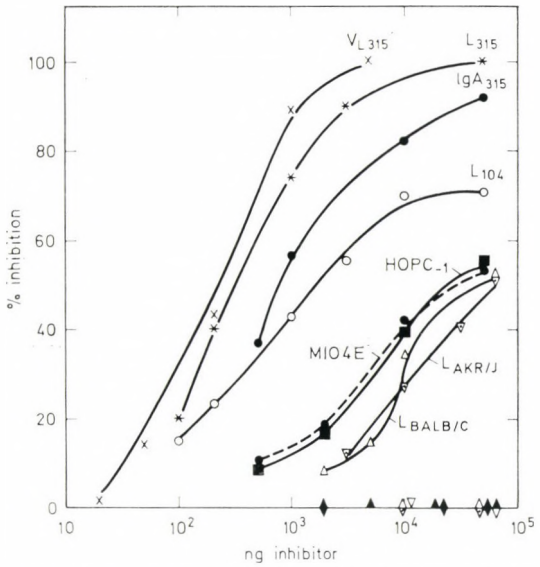

Fig. 3. Radioimmunoassay of anti-V_L antibodies. [^{125}I] V_L (40,000 cpm) was incubated with 0.5 μg anti-V_L antibodies. 60% of the cpm were precipitated when no inhibitor was added. Inhibitors used were as follows: H315 ■, L460 △, L-SJL ▽. Others are indicated in the figure

We have analyzed several antisera for the possibility of detecting activity to V_κ determinants. Most antisera did not react with κ chains or κ-bearing myeloma proteins. In one case, however, an antiserum raised against V_{L315} was found to react with κ chains.

Anti-V_H was obtained by immunizing rabbits with V_{H315} and purifying the antibodies on V_H-Sepharose. Fig. 4 demonstrates the inhibition of the binding of anti-V_H to V_H by various H chains. It is of interest that various heavy chains from different classes like μ(M104), α(X25, M460) and γ(U10) inhibit to a similar extent the binding of anti-V_H to V_H.

The inhibitory capacity of these H chains is approximately 20- to 1000-fold less efficient than that of H315 but, nevertheless, reaches 90 to 100%. Heavy chain of pooled MIgG is the best inhibitor indicating that V_{H315} determinants are quite common in BALB/c serum IgG. Direct binding experiments (not shown) confirm this observation. Another remarkable aspect of the inhibition studies is the lack of inhibition by intact Ig although the H chains derived from this Ig are inhibitory.

Thus, intact M104 or X25 does not inhibit the binding whereas their H chains show inhibition (Fig. 4). In the case of MIgG the intact molecule is a 300-fold weaker inhibitor compared to its H chain. These results suggest that the V_H determinants on various Ig, but not on M315, are exposed only on the isolated H chains, and not on the intact molecule.

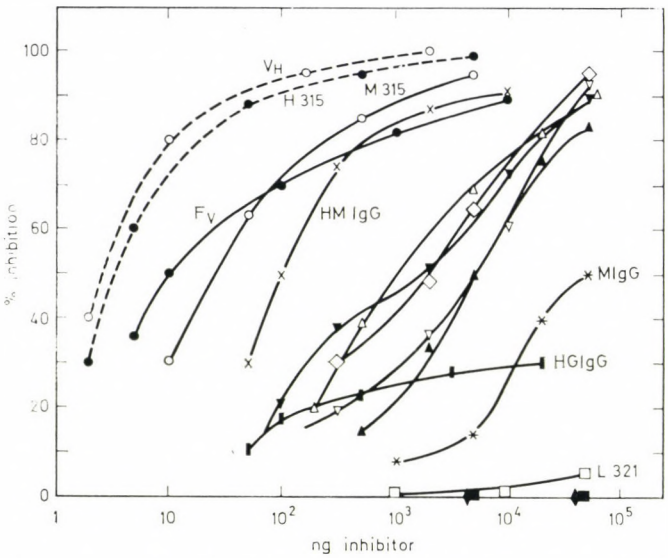

Fig. 4. Radioimmunoassay of [^{125}I]-labelled anti-V_H antibodies. The competition of various antigens with plastic-coated V_{H315} was analyzed. 20 ng of antibodies were used, of which 40% (cpm) were bound when no inhibitor was added. Non-specific binding to FCS-coated plastic wells was less than 0.5%. Antigens used were: V_H ○— —○, H315 ●— —●, M315 ○—○, Fv315 ●—●, HM IgG ×, H104E △, H460 ▼, H15 ◇, H25 ▽, H10 ▲, HGIgG (goat H chain) ▮, MIgG ⚹, M104E □, X25 ◆

In conclusion, the anti-V_{H315} antibodies can be regarded as a general anti-V_H antibody which can be used to detect V_H regions on isolated H chains. This property was utilized in experiments studying the inhibition of antigen binding to purified T cells by anti-V_H or by anti-V_L.

VI. Inhibition of antigen-binding to T cells by anti-V_L and anti-V_H antibodies

In order to test the effect of anti-V_H and anti-V_L on antigen binding by T cells we used the system previously described [13] of binding of [^{125}I]-(T, G)-A – L to T cell in short-term cultures and the binders were enumerated by autoradiography. It was previously shown that soluble macrophage factor (MF) or viral interferon (IF) regulate antigen binding by T cells, and increase the number of binders 3- to 10-fold. MF affects the Lyt-1$^+$ (mostly helper cells) population whereas IF affects the Lyt-2$^+$3$^+$ (mostly suppressor and killer cells) population.

Table 3 demonstrates the effect of anti-V antibodies on antigen binding by T cells and suggests that V_H and V_L are involved in antigen binding on different populations of T cells. Anti-V_H inhibits the effect of MF whereas anti-V_L inhibits the effect of IF.

Table 3

Inhibition of [^{125}I]-(T, G)-A—L binding to non-immunized T cells from C3H.SW mice

Inhibitory serum	T cells ABC/10^4	T cells + MF		T cells + IF	
		ABC/10^4	% inhibition	ABC/10^4	% inhibition
—	17±2	78±5	—	83±5	—
Anti-V_H		22±4	72	80±8	4
Anti-V_L		75±8	4	22±3	74

Analysis of the effect of anti-V regions on T cells previously treated with anti-Lyt and complement (Table 4) provides further evidence that V_H may be present as antigen receptor on Lyt-1$^+$ (helper) cells whereas V_L may exist as antigen receptor on Lyt-2$^+$3$^+$ (suppressor) cells.

Table 4

Inhibition of [^{125}I]-(T, G)-A—L binding to T cells from immunized B6-Ly-1.1 mice

Pretreatment with anti Lyt + C'	Inhibition		ABC/10^4	% inhibition
	α-V_H	α-V_L		
—	—	—	85±10	
α-Lyt-1.1	—	—	39± 6	
α-Lyt-1.1	+	—	41± 6	0
α-Lyt-1.1	—	+	12± 6	70
α-Lyt-2.2	—	—	94± 5	
α-Lyt-2.2	+	—	7± 5	93
α-Lyt-2.2	—	+	72±10	23

This finding suggests a fundamental difference between T and B cells since in the Ig which is the B cell receptor both V_H and V_L are expressed. Selective expression of V_H in the T cell receptor has already been suggested [15]. Our results add to this the possible expression of V_L and suggest that V_H and V_L may be selectively expressed in different T cell classes.

The analysis of V_L dimer described above indicates that the properties of antibody site composed of either V_L dimer or V_H dimer will be different from those of the antibody site composed of V_L + V_H. Some of these properties, such as the conformational flexibility of V_L dimer may be more compatible with the recognition properties by T cells.

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Cancer, Viruses and Environmental Factors*

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The genesis of Burkitt's lymphoma is visualized as proceeding in three steps:

I. Primary EBV infection affects the young child, probably at a relatively high multiplicity. It immortalizes a certain number of B-lymphocytes *in vivo*.

II. This is followed by the impact of an environmental promoting agent, perhaps chronic holoendemic malaria, providing a chronic stimulus to the proliferation of the EBV-carrying preneoplastic cells.

III. Chromosomally abnormal variants appear in the stimulated tissue by chance. After certain types of changes, particularly the 8 to 14 translocation that leads to the 14q+ marker, the affected B lymphocyte would no longer obey the negative feedback controls that would otherwise restrict its proliferation *in vivo*.

The topic that has been assigned to me, expressed in the title of this talk, is much too large to cover in general terms. Rather than attempting it, I shall focus on a case where the interactions of a virus with environmental factors and the role of cytogenetic changes in causing neoplasia are becoming more and more apparent. I am referring to the interaction of Epstein-Barr virus (EBV) with the human B lymphocyte, particularly as it relates to Burkitt's lymphoma.

Epstein-Barr virus (EBV) is a lymphotropic herpes virus in man [11]. Its main target is the human B lymphocyte [35]. Only B lymphocytes and most, if not all, B lymphocytes have specific EBV receptors [22]. It has been recently shown that the complement receptor of the B lymphocyte serves as the receptor for EBV [2, 37, 101, 102]. However, the complement receptors of other cell types, e.g. T cells, macrophages, granulocytes, erythrocytes, do not serve as EBV receptors.

It is interesting that the virus should have "chosen" a pre-existent receptor of the B lymphocyte as its receptor during its evolution. On the one hand, this explains the exclusive infectivity of EBV for the B lymphocyte, among all potential target cells so far tested. On the other hand, it may also give some functional clues. At least some immunologists believe that the complement receptor of the B lymphocyte plays a role in triggering DNA synthesis and opening the way towards blastogenesis, following the attachment of appropriate antigen-antibody complement complexes. It induces DNA synthesis in the infected B lymphocyte

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[18, 66] and activates B lymphocytes to polyclonal IgM production [88]. Induction of cellular DNA synthesis appears to be a prerequisite for EBV-induced immortalization. A variant strain of the virus, derived from the P3HR-1 line, fails to induce DNA synthesis and also fails to immortalize [60, 66]. Prevention of DNA synthesis by cytosine arabinoside completely prevents the induction of EBV-determined nuclear antigen (EBNA) in normal lymphocytes by transforming strains of the virus. EBNA induction is the first step on the way towards immortalization (Ernberg, I. and Eimhorn, L., to be published).

EBNA is a virally determined or virally altered chromosomal protein, the only known viral product expressed in all EBV-DNA-carrying cells, independent of virus production. We have recently found that the amount of EBNA per nucleus is directly proportional to the number of EBV genome copies per cell [13]. Moreover, EBNA seems to be an autonomous function of the viral genome. It is fully expressed e.g. in human/mouse hybrides that are otherwise completely non-permissive and non-inducible with regard to all other EBV-determined functions [43]. Also, EBNA is a DNA-binding protein, a fact that was widely utilized for concentration and purification [4, 51, 56a] and also for the definite demonstration that EBNA was identical with the previously known, EBV-associated complement-fixing soluble antigen. Following concentration and purification, the CF antigen was added to acid-fixed nuclei of various EBNA-negative cells. Brilliant EBNA-specific staining was obtained, both with metaphase chromosomes and interphase nuclei [77].

The autonomous expression of EBNA, together with its DNA-binding properties, raise the question whether it might be a virally determined regulatory protein. The most interesting possibility is that it may play a role in preventing the multiple EBV genomes from entering the viral cycle, a process that inevitably leads to cell death. Repression of the viral cycle is an obvious prerequisite for prolonged viral latency *in vivo* and for the multiplication of established EBV-carrying cell lines *in vitro*.

EBV-DNA- and EBNA-carrying lymphoblastoid cell lines can be regularly established from the peripheral blood or lymph nodes of EBV-seropositive donors, but not from seronegatives [10, 72]. EBV can also transform the B lymphocytes of certain simian hosts [15, 64]. Some of the derived lines can grow progressively after reimplantation and kill the original, autochthonous host [52, 91]. In marmosets [62, 93, 96] and owl monkeys [12] the virus also has a direct oncogenic activity. The induced lymphomas carry the viral genome and contain EBNA.

EBV infects the majority of all adult human populations in all countries [24, 70]. Its seroepidemiology resembles other horizontally transmitted viruses, with the regular presence of passively transmitted antibody in the newborn, its subsequent decline and the reappearance of actively induced antibody after infection. The timing and extent of seroconversion are strongly related to socioeconomic status. In low socioeconomic groups, infection occurs during early childhood, as a rule. It is not accompanied by recognized disease, and the route

of transmission is unknown. Only a minority of young children become infected in high socioeconomic groups where a later (teen-age) infection predominates. Although at least half the teen-age infections appear without recognized symptoms, primary infection is accompanied by heterophile-positive infectious mononucleosis in the other half [14, 25, 28, 69].

Despite the unclear pathogenesis of the infectious mononucleosis syndrome, infectious (transforming) EBV can be regularly recovered from throat washings of patients with the disease [20, 21, 65, 80]. During the acute phase, these patients' peripheral blood contains specific killer T lymphocytes that can lyse EBV genome-positive but not EBV-negative target cells [3, 93]. In parallel, large blast cells appear in the B cell fraction, containing the EBV-determined nuclear antigen, EBNA [48]. Part of the infectious mononucleosis syndrome may reflect an acute rejection reaction against virally converted lymphocytes. Recently, we have examined the lymphoid of a three-year-old girl who died of acute IM. Her thymus, tonsils and spleen contained up to 19% EBNA-positive cells. Her T cell showed only a weak activity against EBV-carrying lines in cytotoxic tests [6]. All this supports the concept that, under normal conditions, a T cell-mediated rejection reaction stops what could otherwise develop into a lethal proliferation of virus-carrying B cells.

Two human neoplasias, nasopharyngeal carcinoma and African Burkitt's lymphoma show a remarkably consistent association with EBV both by serology [26, 27] and by EBV genome tests [55, 75, 107]. African Burkitt's lymphoma can be regarded as the neoplastic proliferation of an EBV genome carrying clone [16] in 97% of the cases [41]. The very rare cases of Burkitt's lymphoma occurring outside the high endemic regions of Africa do not show a similar association with EBV either by serology [54] or EBV genome tests [78], as a rule. Recently, however, five EBV genome-carrying European and American Burkitt lymphomas have been identified, comprising approximately 15 to 20% of all similarly examined cases [105]. Since the histopathological diagnosis of Burkitt's lymphoma is not based on unequivocal criteria, it remains to be seen whether the EBV-carrying and the EBV-negative Burkitt lymphomas represent the same disease. This can only be decided by correlated and, eventually, prospective EBV-related, histopathological and clinical studies.

Like the EBV-carrying established lines, biopsies of African Burkitt's lymphoma carry multiple copies of the viral genome per cell [55, 75, 107]. Part of these genomes exists in a covalently closed, free circular form [1], whereas another part is integrated with the cell genome. The biopsies contain the same type of circles as established lines [1, 38].

EBV-carrying Burkitt's lymphoma cells grow into established *in vitro* lines more readily than explants from infectious mononucleosis or normal seropositive donors. In the majority of the cases studied, the clonal characteristics of established lymphoma-derived lines correspond to the *in vivo* clone [40], but contaminating EBV-positive B cells occasionally can overgrow the lymphoma cells [17]. Representative lymphoma lines differ from "lymphoblastoid lines", i.e.

in vitro EBV-transformed cells and lines derived from non-lymphomatous sources, with regard to a number of morphologic, functional and growth characteristics [71]. Lymphoma lines are relatively uniform, but lymphoblastoid lines show great heterogeneity. EBV-carrying African Burkitt's lymphomas are already uniclonal *in vivo* [16], whereas lines derived from normal donors are polyclonal [5]. The diversity may explain some of the differences. Alternatively, lymphoma development may involve the appearance of a special neoplastic cell type, not present in EBV-transformed normal lymphocyte populations. The latter possibility is supported by the recent observation [33, 57, 104] that a highly specific chromosome-14 translocation can be found in biopsies of Burkitt's lymphoma and derived lines but is not observed in EBV-carrying lymphoblastoid lines of non-lymphoma origin. The same 14q + marker has also been found in a number of other B cell-derived neoplasias [58]. The implications of these findings are discussed in more detail below.

Some EBV genome-negative B-type lymphomas have been established as continuous lines [44], but only with considerable difficulty. The easy overgrowth of EBV-carrying normal cells is one of the main problems. EBV-negative lymphoid lines have never been established from normal tissues, however. Human lymphocytes can probably only grow as established lines if they are derived from a lymphoma or if they carry the EBV genome or both. African Burkitt's lymphoma is the only known condition in which lymphoma derivation and EBV-positive status coincide.

This unique position of Burkitt's lymphoma as the only known EBV-carrying lymphoproliferative neoplasia in man, together with the known transforming and oncogenic properties of the virus, keep EBV as an important aetiologic candidate for oncogenesis. Before accepting that a causative association may exist, alternatives must be considered, however. Numerous early commentators have favoured the "passenger hypothesis". This idea implies that lymphoma cells, arising in EBV-carrying persons for EBV-unrelated reasons, pick up the virus as a passenger, just like normal lymphocytes, carry it along as they proliferate and thereby increase the antigenic load and induce antibody production. However, the very fact that non-Burkitt lymphomas that arise in EBV-seropositive patients do not pick up the virus is in itself a strong argument against this idea. It may be argued that such lymphoma cells are insensitive to EBV infection. However, some of the EBV genome-negative lymphoma lines, derived from EBV-negative Burkitt-like lymphomas that have arisen in seropositive patients could be infected with EBV *in vitro* [45], followed by permanent conversion into EBV-DNA- and EBNA-carrying lines [8, 47, 48]. This finding confirms that EBV-sensitive lymphomacells that arise in seropositive patients do not necessarily become infected by horizontal virus spread *in vivo*, presumably owing to the regular presence of neutralizing antibodies. It also suggests that EBV genome-positive Burkitt's lymphomas originate from a genome-carrying cell. If this speculation is accepted, we are left with essentially two interpretations that are usually referred to as the immunologic and the co-factor hypotheses.

Arguments can be found for both interpretations. Lines derived from *in vitro* EBV-transformed normal lymphocytes can have a neoplastic potential, although we have recently found that EBV-transformed diploid lines of recent origin do not kill nude mice, whereas long-established, aneuploid lines do [73]. Earlier, Nilsson and Pontén [71] showed that EBV-carrying lymphoblastoid lines derived from normal donors or from benign conditions, including infectious mononucleosis, differed from EBV-carrying, Burkitt's lymphoma-derived lines with regard to a whole series of morphologic, functional and growth characteristics.

Perhaps the most important difference lies in the cytogenetic constitution of the BL lines, as contrasted to EBV-carrying lines of non-neoplastic origin. It was originally discovered by Manolov and Manolova [57] and recently confirmed and extended by Jarvis et al. [33] and by Zech et al. [104] that definite chromosome differences exist between normal and lymphoma-derived lines. Normal diploid lines were only found among EBV-carrying lymphoblastoid lines derived from non-Burkitt's lymphoma donors. In contrast, all lymphoma lines so far examined were characterized by various chromosomal anomalies. The majority contained the characteristic chromosome-14 marker, recently identified as an 8−14 translocation [104]. Thus, EBV is fully capable of "immortalizing" B lymphocytes with a normal diploid karyotype *in vitro*, but EBV-carrying lymphomas *in vivo* involve genetic changes in addition, probably of a rather specific kind. Such an interaction of viral transformation and cytogenetic changes is not as unique as it may appear at first sight. There are many experimental examples showing that known oncogenic viruses do not transform all or even the majority of appropriate target cells. Transformability is dependent on an obscure but probably specific "state of competence". Competence may be determined by the differentiation state or the genetic constitution of the cell (or both). The importance of the genetic constitution is emphasized by increasing evidence [49, 53, 67, 76, 92, 100] that both chemically and virally induced tumours may display highly specific chromosomal changes, different from tumours induced by different agents.

Some forms of viral oncogenesis may require specific genetic changes as a prerequisite for full development of cancer *in vivo*. Cells with a normal karyotype possess regulatory mechanisms that could counteract the neoplastic change, even in cells that contain integrated genomes of a potentially oncogenic virus. Basilico's temperature-sensitive transformants [86] exemplify a situation in which a cellular function can influence the phenotype of virally transformed cells. The recent experiments of Melero et al. [59] on DNA-binding proteins in normal and transformed cells are another case in point. Somatic cell hybridization experiment [23, 42, 97] have shown that the highly malignant behaviour of established, polyoma-induced or other tumours could be suppressed by fusion with normal diploid cells.

The importance of cellular genetics for virus-induced neoplastic transformation is also illustrated by the wholly unexpected finding [34, 39, 68, 81, 83, 89, 94, 95] that SV40 transforms with a higher efficiency if the target cells are derived from hosts with a known tendency for increased chromosomal variation or mitotic

anomalies (e.g. Fanconi or Klinefelter syndromes, ataxia-teleangiectasia, or xeroderma pigmentosum). The increased SV40 transformability of normal diploid human fibroblasts with ageing and accumulating chromosomal aberrations may be a further example.

Zech et al. [104] have recently found that the Burkitt's lymphoma-associated chromosome-14 translocation was also present in some EBV-negative Burkitt's and non-Burkitt's lymphomas, but never in EBV-transformed cells derived from normal donors or from patients with infectious mononucleosis. This cytogenetic change may somehow promote transformation to a malignant lymphoma. A relation between abnormal lymphocyte growth and structural rearrangement of the long arm of chromosome-14 is also suggested by recent studies of cells from patients with ataxia-teleangiectasia [58a]. Since a minority of African, EBV-carrying Burkitt lymphomas lack the chromosome-14 translocation [33, 57, 104] (although they have other chromosomal anomalies), the visible manifestation of this particular translocation is not an absolute requirement for the development of full-fledged lymphomas. A similar situation may exist in chronic myelogenous leukaemia where the Ph_1 chromosome (another translocation) is present in the majority but not all the cases.

Studies on the genetics of experimental carcinogenesis provide ample evidence [30, 84] that genetic factors may influence the probability of neoplastic transformation at the level of the target cell itself. Thus, a given genetic change – here expressed by the chromosome-14 translocation – may influence the probability of lymphoma induction by EBV and also by other, as yet unknown agents. This idea does not preclude the possibility that EBV and other agents may occasionally induce lymphomas in the absence of this particular genetic aberration.

A discussion about the possible role of EBV in human neoplasia would not be complete without considering the relation between EBV and nasopharyngeal carcinoma (NPC). In contrast to Burkitt's lymphoma, there appears to be no major geographic variation in the EBV-carrying status of NPC [9, 75, 90] but there is a striking histologic restriction. Only poorly differentiated or anaplastic tumours have been found so far to carry the viral genome [Adams et al., in press]. In contrast to earlier interpretations it is now clear [46, 98, 99] that the viral genomes are not carried by the tumour-infiltrated lymphocytes (largely T cells) [36] but by the carcinoma cells themselves. They also express the EBNA antigen [32, 46, 98]. Recent preliminary evidence suggests that the NPC-associated genome may be slightly different from the Burkitt's lymphoma-associated genome. Pagano [78, 79] found that certain sequences were missing from the EBV-DNA of a Tunisian NPC as compared with a Burkitt's lymphoma-derived viral probe. In two nude mouse carriers of NPC, purified from infiltrating human lymphocytes by heterologous passage, Kaschka-Dierich et al. [38] found covalently closed EBV circles. It remains to be established whether these circles have the same characteristics as the Burkitt's lymphoma-associated circles. If they are different, this may be due to a variation in the virus strains associated with the different individual donors, in analogy with the mo-

lecular variations between different herpes simplex isolates [87], or may have a disease-associated importance.

At this stage, the NPC-EBV relation raises many interesting questions. The exclusive and regular presence of the viral genome in one histologically distinct tumour type, independently of geographic location and high or low endemicity, strongly suggests that the association must have some aetiologic relevance, but it is impossible to state a preference for a causative versus a promoting relation. Genetic factors are known to play an important role in some high endemic ethnic groups, Chinese in particular [31]. In addition to the possible existence of an NPC-associated viral subtype, it would be important to obtain some information about the EBV susceptibility of the normal progenitor cell in the nasopharyngeal epithelium. It would not be surprising if a co-operative interaction of viral transformation and host-cell genetics eventually emerged in this case as well. If so, the picture may resemble the situation postulated above for Burkitt's lymphoma, at least in principle, although the details may be quite different.

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Lymphocyte Activation by Con A*

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Con A can activate cytotoxic effector cells, helper cells and suppressor cells. In all cases Con A activates polyclonally and thus the entire T cell repertoir can be revealed by Con A activation. Nylon purified T cells cannot be activated by Con A. However, in the presence of helper cells, such as adherent cells, or in the presence of serum from several species, Con A activates purified T cells. The serum factors that are responsible for Con A activation of purified T cells are present in the albumin fraction. It is suggested that Ia molecules may be responsible for the ability of Con A to activate purified T cells and a general hypothesis for T cell activation is outlined.

Introduction

Phytohaemagglutinin (PHA) was the first mitogen found to induce lymphocytes into cytotoxic effector cells [5, 6]. At this time T and B cells had not been discovered. It was not until it was found that PHA and Con A were selective T mitogens and LPS and a series of other mainly bacterial substances were selective B cell activators [2, 4] that a detailed study of the mechanism of action of these mitogens for activation of T and B cells could be carried out. This paper will deal with induction of T cells into cytotoxic effector cells, helper or suppressor cells as well as with general problems concerning the mechanism of T cell activation. The literature of the field will not be reviewed; rather we will focus on pertinent findings made in our department.

Induction of cytotoxic effector cells

Figure 1 is the first demonstration of the selective effect of Con A and LPS on induction of DNA synthesis and cytotoxic effector cells [2]. As can be seen Con A was capable of inducing DNA synthesis and cytotoxicity in T cells, both phenomena exhibiting the same dose response curves, whereas LPS only induced DNA synthesis and not cytotoxicity. It was also shown that Con A only activated T cells and LPS only B cells.

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Subsequently, it was shown that Con A could also activate T cells in vivo. Thus, injection of Con A into mice resulted in the appearance of cytotoxic effector cells [1]. It was soon established that Con A induced cytotoxic T cells exhibited specificity in their killing action (Figs 2–4). Cells from Con A injected mice, as well as in vitro Con A activated cells that had been treated with competing sugars to remove membrane bound Con A, were incompetent to kill syngeneic target cells, but killed all H-2 incompatible or semi-allogeneic target cells [12]. However, if Con A was added to the mixture of activated T cells and target cells,

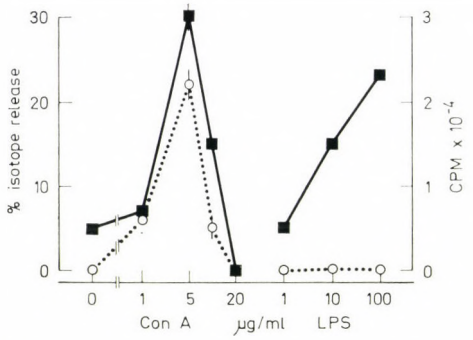

Fig. 1. Induction of DNA synthesis (■—■) and cytotoxicity measured as percent isotope release from ^{3}H-labelled mouse fibroblasts (○···○) with Con A and LPS added to normal mouse lymphocytes

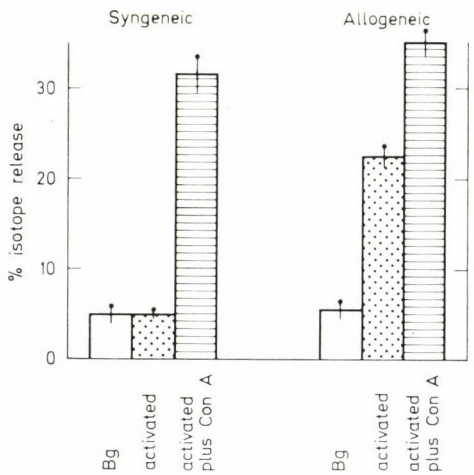

Fig. 2. Cytotoxic response of syngeneic (strain A) and allogeneic (B10.5M) activated or normal (Bg) spleen cells. Cells were activated by in vitro incubation with 5 µg Con A/ml for 48 hours before being treated with alpha-mannopyranoside and set up in the assay system. The normal cells were treated similarly, except that Con A was not added to the incubation flasks. The activated cells were set up with or without Con A in the assay system

the specificity for allogeneic H-2 incompatible targets disappeared and now killing was observed also with syngeneic target cells. This confirms the previous finding that cytotoxic effector cells generated in a MLC reaction could exert cytotoxicity also on syngeneic targets in the presence of agglutinating doses of Con A [3]. Thus, Con A has two properties: 1. to activate T cells and 2. to agglutinate cells.

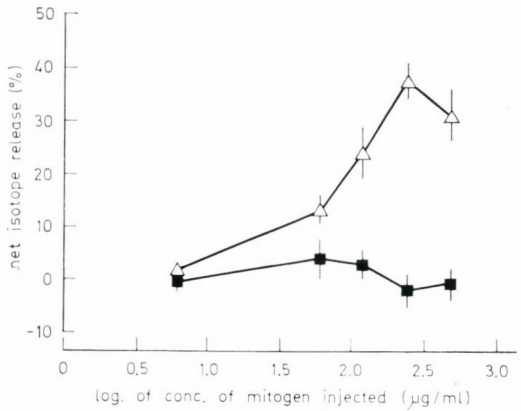

Fig. 3. Net cytotoxic response of spleen cells taken from strain A mice 24 hours after the i.v. injection of different doses of Con A with (△—△) or without (■—■) the addition of 20 µg Con A to the assay system using target cells of strain A origin

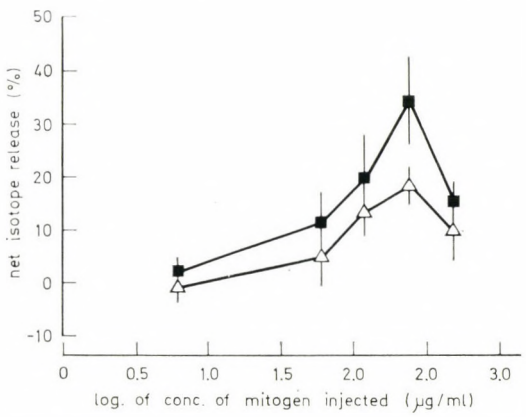

Fig. 4. Same as in Fig. 3, but spleen cells from B10.5M mice were used

Con A activates T cells polyclonally and the activated cells can exert their killing ability when bound to the target cells. In allogeneic situations binding is achieved by T cell receptors, which specifically recognize foreign H-2 antigens. However, binding can also be achieved by adding agglutinating agents, such as

Con A and PHA, when the activated T cells do not possess receptors for the target cells, such as may be the case in syngeneic situations. This demonstrates that killing is non-specific, but requires close contact between target and effector cells. The findings suggest that there exist T cell clones with binding receptors for all allogeneic H-2 specificities, but not for self H-2. However, the latter conclusion may not be valid, as will be discussed below.

It was also studied [11], whether Con A activated T cells from animals made tolerant to H-2 incompatible mice would exert a cytotoxic effect on target cells derived from mice of the tolerated genotype. Tolerance was induced by neonatal injection of spleen cells and was tested with skin grafting. As shown in Fig. 5, there was no difference in cytotoxicity between T cells from untreated

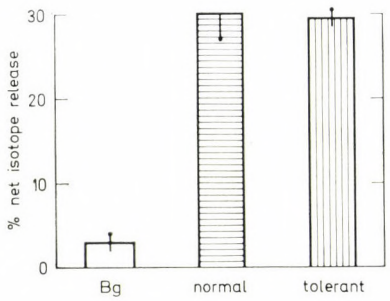

Fig. 5. Percent net isotope release from spleen lymphocytes activated in vivo with Con A. The spleen cells were derived from normal strain A mice, from normal CBA mice and from CBA mice tolerant to skin grafts from strain A. The target cells were YAC tumor cells of strain A origin

and specifically tolerant animals when tested on target cells of the tolerated genotype. This may suggest a fundamental difference between tolerance induction to self antigens and experimentally induced tolerance to foreign H-2 antigens. However, it is possible that technical reasons can account for the failure to detect autoreactive T cells. Since the cytotoxic assay always involves an excess of T effector cells over the number of isotope labelled target cells, the phenomenon of cold target cell inhibition may be involved. If self reactive T cells existed they would have a much greater chance to react with non-labelled autologous lymphocytes than with the labelled target cells and therefore cytotoxicity would be undetectable. In the case of experimentally induced tolerance achieved by establishing a very low level of chimerism, this phenomenon would not operate. The final answer to the question whether self-reactive T cells normally exist in a resting state in the body requires experiments with limiting dilution assays and expansion of individual clones to allow experiments in which cold target cell inhibition would not be equally efficient. As yet this has not been done.

Introduction of helper and suppressor cells

Con A is not only capable of inducing cytotoxic effector cells, but also all other subsets of T cells, such as helper and suppressor cells. Figure 6 shows the first published experiment demonstrating this [2]. In these experiments spleen cells from nude mice were cultured in the presence or absence of sheep red cells (SRC) and the primary immune response to SRC was studied. Nude cells do not respond to SRC by themselves, but in the presence of increasing numbers of Con A activated thymocytes they aquire the ability to respond as cells from normal animals. However, the addition of higher numbers of Con A activated T cells

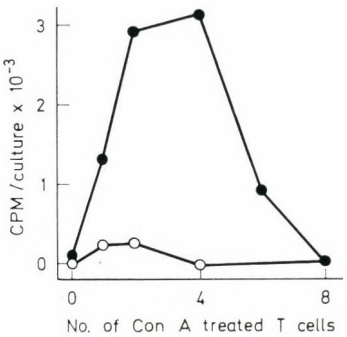

Fig. 6. Reconstitution of the immune response of B cells by thymocytes stimulated by Con A. The thymocytes were cultured for 24 hours with 5 μg/ml of Con A added at the beginning of the cultures (●—●) or 1 hour before harvest of the cells (○—○). Before addition to the B cells the thymocytes were treated with methyl-alpha-D-mannopyranoside to remove Con A

caused a gradual suppression of the response. This phenomenon has been interpreted in different ways. The suppression has been ascribed to induction of suppressor cells or, alternatively, to the supply of too much help, causing a suppression of the response, in analogy with the dose response curves observed with all polyclonal T and B cell activators, which activate at certain concentrations and turn off the response at higher concentrations. Probably both mechanisms can operate in different experimental situations. Thus, it has been shown [7] that the culture conditions are very important for the induction of helper and suppressor phenomena. A certain dose of Con A, which caused marked suppression of a primary response to SRC under optimal culture conditions, was shown to induce help under suboptimal conditions (such as the use of "bad" batches of fetal calf sera for supplementation of the cultures). In this case it is likely that suppression was due to too much help. Conversely, with other concentrations of Con A, it was consistently observed that only suppressor cells were induced, suggesting that Con A only induced suppressor cells [9, 10]. The main variable that determines this difference appears to be the dose of Con A used. Thus, higher doses of Con A seem to preferentially induce suppressor cells, whereas lower doses

induce T cells that function as helper cells under less than optimal culture conditions, or which work as helper cells in low cell numbers and as suppressor cells in higher numbers. Presumably these cells are always helper cells, which can cause a suppressive phenomenon when added in too high numbers.

Mechanism of T cell activation

The mechanism of activation of T cells constitutes a central question in immunology. When B cell activation was studied, it turned out that the use of polyclonal B cell activators gave important information on the mechanism of activation and it was actually concluded that polyclonal and antigen-specific activation was the result of identical triggering signals. Since polyclonal T cell activators, such as Con A and PHA, have been shown to activate all subsets of T cells and within each subset, to reveal all immunological specificities, it seems plausible that the mechanism of T cell activation by mitogens will also be identical to activation by specific antigens.

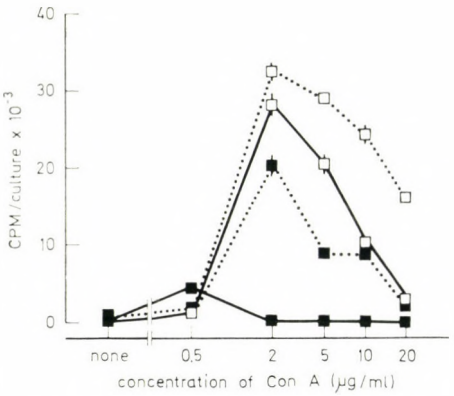

Fig. 7. Sera from different sources support the T cell response to Con A. Nylon wool column (0.6 g of wool) purified T cells from C3H mice were either cultured in the absence of serum (■—■), with 5% mouse serum from ATL mice (■ · · · ■), 5% fetal calf serum (□—□) or 5% human A serum (□ · · · □). Various concentrations of Con A were added to the cultures and DNA synthesis was measured on day 2

Rather surprisingly it was found that nylon wool purified T cells were not activated by Con A to DNA synthesis as shown in Fig. 7 [8]. However, the response could be restored in several ways, such as the addition of adherent cells as well as by serum from mouse, fetal calf and humans. The same number of Con A molecules bound to purified T cells whether they could respond to Con A or not. Since T cells could be made responsive to Con A by a short incubation in serum, it follows that the substances (receptors) responsible for T cell activation by Con

A are passively acquired. This was substantiated by the demonstration that serum pretreated Con A responsive cells lost the ability to respond after treatment with trypsin, but could again be made responsive by addition of serum. Most likely the restoring factor constitutes a very small part of the Con A binding substances on T cells, actually too small for detection with labelled Con A and the substances are trypsin sensitive proteins.

Further analysis established that the albumin fraction, but not the immunoglobulin fraction of serum was responsible for restoration (Fig. 8). A contaminant

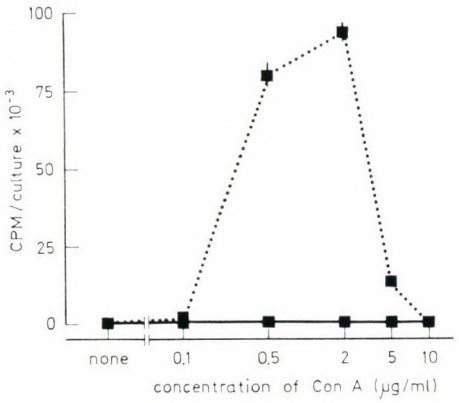

Fig. 8. Human albumin supports the T cell response. Column passed T cells from CBA mice were cultured in medium alone (■—■), with human albumin, human transferrin or human gamma globulin (■···■). All proteins were used at 10 mg/ml and the DNA synthetic response was determined at day 2

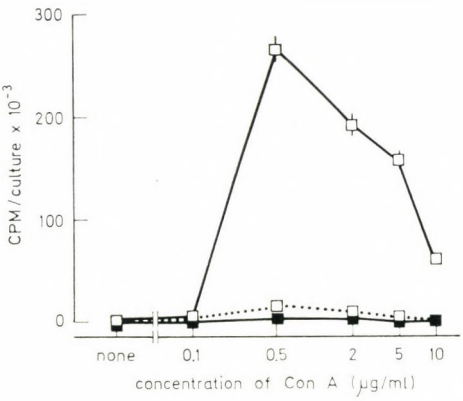

Fig. 9. The helper factor in human albumin is absorbed by nylon wool. Column purified T cells from C3H spleen cells were cultured in medium alone (■—■), or in human albumin (10 mg/ml) that was either unseparated (□···□) or passed through a nylon wool column (□—□). Con A was added and the DNA synthesis was determined at day 2

of the albumin fraction, such as transferrin, could not restore. The observation that albumin, which has been passed through nylon wool, cannot support the T cell response to Con A (Fig. 9), may explain the paradox that T cells in serum lose Con A responsiveness after passage through the column.

Although not yet conclusively demonstrated, it seems possible that Ia molecules may be the factors which mediate restoration of Con A responsiveness. Thus, the cell types and the serum sources that restored responsiveness of purified T cells all contained Ia, such as macrophages, B cells and serum. It has been demonstrated that Ia molecules in serum are present in the albumin fraction. Direct experiments to test whether Ia molecules are responsible or restoration was carried out by adding the IgG fraction of anti-Ia sera to cultures of purified T cells mixed with Con A and serum from the appropriate strain have not yielded conclusive results. The general findings have been that antisera affect the dose response curve to Con A in such a way that higher Con A concentrations are needed for optimal activation and usually the antisera are lowering the level of the maximum DNA synthesis.

It is an attractive hypothesis that Ia molecules are responsible for T cell activation. This hypothesis predicts that T cells would possess receptors for Ia molecules. Con A would activate T cells by binding to the sugars present on Ia molecules and stabilize their interaction to the receptors on T cells. In antigen induced T cell activation, the specific antigen-binding T cells would bind antigen present on Ia containing macrophages or B cells and thereby the T cells would be exposed to triggering concentrations of Ia molecules.

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Immune Complexes and Triggering of Cellular Mechanisms*

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Although there is growing awareness of the involvement of immune complexes in many diseases, little is known yet about their mode of interaction at the cellular level. Inevitably, therefore, the emphasis in this paper is on theoretical aspects of this fascinating topic; although, the ideas discussed are based on the one hand on a growing understanding of the molecular basis of mast cell triggering resulting from anaphylactic antibody-antigen interaction; and on the other from work in progress in my laboratory on the role of the antibody in the in vitro triggering of macrophages by antigen-antibody complexes.

Crucial questions which are discussed in considering possible mechanisms of interaction between immune complexes and target cells include the possibility of distinguishing between receptors for monomer and aggregated antibody and between aggregate binding and target cell triggering functions. In other words, are certain types of antigen-antibody complex capable of directly triggering nonsensitized target cells via a distinctive activation site; or is the Fc receptor multi-functional, with triggering merely involving an increase in association brought about by the cross-linking of antibody molecules by antigen?

Introduction

Although there is a growing awareness of the involvement of immune complexes in the underlying immunopathology of many disease states, little is yet known about their precise mode of action at the cellular level. Indeed, is it conceivable that, despite their frequent detection in the circulation of patients with conditions such as nephritis, S.L.E. and rheumatoid arthritis, pre-formed complexes have little to do with the primary initiation of the observed tissue damage?

A definitive answer to such a key question will only be obtained from in vitro studies of the direct effect of complexes on appropriate target cells, under conditions approaching those observed in vivo. Ultimately, it will be essential to test purified immunopathogenic complexes in such systems. But, in the meantime it is necessary to define the factors which influence triggering by immune complexes, using well characterised experimentally produced antibody-antigen complexes (and aggregated immunoglobulin analogues of these) as test substances.

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In this connection, it is worth bearing in mind that all immunological trigger processes mediated through cytophilic antibodies – and potentially, therefore, also through pre-formed antibody-antigen complexes – possess certain important features in common whether the target cell be a macrophage, a lymphocyte or a mast cell. For, in each case, antibody bound to the target cell via Fc receptors reacts with specific antigen to bring about the release of mediators, by means of energy and cation dependent active secretory processes.

When one considers the nature of the primary triggering stimulus involved, several crucial questions come to mind; particularly in relation to triggering by antigen interaction with antibody sensitised cells as opposed to that initiated by pre-formed antibody-antigen complexes (in the case of macrophages: the difference between triggering by the cytophilic and opsonic pathways). For instance, are certain types of Ab-Ag complex capable of directly triggering non-sensitised target cells; or, alternatively, is the Fc receptor multi-functional, i. e. involved in primary antibody binding as well as subsequent target cell triggering? In the latter connection, is it possible, therefore, to distinguish between receptor for monomeric and aggregated antibody?

I am going to put forward the proposition that the findings from studies undertaken by our own and other laboratories during recent years on the molecular basis of anaphylactic antibody-antigen triggering of mast cells and basophils suggest a useful way of considering the mechanism of antibody-antigen triggering of other types of target cells, such as macrophages and neutrophils. And that it might be preferable to consider the possibility that here, too, Fc binding and target cell triggering functions can be separated. In taking this approach, I shall pay particular attention to the question of triggering by *pre-formed* immune complexes (and aggregated immunoglobulin analogues of these); and, inevitably, it has not proved possible to ignore the contribution of complement activation by both the classical and alternative pathways.

1. *Current thoughts about the manner in which anaphylactic antibody-antigen interaction triggers mast cells*

An important point, which deserves re-emphasizing, is that in addition to the regular cell bound antibody-antigen release process there are many other (artificial) ways of effecting triggering; all of which likewise involve the active secretion of histamine and other mediators in the absence of cell lysis, as indicated by no loss of cytoplasmic LDH [12, 13]. These alternative trigger processes rely on the cross-linking of the sensitizing antibody by antiantibody (as in the reverse passive anaphylactic reaction) or lectins (such as concanavalin A); or the direct stimulation of non-sensitised mast cells by incubation with basic polypeptides and other types of basic substance (eg. Compound 48/80), and with preformed anaphylactic antibody-antigen complexes as well as pre-aggregated anaphylactic antibody (e.g. IgE) or its Fc fragment [7]. Included, too, in this latter category are the C3a and C5a anaphylactoxic fragments which are also basic polypeptides [17].

Consequently, if my central tenet is acceptable, namely that all of these triggering processes are acting via essentially the same primary pathway, it follows that the elucidation of the mechanism whereby combination of cell bound sensitising antibody with specific antigen leads to mediator release could at the same time throw light on the manner in which pre-formed antibody-antigen complexes accomplish this. It is, therefore, worth outlining briefly our current views on the multiple functions of the cytophilic antibody in regular antigen-induced mast cell and basophil triggering.

Following our initial demonstration [14] based on inhibition studies with cleavage fragments of myeloma IgE, that the mast cell binding site(s) were located in the Fc region of reaginic antibodies, we have obtained increasing evidence in support of our long standing contention that subsequent bridging of the cell band antibody molecules by antigen (allergen) induces within them a conformational change which brings *separate* Fc-located effector sites into the correct position to trigger complementary activation sites on the mast cell membrane. Moreover, our more recent studies (in collaboration with colleagues at the Beecham Research Laboratories) involving the induction of histamine release from *normal* mast cells by synthetic peptides representative of a human IgE Fc sequence, have suggested that such an effector site incorporates a cluster of basic amino acid residues (which supply a "pulse" of positive charge to the appropriate target cell membrane penetrating potential).

At the cellular level, there is growing reason to believe that this type of antibody-antigen trigger process leads to the opening of a Ca^{++} ion gate [5]; and we have substantial new evidence to suggest that a Ca^{++}, Mg^{++}-activated ATPase located in the outer membrane of the mast cell might well be implicated in such a process [2]. This selective triggering of an α-adrenergic-like receptor, which is accompanied by β-adrenergic receptor triggering leading to cyclic AMP production (and consequent modulation of the release of histamine) is, however, not unique to mast cells; on the contrary many other types of cell triggering seem to involve these two major forms of receptor-ligand interaction [10]. As far as the present discussion is concerned, the question arises as to whether similar types of receptors are involved in other immunological trigger processes, involving other types of target cell; and, more particularly, whether antigen-antibody complexes are capable of stimulating them – possibly in an analogous manner to that envisaged for mast cell triggering (as just described).

2. *The role of cytophilic antibody in the triggering of phagocytic cells*

Although there is evidence that at least some of the features which I have attributed to the immunological triggering of mast cells and basophils are demonstrated also by lymphocytes, whether they be B-cells stimulated by antigen or lectin, or K-cells sensitised with cytophilic IgG antibody, the consideration of the role of the antibody in phagocytic cell triggering is more relevant to the present discussion; in view of the probability that much of the tissue damage en-

countered in the so-called immune complex diseases can be attributed to the non-physiological release of lysosomal enzymes.

The question arises as to what type of immune complexes are involved in these undesirable release processes; and by what mechanism are they exerting their effect at the cellular level? In other words, which types of antigen-antibody complex are likely to be immunopathogenic; and why are these not handled by the body in the normal way, i.e. taken up cleanly by tissue macrophages and circulating neutrophils, and degraded intracellularly? There is plenty of circumstantial and direct experimental evidence to indicate that antibody-antigen complexes and aggregated forms of immunoglobulin are handled in a different way to monomeric antibody. And, in this connection, it is interesting to note that we showed some time ago that a preparation of human IgG rich in dimer was catabolised by rabbits at a similar rate to monomer human IgG [3]. Moreover, we have shown, more recently in collaboration with Dr. E. J. Holborow and colleagues [4], that similar structural requirements could govern the localisation of IgG aggregates in the germinal centres (of mice). Thus, it was found that at least trimerisation of the human IgG must occur before any germinal centre trapping is manifest. Significantly, too, this appears to be the minimum aggregated form of IgG capable of activating the classical complement pathway; and it might be argued that this reflects a requirement for at least one of the IgG molecules to be "cross-linked".

Although we have suggested that such aggregated forms of IgG could be transported to germinal centres via a C3 receptor on the membranes of mobile cells (and are then released with loss of complement); it is conceivable that similar structural requirements govern their subsequent fixation to dendritic macrophages, where the activation of the classical complement pathway could be involved. Indeed, this is in line with the observation of Waldmann and Ghetie [16] that only aggregated IgG is effectively endocytosed by macrophages; and is consistent with the known enhancement of phagocytosis via the opsonic pathway which results from C1 activation.

At the cellular level, there is a growing number of reports of studies on the nature of the binding of IgG to macrophages and neutrophils. Interestingly, only those sub-classes of human IgG which show substantial activation of the classical complement pathway (i.e. IgG1 and IgG3) have been found to bind human monocytes. It should be recognised, however, that there is some controversy about the location of the macrophage and monocyte binding site within the Fc regions of such molecules. We ourselves have found from rosetting inhibition studies that such a site is located in the C_H3 domain of rabbit IgG molecules [15], which is consistent with the findings of Yasmeen et al. [18] for human IgG molecules; and, interestingly, agrees with the findings from MacLennan's laboratory on the location of K-cell binding sites of rabbit IgG antibody directed against target liver cells [9]. On the other hand, Alexander and associates [1] have found guinea pig IgG2 to bind to macrophages through sites located in the C_H2 domain.

It is possible that these apparent discrepancies to the so-called "Domain

Hypothesis", which favours target cell binding sites being located within the C_H3 domain of IgG molecules, will ultimately prove attributable to technical factors. But, to my mind, a far more fundamentally controversial issue concerns the nature of the antibody-receptor interaction; and in particular, the effect on this of pre-aggregation of the antibody molecules (either by polymerisation or cross-linking by specific antigen to form soluble complexes).

Debate on this crucial question has been raging for some years now, between the "enhanced binding" and "conformational change" camps. Indeed, it is almost ten years since Phillips-Quagliata and colleagues [11] published in *Nature* a report of their studies on the mechanism of binding of penicilloyl hapten-antibody complexes to rabbit and guinea pig macrophages and neutrophils, in which they observed that only complexes containing *polyvalent* hapten significantly and consistently increased the binding of antibody over that which they demonstrated in the absence of antigen. They concluded from these observations that enhancement of binding seemed more likely to come from an increased energy of binding resulting from the summed effects of individual binding sites (plus an entropy factor). Although they did concede that an allosteric change brought about by the aggregation of the antibody could not be excluded; and that only large antigen molecules were capable of inducing such a conformational change on interaction with specific antibody.

In this connection, it could be of some significance that in our early studies on antigen-induced conformation change in rabbit IgG antibody molecules, based on both the formation of complex-specific antigenic determinants and optical rotatory dispersion measurement, Henney and I [6] observed what appeared to be an increase in conformational change with the size of the antigen employed. Of course, at that time we were using relatively crude physio-chemical procedures to demonstrate such antigen-induced changes; but, it is worth noting that the subsequent application by other laboratories of more sophisticated techniques (such as measurement of circular polarized fluorescence) has provided further evidence that combination of rabbit IgG antibody with specific *polyvalent* antigen results in a structural change within the Fc region.

What is the potential significance of this as far as the triggering of cells by immune complexes is concerned? As I indicated earlier, we feel that it could be a crucial event in the mobilisation of an effector group within the Fc region of IgE antibody molecules which is responsible for affording the primary trigger process to sensitised mast cells. But, does an analogous antigen-induced conformational change play any role in the triggering of other types of target cells such as macrophages by IgG antibody molecules? One possibility is the induction of a change in intramolecular C_H2 domain spacing (suggested from X-ray data); which is thought to be a consequence of antigen binding by IgG antibody leading to classical complement activation. (Although it should be recognised that in complement mediated *lysis* of target cells, the IgG antibody molecules are directed against membrane antigenic constituents thereby leaving C_H2 domain sites readily accessible for C1q activation.)

Turning to recent experimental observations relevant to this crucial question, as to whether the antigen-induced conformational change of cytophilic antibody plays any part in macrophage interactions, the recent findings of Leslie and associates [8] on the binding of immune complexes of guinea pig IgG2 antibody molecules to homologous peritoneal exudate cells are particularly illuminating. These carefully executed quantitative studies, performed on complexes of different sizes and composition, suggest that enhancement of binding shown by complexed antibody (which was greatest in the antibody-antigen ratio optimal for immune precipitation) was attributable to a co-operative action of multiple Fc-sites rather than to any conformational change.

It seems to me, however, that such observations, important as they are, do not necessarily preclude the possibility that a second target cell membrane activation site distinct from the cytophilic antibody receptor might be involved in phagocytic cell triggering; and that this might be analogous to the site which we think is implicated in mast cell triggering by antigen cross-linking of cell bound anaphylactic antibody and by preformed complexes and aggregates of anaphylactic antibody (in the manner outlined earlier). Moreover, it is conceivable that the unwarranted triggering of such an activation site could be involved in the premature release of lysosomal enzymes, in frustrated phagocytosis situations that could be encountered in immune complex diseases.

David Riches, who is undertaking a PhD research project in my laboratory, has attempted to obtain direct evidence in support of this idea. He has been studying the capacity of pre-formed IgG antibody (rabbit)-antigen complexes and aggregates of IgG (human) to induce the selective release of lysosomal enzymes (β-glucuronidase and β-galacturonidase) from mouse peritoneal macrophages in vitro, i.e. in the absence of parallel release of a cytoplasmic marker, LDH; in the presence and absence of an added complement source. Briefly, we have found that no soluble complexes of rabbit antibody with BSA antigen, nor soluble aggregates of human IgG (of the type mentioned earlier which activate the classical complement pathway, i.e. trimer and above), effected release of lysosomal enzymes either in the presence or absence of complement (in the form of fresh guinea pig serum). On the other hand, insoluble antigen-antibody complexes were capable of initiating the selective release of substantial amounts of lysosomal enzymes; and this capacity was not greatly reduced by excluding added complement from the system. Moreover a similar release process was initiated by incubation of the mouse macrophages with zymosan particles in the presence or absence of added complement.

An obvious interpretation of these data is that the pre-formed insoluble antibody-antigen complex, like the zymosan particles, are activating C3 complement component produced by the macrophages; which in turn is leading to the triggering of the C3b receptors on the target cell. So, in a sense, there is an analogy with the mast cell triggering mechanism which I proposed earlier. For presumably here, too, initial binding of cytophilic antibody to Fc receptors occurs; but it seems that this facilitates the activation of C3 by the large (insolubilised) immune

complex culminating in the triggering of the C3b receptor on the macrophage membrane. In other words, the premature release of lysosomal enzymes – as with the release of histamine from mast cells – appears to require the triggering of a target cell membrane site which is *distinct from the Fc receptor*; but, in the case of macrophage triggering, this process is only accomplished through the expediency of ancilliary alternative complement pathway activation.

This suggestion raises many important questions which will not be finally resolved until the in vitro systems have been refined to an extent which will permit the precise delineation of the contribution of target cell-derived complement components to the immune complex initiated triggering process. And, as mentioned in the introduction, it will be important to study the direct lysosomal enzyme releasing capacity of the so called "immunopathogenic" complexes in such systems. In this latter connection, the potentially unique contribution of anti-γ globulin antibody could prove to be well worth investigating.

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The Biological Role of Immune Complexes

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The biological role of immune complexes (IC) is reviewed. The mechanism of IC elimination, the influence of IC composition on their biological properties as well as on their elimination are discussed. The regulatory effect of IC in immune phenomena is briefly summarized.

Immune complex (IC) formation is an integral part of humoral immune response, for ICs are formed whenever free antigen is present together with the corresponding antibody. The circulating IC level depends on the ratio of IC formation to IC elimination. Under normal conditions, IC concentration does not reach the threshold level detectable by the methods available. Thus, a failure to detect IC does not mean that there is no IC in the circulation. This is important because in IC diseases circulating (and deposited) ICs are sought ever more frequently and it is sometimes forgotten that the presence of IC is concomitant to the normal immune response. Furthermore, it should be remembered that an antigen–antibody reaction in itself does not indicate the destruction and elimination of the antigen. These processes require an activation of the so-called effector functions. Accordingly, immunohomeostasis is ensured through ICs, at least in this relation.

All this shows the biological importance of ICs in maintaining an immunological equilibrium. Obviously, an ever growing body of evidence has supported the view that the activation of important control mechanisms may be attributed to ICs. The present paper is aimed at reviewing the relationship between ICs and effector functions as well as the biological role of ICs. Since in the last years several excellent reviews have been published on the subject, it seems superfluous to discuss all the details.

Formation and elimination of ICs in vivo

In the induction of an immune response, the interaction between antigen and antigen-recognizing receptors is the first step. These receptors are immunoglobulin molecules on the membrane surface of B lymphocytes and immunoglobulin-like structures on T lymphocytes and macrophages [2]. The receptor – antigen interaction itself can be regarded as an IC formation, for complexes detaching from the cell surface play an important role in the cell–cell interactions

taking place in the central phase of immune processes. There are receptor structures capable of recognizing and fixing such complexes on the membranes of T-helper (T_H) cells as well as of B cells. Interactions between these complexes and receptors indicate the transmission of information on the antigen, i.e. the stimulus necessary for the differentiation of antibody-producing cells.

Little is known about these complexes; it has been shown that immune responses are considerably influenced by some of their properties [3]. Antibody formation starts as a result of these cell interactions. It should be noted that the humoral immune response is extremely heterogeneous. Even antigens of very simple structure may induce the formation of antibody molecules highly variable in structure. The heterogeneity manifests itself in the different class and subclass properties of antibody molecules, in their avidity varying during immune response, in their precipitating or non-precipitating character, etc. Antibody heterogeneity depends, to some extent, on the structural properties of the antigen. An antigen may represent a number of determinant groups (haptens), each reacting with a well-defined antibody molecule specifically. Therefore, interactions occur between two heterogeneous systems and the composition, and consequently the biological effect, of the ICs formed during an immune response are continually changing.

The fate of an IC may be of two main types. IC-binding receptors are borne by many different cells, and ICs may react with these receptors. The cell function is usually affected by the IC – receptor interaction, which may represent a negative or positive signal inducing or inhibiting a number of important biological mechanisms. On the other hand, IC formation is a prerequisite of the triggering of effector mechanisms resulting in antigen destruction and elimination. Of the effector mechanisms, the most important is the complement system. Its activation leads to lysis of the cellular antigen or to formation of smaller inactive ICs. ICs may attach to Fc receptor (FcR) or, through the complement component C3b, to the C3b receptors of phagocytes. These interactions may considerably increase the efficiency of phagocytosis and, consequently, the elimination of ICs.

Since ICs of variable composition are formed during an immune response, it is important to know how the biological activation and the elimination of these are influenced by the special properties of the IC components. It is of special interest that an imbalance of IC formation and elimination may lead to pathological processes.

How are the biological properties of an IC influenced by the antigen?

An antigen is composed of a carrier and one or more hapten group(s) on the surface of the carrier. Induction of an immune response requires both hapten and carrier while only the hapten groups participate in the interaction with antibody. Hapten groups are bound to the antigen-binding sites present on the surface of antibody molecules. The fate and the function of an IC are substantially influenced by some properties of the antigen, namely (a) size, (b) hapten number

and heterogeneity, (c) site and mode of administration, (d) thymus dependence or independence, and (e) further biological properties.

As to the size of the antigen, small molecules, being eliminated rapidly, are less effective than larger ones. The valency of the antigen is a more essential factor. A univalent antigen possesses only one determinant per molecule, i.e. it cannot react at a given time with more than a single antibody molecule or antigen-combining site. A multivalent antigen, on the other hand, has more than one binding sites. Obviously, IC formation is influenced by the valency of the antigen. The structure, the number and the density of determinant groups all are very different. Owing to repeated identical structural units, polysaccharide antigens are multivalent, yet they react only with a homogeneous population of antibody molecules. In contrast, the polypeptide chains of a protein antigen may bear determinants different in structure (e.g. A, B and C determinants); an antiserum raised against such antigens may contain antibody to each of the determinants (anti-A, anti-B and anti-C). The protein behaves as a multivalent antigen against such antisera and thus several kinds of antibody may be involved in the resulting IC. A single protein molecule interacts as a univalent antigen with an antibody population produced against one of its determinants. If an antibody specific for one of the determinants (e.g. anti-A) is isolated from the above-mentioned mixture and mixed with the antigen, only soluble IC will be formed, whereas the antigen will be precipitated by the original antiserum. In other words, an antigen bearing more than one kind of determinant behaves as a multivalent antigen against an antiserum representing many specificities, but it behaves as a univalent one in interactions with an antibody specific for one of the determinants. As regards valence, even the size of the antigen is of importance. The albumin molecule, the haemocyanin molecule and a bacteriophage bind 8, >200 and nearly 2000 antibody molecules, respectively.

The immune response and some properties of the ICs are considerably influenced by the dose of the antigen. It is well known that small and extremely large doses induce tolerance. Furthermore, after the organism has been invaded by the antigen, the antigen/antibody ratio is continually changing. Initially, the antigen is in excess, then, parallel with antibody formation, free antigen declines until an equivalence and, at last an antibody excess is reached. Accordingly, ICs are formed first in antigen excess, then in equivalence and at last in the presence of antibody excess. Quantitative precipitation assays *in vitro* have shown that the solubility of an IC is determined by the ratio of the antigen to antibody of which the IC is composed. The ICs tending to precipitate are eliminated easier than the soluble ones. The affinity of the antibodies is also influenced by the dose of the antigen (see below). In conclusion, the antigen/antibody ratio is highly dependent on the phase of the immune response in which the IC was formed and the mode of activation of effector functions depends on the IC composition.

The class, or subclass, of the induced antibody also depends to some degree on certain properties of the antigen [5–7]. This is of importance because the biological effect of an IC may be determined by the class of the involved anti-

bodies; e.g. the anti-dextran and anti-laevan molecules are of the IgG2 subclass, the anti-D molecules of the IgG1 or IgG2 subclass, and all anti-I specificities are of the IgMK class.

Thymus dependence or independence is a further interesting property of the antigens. The great majority is thymus-dependent; in the recognition of these and in the initiation of the corresponding immune response, T lymphocytes dominate. The T-independent antigens are linear polysaccharide molecules composed of repeated units of identical structure. Thymus dependence indicates participation of T_H cells in the initiation of antibody response and this participation manifests itself with the formation of antibodies of different classes [8]. Thymus-independent antigens directly stimulate B cells bearing the antigen-recognizing sIg of the IgM class and induce the production of IgM antibodies showing limited heterogeneity [9].

The fate of antigens may be influenced by their further properties as well. Some antigens themselves are capable of activating the complement system even if they are incorporated in an IC [10].

How are the biological properties of ICs influenced by antibodies?

Antibodies are proteins of dual function. Recognition and fixation of an antigen are specific functions taking place in antigen-binding sites in the domains containing variable sequences. Initiation of the effector mechanisms, on the other hand, is the function of the domains containing constant segments, i.e. to the Fc part of the immunoglobulin molecule [11]. It is of great importance from the aspect of antigen destruction and IC elimination that the native antibody molecule is unable to initiate effector functions. Activation of the classical pathway of the complement system as well as attachment to receptor structures need immunoglobulin present in the form of IC (FcR on the membrane of macrophages and of mast cells may interact even with native antibody molecules). The antigen–antibody reaction leads to changes in the antibody molecule which are prerequisites of effector functions. The nature of the antibody changes is still unclear. Some authors postulate conformational changes in the antibody molecule as a result of which hidden groups able to activate effector functions become exposed (conformation model). According to others, antigen fixation is accompanied by a dislocation of the Fab parts of the Y-shaped molecule and a consequent change in the angle enclosed by the Fab and Fc parts may be related to the activation of the effector mechanism (distortion model). A third model (association model) regards association of several antibody molecules as a prerequisite of effector stimulation. Accordingly, IC formation would include two types of linkages between antibody molecules, *viz.*, the antigen, while reacting with the antigen-binding sites of antibody molecules, would bind these molecules together through their Fab parts (primary interaction); thus, antibody molecules come close to each other and may interact *via* their Fc parts. The latter interaction, i.e. secondary fixation between antibodies, would be a prerequisite of further phenomena such as the

formation of the lattice structure characteristic of ICs, activation of the complement system *via* the classical pathway and interaction with single FcRs [12]. In experiments using bivalent haptens we could show the importance of the secondary interactions in the activation of the classical pathway of the complement system since complement activation required complexes each containing at least four antibody molecules although we could not exclude the possible role of distortion in the complement activation [13].

In the dynamic changes of the compositional and functional properties of the ICs formed during the immune response (see above), antibodies play a considerable part.

The class and subclass characteristics are determined by the constant sequences of the heavy chains. Since the groups co-operating in the initiation of effector functions are located in the constant domains of the immunoglobulin molecules, there is an essential difference between classes, and also subclasses, in the complement-fixing and cytophilic properties of the antibody molecules [11]. The complement system, e.g., is activated well by IgM, IgG1, IgG2 and IgG3 antibodies and fixation of IgM, IgG1, IgG3 and IgE antibodies to certain FcRs is well known. Therefore, the class (and subclass) properties of the antibody component of an IC cannot be neglected with respect to the fate and biological function of the IC. In the case of T-dependent antigens, as it is well known, formation in the course of primary immune response of IgM antibodies is switched over into IgG ones of identical specificity.

The composition and the functional properties of the IC are considerably influenced by the phenomena called maturation and degeneration of the immune response. The antibodies formed in different phases of the immune response are different in both avidity and specificity.

In the course of immunization with an antigen of complex structure, the antibodies interacting with different antigen determinants appear at different times. The avidity of an antibody depends on the time elapsed between the injection of the antigen and the formation of the antibody. In general, late antibodies are more avid than early ones, a phenomenon called maturation of immune response. It is postulated that in the early stage of an immune response, when the antigen concentration is high, mainly those antibody-producing cells are stimulated which possess low-affinity antigen-recognizing sites on their membranes. Later, when the antigen concentration is declining, the antigen will be bound by cells bearing receptors of higher affinity [14].

Early and late antibodies differ from each other in specificity as well. There is an inverse relation between avidity and specificity; late antibodies interact not only with the antigen that was used in immunization, but also with a number of antigens similar in structure. Even memory cells bearing receptors of high affinity are stimulated by cross-reacting antigens. The decline in specificity in the course of immune response is designated as degeneration of immune response [15]. Therefore, ICs may contain, besides the immunogen, related antigens.

The majority of antibodies are of precipitating property, they form a precipi-

tate when mixed with the antigen. However, in the late stages of the immune response non-precipitating antibodies are also formed continually. These make up 10 to 15% of the whole antibody population. The ICs containing such antibodies are always soluble, consequently, they may be more pathogenic than those containing precipitating antibodies. The structural characteristics responsible for the lack of precipitating capacity of the non-precipitating antibodies are unknown. Since some of these antibodies show a high affinity to antigens, it has been postulated that both of their antigen-combining sites bind to the same antigen and thus they do not keep two antigens together, which would be a prerequisite of precipitation. This phenomenon is called monogamic bivalence [16]. On the basis of other experimental evidence, the non-precipitating property has been attributed, especially in the case of large antigens, to differences in affinity between the two antigen-binding sites of the same antibody molecule [17].

Dynamic changes in the IC composition in the course of immune response

It may be concluded from the foregoing that during immune response ICs are continually formed and the composition of the newly formed ICs is continually changing. The conclusion may be summarized as follows.

(a) The antigen/antibody ratio is declining; antigen excess is followed by equivalence and, later, by an antibody excess.

(b) The class and subclass property of the antibody component is changing.

(c) The antibodies to be included into ICs are changing in avidity and affinity; antibodies of low avidity and high specificity are gradually replaced by those of higher avidity and lower specificity.

(d) ICs containing non-precipitating antibody are continually formed; initially, due to the antigen excess, there is no precipitation, i.e., one should reckon with formation of soluble ICs in the course of every immune response.

(e) Our environments are rich in antigens; immune responses are therefore always taking place in an organism. Consequently, ICs of different compositions are present simultaneously.

The question arises how the elimination processes and the functional properties of ICs depend on the composition of the IC.

How are the elimination mechanisms influenced by the IC composition?

The complement system plays the central role in the elimination of ICs. The chain reactions of the IC-activated complement components contribute to the destruction of the antigen on the one hand, and mobilize the cells necessary for IC elimination, on the other. Thus, antigen elimination is promoted by the complement system. As to details of the mechanism, I refer to an up-to-date review [18].

Complement-dependent complex release activity (CRA) is a phenomenon described most recently. It may play an important role in IC elimination [19, 20].

CRA is a triphasic reaction. In the first phase, C3 convertase attaches to precipitated ICs. The process starts in the classical pathway of complement activation, but the alternative pathway contributes to the C3 convertase – C3 reaction. In the second phase, IC-C3 convertase complex interacts with C3 molecules and splits off the latter. Subsequently, C3b fragments are intercalated into the IC lattice *via* their labile binding sites. In the third stage of CRA, small ICs are released from the loosened lattice. Each small IC contains one molecule of C3b. The final result of the CRA is that soluble, but inactive, ICs arise from large, functionally active ones. The role in formation of small IC of C3b incorporation into the lattice has been confirmed by us [21].

In IC elimination, phagocytes, first of all the mononuclear phagocyte system (MPS) and also neutrophils, play an important role. The phagocytosis of antigens is considerably supported by binding to antibody (opsonization). In this process, the antibody-coated antigen attaching to FcRs of cells enhances the phagocyting capacity of the cells. The consequences are similar if the IC activates the complement system. In such cases antigen antibody-C3b complexes are formed, which are then bound to C3bRs of cells and thus exert an opsonizing effect. Antigen elimination is similarly promoted if an antigen activates the alternative pathway without being bound to antibody; in this case, too, C3b binds to the surface of the antigen. (It deserves mentioning that further, non-immunoglobulin-specific, clearance processes also participate in IC elimination.)

The IC elimination processes are substantially influenced by the composition of the IC. The variability of the complement-activating capacity and of the cytophilic property by antibody class and subclass has been mentioned above. The elimination of ICs containing complement-non-activating or non-cytophilic antibodies, the biologic activity of such ICs and, consequently, their pathological role, differ from those of differently composed complexes. This subject needs further elucidation [22, 23].

Since the antibodies formed in different stages of the immune response are different in avidity, the question has emerged of the relationship between avidity and effector functions. Early data on the subject were contradictory [24, 25]. We produced antibodies in rabbits against ovalbumin and human serum albumin and isolated low- and high-avidity populations of antibodies. We failed to show any correlation between avidity and precipitation capacity. A close positive correlation was, however, found between avidity and complement-activating capacity for populations obtained from the same antiserum. In further experiments, the complement-activating properties of soluble and insoluble ICs formed at various antigen/antibody ratios were examined. At moderate antibody excess almost the whole complement-activating capacity was recovered in the insoluble fraction while the soluble complexes formed in the zone of a moderate antigen excess showed a considerable complement-activating capacity [26]. These observations suggest that the antibodies of high avidity, i.e. those formed in a late phase of the immune response, activate the complement system more efficiently than do the early antibodies. Therefore, the late phase favours the effector functions and

antigen elimination. In spite of their lower complement-activation-inducing capacity, early soluble complexes, containing antibodies of low avidity, are capable of inducing the effector functions including complement activation and the consequential complement-mediated tissue damages.

We have examined the influence of IC composition and antibody avidity on the CRA. The precipitate formed in the presence of slight antibody excess was dissolved faster than the slowly dissolving precipitate formed at equivalence or slight antigen excess. This interesting observation suggests that the ICs formed in an early phase of the immune response activate the effector mechanisms less effectively than those formed later. We found, however, no positive correlation between the avidity of IC-included antibodies and the rate of IC dissolution. This observation agrees well with the association model of complement activation. If the lattice formation within IC was related only to the primary binding, the IC formed with high-avidity antibodies should show a weaker tendency to dissolve in the CRA test than do the ICs containing antibodies of low avidity. Our results suggest that mainly the secondary linkages developed between the Fc parts of antibody molecules were opened by the C3b components inserted in the lattice. These linkages do not depend on the avidity of the antibodies. Presumably, the secondary interactions are not only opened by the very strongly bound C3b, but they are also prevented from being newly formed; the inactivity of the small ICs formed during the reaction might be explained in this way [13, 26].

As to the attachment of ICs to FcRs, there is no doubt that this interaction is influenced by some properties of the IC. The correlation between the size of the IC and the rate of elimination *via* MPS is well known. It is influenced by the saturability with ICs of the MPS and the nature of the antigen, e.g. free single-stranded DNA is eliminated from the circulation faster than the same DNA bound to an antigen in the form of an IC. In current investigations, we found a parallelism between the complement-activating capacity of ICs formed at various antigen/antibody ratios and their binding to the FcRs of peripheral mononuclear cells.

In conclusion, the effector-activating capacity of ICs is considerably influenced by their composition. The ICs formed during the early phase of immune response are less active in initiating elimination mechanisms than those formed at a later phase and thus containing antibodies of higher avidity.

Functional activity of ICs

The biological importance of the antigen–antibody fixation and of the activating properties of ICs should, first of all, be sought in destruction and elimination of non-self structures threatening the organism. Furthermore, the role of ICs in the control of immune processes should be considered.

Speaking of ICs we usually think of the complexes resulting from the interaction of antigens with circulating antibodies and, owing to their pathological significance, ICs deposited or formed in tissues are also often mentioned. Owing

to the regulative role of ICs, further structures which may bind, or become components of, ICs should also be taken into account. These are (1) the antibody components of the cell membrane, (2) membrane structures as antigens, and (3) immunoglobulin-specific and IC-specific receptors.

1. *Antibody components of the cell membrane.* The antigen-recognizing receptors of T and B lymphocytes are antibody molecules or other structures containing structural elements of immunoglobulins. The latter, too, contain the variable sequences determining the antigen-binding site, i.e. the sequences determining the so-called idiotypic properties. These receptors, interacting with antigen, may form IC on the cell surface, thus influencing cell functions. Furthermore, ICs formed on the cell surface may detach from the cell membrane and, attaching to the corresponding receptor of other cells (e.g. macrophages) may support the cell interactions of the central phases of the immune response.

2. *Membrane structures as antigens.* Principally, under appropriate conditions, any membrane structure may behave as an antigen and form an IC with antibodies reacting with it on the cell surface, e.g. idiotype determinants of membrane immunoglobulins (sIg) may bind to anti-idiotype antibodies or tumour-specific antigens of tumour cells may interact with the corresponding antibodies. Such ICs, too, may detach from the cell surface. Obviously, structures shed from cells (shedding phenomenon) may also form ICs with circulating antibodies.

3. *Immunoglobulin-specific and IC-specific* receptors. Receptors capable of binding immunoglobulins or ICs are found on the surface of many kinds of cell. The FcRs of macrophages may fix native IgG or IgG as IC component with high affinity, and ICs containing IgG and/or IgM are bound by the FcRs of many varieties of cells. Also C3bR, a receptor reacting with ICs containing the C3b complement component, has been demonstrated on various cells.

The regulatory role of ICs, *viz.* those interacting with cells, formed on the cell surface or detaching from the celll membrane, has been supported by an ever growing body of evidence. Some examples are presented in the following.

The idea that IC formation is a prerequisite of immune response was often suggested. Jerne [34] was the first to suggest that initiation of an immune response requires natural antibodies to co-operate. Antigens bound to the latter form ICs, these are incorporated into phagocytes, and this was thought to be the first step of antibody production. This early form of the selection theory has been questioned by numerous authors. Even the existence of "natural" antibodies, i.e. antibodies requiring no antigenic stimulus to be formed, was not generally accepted until recently. Today natural antibodies are spoken of again (owing to the demonstration of sIg-shedding requiring no antigenic stimulus and of the multispecificity of antigens) and the role of ICs in the initiation of humoral immune response has been proved.

The view that initiation of antibody production, either *in vivo* or *in vitro*, needs the presence of the C3 complement component, has been supported by many literary data. The same is valid for the formation of B memory cells. The phenomenon is related to ICs containing C3, which attach to the C3bRs of dendritic

cells located in lymph nodes. Germinal centres, and thus memory cells, are not formed without such interaction [27, 28].

FcRs, which have IC as ligand, also play an essential role in the control of the function of immunologically competent cells. The function of B lymphocytes is inhibited by large or immobilized ICs. This inhibition manifests itself with a reduction of cell proliferation and an inhibition of antibody formation. Interestingly, the same inhibition ensues when the antigen binds to cellular sIg (recognizing receptor) and the same antigen molecule is interacting with another IgG molecule coming from the circulation. In such cases, an IC is formed on the cell surface and the same IC will be bound to an FcR of the same cell through the Fc part of the IgG component of the IC [29]. Accordingly, different receptor structures may interact with each other *via* IC and the cell function may be altered by these interactions. We have reported on the complement-mediated inhibition of FcRs. It was shown that IC–FcR-binding is prevented by complement components attached to the membrane [30, 31]. We have suggested that the inhibition takes place *in vivo* as well, under suitable conditions.

An increase in the level of a given specificity is well known to be followed by a decrease in the rate of production of antibodies of the same specificity. It was suggested that ICs may contribute to this feedback-type phenomenon, too. This assumption has, however, been disproved.

It has been reported that in the presence of particulate antigens or adjuvants a factor is released from thymocytes; this factor induced cytophilic antibody formation in the presence of antigen. The IC thus formed binds to some spleen cells and stimulates them to turn into helper function. Such a factor appears in the blood of animals immunized with Freund-adjuvated antigen, as soon as 6 hr after injection. The helper function is therefore mediated by the antigen-induced factor and IC [32].

Specific blocking factors (SBF). The function of SBF in the immune response to tumour cells is well known. All humoral factors specifically inhibiting the immune response to tumour antigens are called SBF. ICs play a central role in the blocking phenomenon. They are formed from tumour-specific antigens shed from tumour cells and the corresponding antibodies. They may equally attach to target cells and effector cells, thus inducing their inhibition. Such ICs, when stimulating T cells may, on the other hand, induce production of blocking factor. The SBF molecules thus formed may form IC with antibodies and thus exert a suppressor effect through T cells [33].

The regulation of immune response results from genetically controlled interactions in which T lymphocytes play the primary role. These interactions are positively and negatively influenced by T_H and T_S cells, respectively. Recent studies have proved the dominance of suppressor cells in this regulation. The network theory of immune regulation was developed by Jerne [34]. In the network, like in other cybernetic systems, stability primarily depends on suppressive effects. Antigen-specific and immunoglobulin-specific T_S cells are equally known. The

suppressive, consequently regulative, role of T_S cells has been described in numerous processes.

Allotype suppression is a special case of the regulation of immune response. If an immune serum specific for an allotypic determinant is given to an experimental animal or foetus, the formation of immunoglobulin of the corresponding allotype stops. The phenomenon is related to T_S cells [35].

Idiotype suppression [36] is one of the most important control mechanisms of immune response. Antibodies produced (or formed) against idiotypic determinants of antibody molecules stop the formation of immunoglobulin molecules possessing the corresponding idiotype property. Investigations into idiotypic suppression have gained special importance in the light of Jerne's network theory. On the effect of an antigenic stimulus, antigen-specific T_S and T_H cells are equally formed and the start of antibody production induces formation of antibodies of anti-idiotype specificity. This will induce development of T_S cells bearing idiotype-specific receptors. It may be concluded that the regulation of antigen-induced immune response manifests itself at several levels. As the T_S cells induced by the antigenic stimulus have disappeared, T_S cells interacting with the idiotype determinants of antibodies take over the regulation of the immune response, i.e. the immune response is regulated by ICs in the form of idiotype – anti-idiotype interaction.

The above examples show that the T-cell-dependent regulation and the role of ICs are closely connected phenomena. A number of relations have already been clarified, yet, the whole picture is still unclear. Although antigen – antibody-binding has been investigated since the earliest times of immunological research, most of the data are to be reconsidered in the light of the new knowledge of antigens, antibodies and cell interactions.

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Clinical Significance of Circulating Immune Complexes

Effect of Plasmapheresis*

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1. The tissue damage in immune complex diseases is most probably caused by actively deposited immune complexes. But even in this disease entity circulating immune complexes may lack the capacity to trigger inflammatory reaction.

2. Potentially pathogenic immune complexes may have beneficial effect.

3. In many clinical disorders the high incidence of circulating immune complexes is most probably secondary to the underlying disease. In sarcoidosis, Crohn's disease and in some other chronic diseases the tissue damage togetherwith an impaired function of the mononuclear phagocytic system is responsible for the circulating immune complexes.

4. The incidence of circulating immune complexes is high in different malignancies. The size and composition of the immune complexes change in the course of the disease. The presence of circulating immune complexes in malignancy is most probably due to their impaired elimination.

5. Methods applied for the detection of circulating immune complexes are all indirect assays and thus reveal *presumed* immune complexes. Direct techniques of measuring and characterizing immune complexes and methods giving better insight into their *in vivo* activity are badly needed.

6. Intensive plasmapheresis can be successfully applied in the treatment of some fulminant immune complex diseases and in disseminated melanoma. The dramatic response to plasmapheresis may be due, apart from the removal of circulating immune complexes, to the simultaneous reduction of other plasma constituents with high biologic activity and/or to the replacement of low affinity Ab-s with their high affinity counterparts.

The formation of immune complexes is an integral part of the immune response, a physiological component of the elimination of antigens. No wonder that immune complexes are continuously formed in health and disease. Most of the circulating immune complexes (CIC-s) are rapidly cleared by the mononuclear phagocytic system especially by the Kupffer cells of the liver [22, 48]. Both the triggering of the immune complex elimination and the pathogenic effect of the complexes depend on their composition, i.e. the ratio of antigen to antibody and the concentration of both, the quality of the antigen: molecular weight, number and density of the antigenic determinants within the molecule, routes and timing of entrance of the antigen, genetic make-up of the host, the specificity, avidity,

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class and subclass of the antibodies produced, the size and lattice formation capacity of the immune complex. Under physiological conditions the stimulation of immune complex-eliminating mechanisms by the complexes themselves (C activation, CRA, immune adherence → phagocytosis) results in a dynamic equilibrium of immune complex formation and elimination.

The pathogenicity of immune complexes resides in:

1. their capacity to trigger a sequence of humoral and cellular events leading to an acute inflammatory reaction, tissue damage and systemic reactions (resulting in the most severe cases in disseminated intravascular coagulation and shock);
2. their capacity to modulate the function of other immunological effector systems.

Immune complexes have been indicated in human disease by their detection in body fluids or at the site of tissue injury. The development of new techniques for the detection of immune complexes in biological fluids [30] gave rise to a number of investigations, as a result of which circulating immune complexes had been demonstrated in a variety of clinical disorders, frequently without any signs or symptoms of immune complex disease. All these methods are indirect assays and thus detect *presumed* immune complexes. For the sake of simplicity the qualification "presumed" will be further on omitted.

This paper is focussed on the problem as to whether these circulating immune complexes are pathogenic factors in the development of, or rather secondary to the underlying disease. To this end I will discuss the clinical significance of immune complexes under three headings:

1. Diseases which are most probably caused by immune complexes.
2. Potentially pathogenic immune complexes with beneficial effects.
3. Clinical disorders with high incidence of circulating immune complexes, most probably secondary to the underlying disease.

The therapeutical effect of plasmapheresis will be discussed only briefly.

1. Diseases which are most probably caused by immune complexes

This group of disease is called "immune complex diseases". Most of our knowledge of this disease entity has been derived from serum sickness, from the local Arthus phenomenon and drug allergy. There are suggestive clinical and histopathological similarities to the former disorders, as well as evidence of circulating immune complexes in some patients with rheumatoid arthritis, systemic lupus erythematosus, hyperglobulinaemic purpura, cryoglobulinaemia, Henoch – Schönlein's disease, different types of glomerulonephritis and vasculitis, allergic skin necrosis, *hyperacute viral infections* (HB infection, dengue, haemorrhagic fever)

and *chronic viraemias* (lymphochoriomeningitis, sclerosing panencephalitis, HB and Epstein–Barr virus infections), *bacterial* (poststreptococcal glomerulonephritis, leprosy, etc.), *protozoan* (malaria, trypanosomiasis) and *helminthic* (schistosomiasis, onchocerciasis) infections.

Circulating immune complexes may be formed by antibodies reacting with soluble non-cell-bound antigens or by antibodies reacting with an antigen which is an integral part of, or passively attached to, a membrane and the resulting complex is being shed secondarily into the circulation. Large and/or C-fixing complexes are rapidly cleared by the mononuclear phagocytic system. It has been suggested that smaller and non-C-fixing complexes may be trapped and fixed on different filtering structures (like the spleen, renal glomeruli, blood vessel walls, synoviae or chorioid plexus) and give rise at these sites to the well-known localized or multiple immune complex disease symptoms. Some recent data are, however, in contradiction with this widely accepted hypothesis as far as they render evidence of the complete inactivity of the circulating immune complexes in rheumatoid arthritis, SLE and autoimmune diseases. It is claimed that circulating immune complexes lack the capacity of triggering inflammatory reaction [1].

Immune complexes cause tissue injury by activation of plasma components and react with cells bearing Fc and/or C receptors, i.e. platelets, granulocytes, mononuclear phagocytes and lymphocytes.

Immune complexes induce platelet aggregation and release resulting in formation of microthrombi. Immune complexes activate the C system through both the classical and alternate pathways (but most probably the activation of the classical pathway is principally involved in the development of human diseases). Complement activation results in increase of vascular permeability, chemotaxis, immune adherence and release of proteases, basic proteins, superoxides, singlet oxygen and peroxide from the granules of granulocytes, macrophages and release of histamine from mast cells. The release of protease and free radicals leads to tissue injury and necrosis. The procoagulant released from platelets and granulocytes triggers intravascular coagulation. Collagen-associated mucopolysaccharides exposed in the inflammatory process of vascular basement membranes and the proteases released by neutrophils and macrophages activate the Hageman factor (XII) pathways: the kinin-forming, intrinsic clotting and fibrinolytic systems and the C system. This indirect activation of the C system may be involved in the development of tissue injuries triggered by non-C-binding immune complexes, which interact with the Fc receptors of granulocytes, inducing release of their granules. In the absence of marked deficiency of granulocytes, macrophages and platelets, immune complexes will not cause either inflammatory reactions or tissue injury.

2. Potentially pathogenic immune complexes with beneficial effects

I will deliberately skip here the regulatory role of immune complexes in the immune response and their physiological role in the elimination of antigens. I want to discuss, however, the beneficial effects attributed to immune complexes

in pregnancy, in tissue transplantation, in paroxysmal nocturnal haemoglobinuria and in malignancy.

On the one hand, immune complexes may be pathogenic factors in pre-eclampsia, especially in view of the observation that deposits of IgG and C3 have been detected in the renal glomeruli of normal pregnant mice and guinea pigs [45].

On the other hand, it had been suggested by Temerius et al. [41] that immune complexes may act as blocking factors in pregnancy. It is presumed that the antigenic part of the inhibitory complexes originates from placental or foetal tissues [23]. Recent findings of Murgita et al. [26] favour, however, the possibility that α-foetoprotein serves as an agent to protect the mammalian foetus from graft-versus-host and host-versus-graft reactions through the selective induction of suppressor T cells.

In organ transplantation beneficial effect is attributed to antibody complexing with antigen shed spontaneously or stripped by antibody from the surface of the graft.

Immune complexes have also been reported to be particularly efficient in inducing the production of anti-idiotypic antibodies, which impair specifically graft-versus-host reactivity in animal experiments.

On the contrary, Ooi et al. [29] gave evidence in a limited number of patients of a correlation of increased ^{125}I-C1q-binding activity with acute kidney graft rejection characterized by fibrin deposition in the kidney. These contradictory effects may reside *inter alias* in differing compositions of the circulating immune complexes. In antigen excess the blocking activity may prevail, while antibody excess may enhance rejection of the graft.

I will now report about a patient in whom the occurrence of a severe but transient complex disease coincided with the cure of a chronic underlying disease.

Paroxysmal nocturnal haemoglobinuria (PNH) was diagnosed in the proposita at the age of 16. She had a very high percentage (70%) of C-sensitive red cell population with an extremely shortened life-span, very frequent and severe haemoglobinuria, anaemia, leukopenia and thrombocytopenia with some thrombotic episodes. The washed erythrocyte concentrate need was high. In spite of regular substitution therapy she had to be hospitalized frequently during the first nine years of her disease, when she suddenly showed typical signs and symptoms of an acute rheumatoid arthritis. Two weeks after the onset of this second disease, characterized by the appearance of high levels of rheumatoid factor and antinuclear factor, all signs and symptoms of PNH subsided and during the following ten years she never again had haemoglobinuria. The rheumatoid arthritis responded well to steroid and penicillin treatment. To our surprise her C-sensitive erythrocyte population disappeared completely. She is still regularly controlled, she had an intercurrent stillbirth but since 1972 she is completely healthy without any treatment. Rheumatoid factor cannot be detected since 1973 and antinuclear factor since 1976 (Fig. 1).

Paroxysmal nocturnal haemoglobinuria is an acquired intrinsic erythrocyte membrane defect, due to a somatic mutation. Normal human erythrocytes are

extremely insensitive to complement, but the "sensitive" fraction of PNH cells may be twenty to thirty times as vulnerable. PNH cells are not only susceptible to acid lysis and antibody-mediated lysis but also to any activator of the alternate or classical pathway of complement. PNH in those patients who have a large proportion of C-sensitive cells and typical pattern of haemoglobinuria has a grave prognosis. Although some very rare cases had been reported where the disease gradually ameliorated after many years and in two patients of the long survivors of Carache [2] and in two out of the more than hundred PNH patients

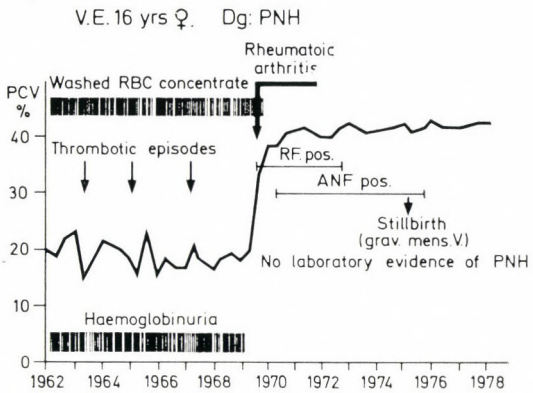

Fig. 1. Clinical course of a patient with paroxysmal nocturnal haemoglobinuria from the detection of disease (1962) until 1978

of Dacie (personal communication) all evidence of the disease disappeared. In the light of these extremely rare cases, it cannot be excluded that the coincidence of the cure of PNH and the manifestation of an immune complex disease was merely by chance. But this is most improbable taking into account the sudden disappearance of the PNH cells of our patient in contrast to the gradual amelioration of the unique cases of cured PNH patients reported. It is much more plausible that the circulating IgG-anti-IgG complexes were involved in the elimination of the C-sensitive PNH population. It is claimed that circulating immune complexes in rheumatoid arthritis lack the capacity to activate C, but as mentioned before, PNH cells are twenty- to thirtyfold more C-sensitive than their normal counterparts.

3. Clinical disorders with high incidence of circulating immune complexes most probably secondary to the underlying disease

Circulating immune complexes and immune complex deposits in tissues have been found in several chronic diseases of unknown origin, e.g. in subacute sclerosing panencephalitis, multiple sclerosis [39], amyotrophic lateral sclerosis

[28], in atherosclerotic vascular diseases [6], in sarcoidosis [8, 14], ulcerative colitis and Crohn's disease [13]. The presence of immune complexes in the sera of patients with these chronic diseases is most probably secondary to tissue damage together with an impaired function of the mononuclear phagocytic system. In sarcoidosis [7] and in Crohn's disease [35] a marked uniform failure of neutrophil migration into skin windows has been observed. Secondary infections, breakdown products of inflammatory tissue damage may overload the mononuclear phagocytic system. Treatment of the underlying disease by steroids and/or other immunosuppressive agents may contribute to the impairment of the mononuclear phagocytic system.

The presence of immune complexes in human leukaemia and lymphoma has been suggested by the finding of renal glomerular deposits of Ig and C in some patients [38]. In recent years a number of reports have been published demonstrating the presence of immune complexes in the circulation of tumour-bearing animals and in patients with different malignant diseases, e.g. solid tumours, leukaemia and malignant lymphomas [3, 4, 9, 10, 17, 21, 27, 32, 34, 43, 44].

The presence of immune complexes in the circulation of patients with malignant diseases is of special interest since the first reports of Hellstrøm and coworkers proved evidence of a specific blocking effect of tumour Ag-Ab complexes on cell-mediated tumour immunity as measured in their *in vitro* system [36]. Immune complexes, however, depending on their composition may enhance or suppress lymphocyte activation and modulate functions of both B and T lymphocytes. Recent data have shown that specific blocking factors include, apart from free tumour antigen and Ag-Ab complexes, probably specific immunosuppressive molecules formed upon contact between suppressor T lymphocytes and tumour antigens (or complexes) [11].

Despite the attempts to characterize the antigen in the circulating immune complexes in malignant diseases, still very little is known about their composition and *in vivo* significance.

In collaboration with Drs. Carpentier, Lambert and Miescher we have investigated 220 sera of 86 patients with leukaemia, malignant lymphoma and other haematological diseases for the presence of circulating immune complexes by three different methods: The ^{125}I-labelled C1q-binding test [49], the complement consumption test [16] and the C1q solubility test, based on the method of Johnson et al. [16] and modified by us for quantitative estimation.* All patients were treated at the Department of Haematology of the National Institute of Haematology and Blood Transfusion, Budapest. This gave us the opportunity to correlate the slightest changes in their clinical course or medication with changes in the level of circulating immune complexes (Table 1).

Values increasing by more than 2 SD as compared with normal sera in at least two out of three tests were recorded in 18 of 27 patients with acute myeloid leukaemia (AML), in all 4 patients with acute lymphoid leukaemia (ALL), in 7

* For detailed clinical data and laboratory results see [24].

Table 1

Results of parallel measurements of ^{125}I-C1q binding and complement consumption activities and C1q-solubility in leukaemia patients

Type of leukaemia	No. of patients tested	Positive results in*			
		three tests	two tests	one test	neither
AML	27	10	8	9	0
ALL	4	2	2	0	0
CML					
Blastic crisis	11	4	3	4	0
CML	11	0	6	3	2
CLL	22	4	9	7	2
Total	75	20	28	23	4

* Values increased by more than 2 SD values as compared with normal sera

of 11 patients in blastic crisis of chronic myeloid leukaemia (CML), in 6 of 11 patients in the chronic phase of CML and in 13 of 22 patients with chronic lymphoid leukaemia (CLL), altogether in 48 of 75 leukaemic patients (64%).

In longitudinal studies (3 to 10 samples from the same patient) positive results in at least two tests were recorded in 28 (88%) of 32 leukaemic patients.

These results are in good agreement with earlier reports of Heimer and Klein [10], Oldstone et al. [27], Carpentier et al. [4], Theofilopoulos et al. [44].

We could confirm the earlier findings of Carpentier et al. [4] demonstrating higher incidence of positive results of C1q-binding test and higher C1q-binding activity in untreated patients and in patients in relapse. The incidence of positive C1q solubility test showed the same good correlation with the clinical stage, while the results of the complement consumption test showed no significant correlation

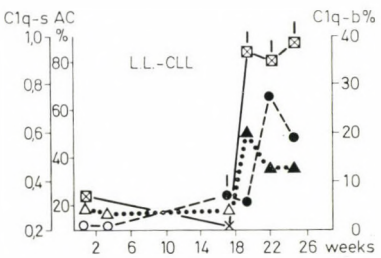

Fig. 2. Serial measurement of serum ^{125}I-Clq-binding and complement consumption activities, and Clq solubility in a patient with chronic lymphoid leukaemia. Effect of infection: ^{125}I-Clq-binding (Clq-b) test = ... Complement consumption (AC) test = — — —. Clq-solubility (Clq-s) test = - - - - -. Positive results (increased by > 2 SD values as compared with normal sera = ▲ ⊠ ●. Negative results = △ × ○. I = intercurrent infection

with remission or relapse (Tables 2 and 3). The concentration of circulating immune complexes showed a decreasing tendency after chemotherapy. Intercurrent infections were best reflected by the complement consumption test (Fig. 2), but no significant correlation could be detected either between circulating immune complex levels and infection, or between circulating immune complex levels and bleeding episodes (Figs 2 and 3).

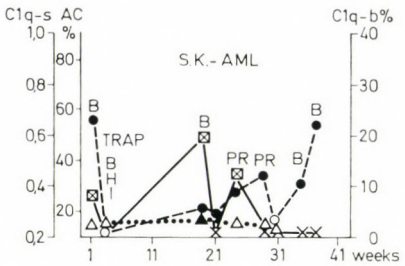

Fig. 3. Serial measurement of serum ^{125}I-Clq-binding and complement consumption activities, and Clq solubility in a patient with acute myeloid leukaemia. Results obtained in different stages of the disease. Fluctuation in the results of the different tests: B = blastosis, PR = partial remission, H = haemorrhage, I = intercurrent infection, TRAP = abbreviation of treatment protocol

Table 2

Correlation between the stage of leukaemia and the incidence of positivity of the tests for the detection of circulating immune complexes

Stage of leukaemia	Incidence of elevated		
	^{125}I-C1q binding activity	complement consumption activity	C1q-solubility
	No. of sera	No. of sera	No. of sera
Blastosis	10/36	24/41	33/41
Remission	1/19*	14/25	10/25*

* $P < 0.01$, significant difference calculated by χ^2-test

We have found very definite fluctuations in the levels of circulating immune complexes in the serial studies of our leukaemia patients. This is in good agreement with the findings of Teshima et al. [43] and Rossen et al. [32] in cancer patients and with those of Mohammed et al. [25] in coeliac disease.

Evaluating our longitudinal studies two types of fluctuations in circulating immune complex level could be revealed:

1. Changes which could be correlated with the stage of leukaemia and/or with the presence of intercurrent infections.

Table 3

Correlation between the stage of leukaemia and the concentration of circulating immune complexes according to the results of the three applied tests

Stage of leukaemia	^{125}I-C1q binding activity	Complement consumption activity	C1q-solubility
	Mean ± 1 SD	Mean ± 1 SD	Mean ± 1 SD
Blastosis	5.54 ± 5.29	16.5 ± 16.4	0.41 ± 0.18
Remission	3.03 ± 0.82*	13.7 ± 14.7	0.32 ± 0.36

* $P < 0.05$, significant difference calculated by *t*-test

2. The other type of fluctuation seems to be completely independent of the clinical course. The fluctuations detected by the three different assays did not run parallel on several occasions. The peaks of the curves did not coincide and the curves often crossed each other (Figs 3 and 4).

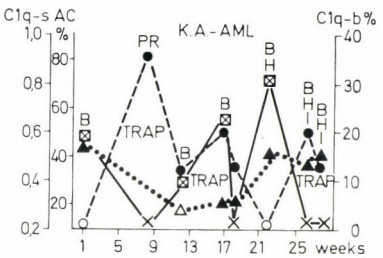

Fig. 4. Serial measurement of serum ^{125}I-Clq-binding and complement consumption activities, and Clq solubility in a patient with acute myeloid leukaemia. Results obtained in different stages of the disease. Fluctuation in the results of the different tests: B = blastosis, PR = partial remission, H = haemorrhage, I = intercurrent infection, TRAP = abbreviation of treatment protocol

Considering the varying sensitivity of the different assay systems to immune complexes of different size and composition [5, 13] our results suggest that *the composition of the circulating immune complexes* changes in the course of leukaemia. These changes may be due to the fact that the antigenic component of circulating immune complexes may be of very different origin during the course of leukaemia. Apart from the postulated but never proved *leukaemia-specific antigens*, *phase-specific* (embryonic) *tumour antigens*, *nuclear antigens* expressed in proliferating cells [18, 33, 37], *microbial antigens*, *HL-A antigens* of transfused cells, *drug antigens*, etc. may form the antigenic component of the circulating immune complexes.

Lack of correlation between the presence of circulating immune complexes and clinically evident infections speaks against the microbial origin of the com-

plexed antigen. Clinically inapparent bacterial, slow virus or fungal infections, however, cannot be excluded.

Any long-standing infection or tissue injury may give rise to circulating immune complex formation. The levels may remain high because the elimination of the complexes is severely handicapped in relapse of leukaemia and other malignant diseases by the depletion and functional impairment of phagocytes. The persistence of circulating immune complexes may also be favoured by the production of low avidity antibodies, due to the immunodeficiency of leukaemia patients, especially those treated by cytostatics. The C-activating capacity of antibodies with low avidity is low as well and thus the solubilization rate of the complexes is decreased. I hold the opinion that *decreased elimination of circulating immune complexes accounts for the strikingly high incidence of circulating immune complexes in malignant diseases in relapse.*

Carpentier et al. [3] presented impressive data showing that patients with AL and circulating immune complexes have a worse prognosis than those without such complexes. I have the feeling that these results of real practical importance mirror the fact that leukaemia patients with highly impaired function of the phagocytic system have a very bad prognosis. This is why AML-s and in general adult leukaemias have a much worse prognosis than the most common 0 cell AL-s of infancy and childhood. Our further studies will be carried out in this line.

The effect of plasmapheresis

Plasma exchange may help to deplete circulating immune complexes or alter the equilibrium between soluble Ag and Ab, which cause complexes to form and circulate. Very impressive results had been obtained by intensive plasmapheresis treatment, combined with cytostatic drugs and steroids in patients with Goodpasture syndrome and in those with fulminating immune complex-crescentic nephritis still retaining some renal function. The level of anti-glomerular basement membrane antibody decreased, renal function ameliorated and pulmonary haemorrhage was rapidly controlled [19, 20]. Even those patients responded well to repeated plasma exchanges whose circulating immune levels had not been influenced by prior treatment with immunosuppressive drugs [31]. The renal function deteriorated in the patients of Walker et al. [47] after plasmapheresis was stopped. One of these patients responded to a second course of plasmapheresis but failed to respond to a third. It is claimed that a continuing treatment for a period of weeks is needed to permit prolonged remission.

Clinical and immunochemical improvement was produced by plasmapheresis in patients with SLE with detectable circulating immune complexes.

Using a ^{51}C-release cytotoxicity assay Hersey et al. [12] reported that intensive plasmapheresis resulted in removal of serum-blocking activity in patients with disseminated melanoma. Post-plasmapheresis sera from several patients also increased cell-mediated cytotoxicity by induction of antibody-dependent killing.

It is claimed that this effect may have resulted from removal or alteration of circulating immune complexes. We have observed striking regression of cerebral lesions following intensive plasmapheresis in a patient with disseminated melanoma. Israel et al. [15] found partial regression of evaluable lesions in a marked percentage of patients with disseminated tumours. They also attributed these results to the removal of "blocking factors", but suggest that these blocking factors are rather "non-specific" acute phase reactants or "sialoglycoproteins".

Venge et al. [46] reported on the disappearance of inhibitors of PMN migration and quick recovery following intensive plasmapheresis in a patient with severe thermal injury.

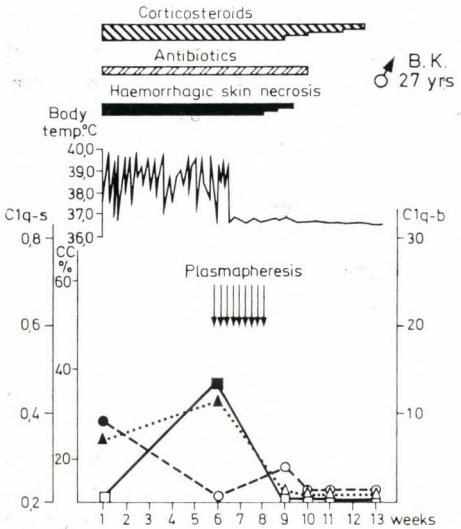

Fig. 5. Clinical course of a patient with disseminated allergic haemorrhagic necrosis. Effect of intensive plasmapheresis

Talpos et al. [40] report on the beneficial results of plasmapheresis in five patients with severe Raynaud's disease.

We had observed a most impressive cure after intensive plasmapheresis in a patient with huge disseminated allergic haemorrhagic skin necroses. The patient did not respond at all to corticosteroids and to the most potent combinations of antibiotics. He had septic fever, new deep haemorrhagic skin necroses appeared and he had high circulating immune complex levels according to the C1q-binding and complement consumption assays throughout the four weeks preceding plasmapheresis. Circulating immune complexes disappeared in parallel with the dramatic cure of the patient. The deep postnecrotic wound had been covered successfully with skin transplants (Fig. 5).

When evaluating all these striking immunochemical and clinical improvements it has to be taken into account that generally only a few patients, often a single patient, were included in the studies. Adequate control information is lacking. The clinical course of the immune complex diseases and malignant diseases varies greatly. Intensive plasmapheresis is often combined with immunosuppressive treatment, which alone gives good results in some of the reported diseases.

In addition, plasmapheresis (or more exactly plasma exchange) is a crude method of the removal of macromolecules. Intensive plasmapheresis results simultaneously with the removal of circulating immune complexes in a depletion of immunoglobulins, complement components, acute phase reactants and clotting factors of the patient.

When immune complex disease developed in an immunodeficient patient it may result in a low affinity antibody response to an as yet unidentified antigen. If this antigen is at the same time a common immunizing agent in the given population, then the fresh frozen plasma used for replacement of the removed plasma may contain high affinity antibody. This latter may play a decisive role in the elimination of the disease-causing antigen.

In implicating clinical significance to circulating immune complexes fact and fancy have often been confused. Clinicians using experimental treatments like plasma exchange imagined sometimes that they were interfering with the immune system along lines that followed logically from hypotheses based on experimental *in vitro* observations. The aim of my lecture was to consider – as far as they are understood – the rather complex interrelationships between circulating immune complexes and the pathologic alterations attributed to them.

The final goal would be to remove pathogenic immune complexes and other tissue-damaging plasma components by a method which is more selective than plasmapheresis. To state Terman et al. [42] presented a method for the selective removal of antigens and immune complexes from the circulation of experimental animals with extracorporeal immunosorbents. This technique has not yet been applied in man. Another important aim would be to replace the low affinity antibodies by high affinity antibodies directed against the disease-causing antigen. Other depleted plasma components (like C components) involved in the clearance of immune complexes or those having an inhibitory effect on serine proteases should also be substituted. In malignant diseases the removal of factors inhibiting cellular response and/or physiological pathways of cell proliferation and maturation will be needed.

The prerequisite of all these is the development of methods for the detection and identification of the disease-causing antigen and further development of direct techniques of measuring and characterizing immune complexes. Methods which give a better insight into the *in vivo* activity of circulating immune complexes are badly needed. No consultation on hypotheses will help without the knowledge of these hard facts. We need to be very critical if we do not want to fall from the heights of enthusiasm to a stony ground.

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The Biological Role of the Complement System and the Clinical Importance of Complement Measurements

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Properties of serum proteins belonging to the complement system, two pathways of the complement activation (classical and alternative pathway) as well as the physiological role of the complement system are discussed. Complement has essential importance in some physiological processes: In the induction of the humoral immune response, in the elimination of immune complexes and in the protection against bacterial and viral infections. After a short discussion of the genetics of the complement system, the principle and possibilities of clinical applications of the complement measurements are described. Finally, different approaches to the therapeutic manipulation of the complement system are discussed.

Elucidation of the most important mechanisms of complement activation in the 'sixties and in the early years of the present decade has opened a new era of complement research. Having understood that numerous molecular processes take place in the course of complement activation, complementologists have turned to the physiological and pathological role of the system. The aim of the present paper has been to review the recent knowledge of the biological role of the complement system and to point to the usefulness of complement investigations in clinical medicine. Finally, I wish to outline the results and perspectives of the therapeutic use of influencing the complement system. As to further details of the activation mechanisms, I refer to recent reviews [27, 38]. In the present paper, only the essentials of these mechanisms will be described.

I. Structure and function of the complement system

A) Definition of the complement system

Owing to its complexity, the complement system cannot be defined in a single sentence. It consists of proteins which are present in the blood plasma and other body fluids (CSF, synovial fluid, etc.). Under normal conditions, these proteins are present in an inactive state and need special substances for being activated. The complement components activate one another, in general enzymatically, in a well-defined order. As a result of this cascade of reactions, biologically active substances appear in the blood and other body fluids, and the active substances

may induce various reactions resulting e.g. in an increased vascular permeability at, and attraction of neutrophils to, the site of complement activation. Moreover, cells react with complement proteins, whence some properties of the cell surface will be changed, e.g. the affected cells will be capable of attaching to the surface of macrophages, leucocytes and platelets. In this way, the complement may form intercellular bridges. The cells on the surface of which complement activation has reached its end, usually die.

According to present knowledge, complement plays an important role in starting immune responses to the majority of antigens. In the absence of complement, the organism is unprotected against bacterial infections and its defense mechanism against certain viral infections is impaired. Under pathological conditions, complement contributes to the development of inflammations of both immunological and non-immunological origin.

The whole complement system is present in the blood of all vertebrate animals except the *Chondrostei*.

B) The serum proteins of the complement system

The complement system may be activated in two main pathways. This duality is reflected by the designation of the serum proteins called complement components. The components of the classical pathway (CP) are designated by C and a suffixed Arab number from 1 to 9. The first complement (C1) is a complex consisting of three different serum proteins, namely C1q, C1r and C1s; it is held together by Ca^{++}. According to the generally accepted nomenclature, a horizontal bar over the suffix indicates that the component is in an enzymatically activated state, e.g. active $\overline{C1}$ is designated as C1. In the course of activation, most of the complement proteins split into two fragments; these are designated with a and b, respectively, written after the Arab number.

The components of the alternative pathway (AP) are designated with capitals. Activated state and fragments are designated as in the CP. C3 and C3b participate in both pathways.

Table 1 shows the essential properties of the components of the complement system. C3 deserves special emphasis. This component, whose serum concentration is outstandingly high, plays a key role in both mechanisms.

C) The process of complement activation

In the following, the processes of both main pathways are outlined. First of all, I present in brief the most important rules of activation.

1. Activation of the complement system causes biological alterations even if the process does not reach its end; such changes may ensue even in the early phases of activation. Furthermore, besides the two main mechanisms, there are other possibilities of activation, and in such cases activation may be started at steps other than at which the two main mechanisms started; e.g. C3 can be acti-

Table 1

Physicochemical properties of the proteins of the human complement system

Protein	Molecular weight × 1000	Electrophoretic mobility	Mean serum concentration mg/dl
I. Proteins of the classical pathway			
C1q	400	γ_2	18
C1r	180	β	7
C1s	86	α_2	11
C4	206	β_1	64
C2	117	β_1	2.5
C3	180	β_1	160
C5	180	β_1	8
C6	95	β_2	7.5
C7	110	β_2	5.5
C8	163	γ_1	8
C9	79	α	23
II. Proteins of the alternative pathway			
IF (initiating factor)	150	γ_1	—
P (properdin)	184	γ_2	2.5
C3	180	β_1	160
C3b	171	α_2	—
C (C3 proactivator)	93	β	20
$\overline{D}$ (C3 proactivator convertase)	24	α	—

vated directly by proteolytic enzymes and the activation may proceed as if C3 had been activated *via* either CP or AP.

2. Two types of biochemical reaction, *viz.* protein–protein interaction and enzymatic proteolysis play the main roles in complement activation. The former takes place in each phase, the latter in the form of limited proteolysis in each step except those in the last stages of activation. Limited proteolysis means that only a single peptide bond is cleaved. Thus, two fragments arise, the one twenty to thirty times as large in molecular weight as the other. The smaller fragment may be a biologically active substance which exerts is effect in the fluid phase, i.e. in the blood or body fluids. The larger fragment may attach to cells or substances like immunoglobulins. It interacts with the component next in sequence through its enzymatically active site which accounts for the limited proteolysis of the subsequent step. The cell-attached complement fragments come into close relation with the cell membrane, and strong hydrophobic interactions arise between membrane proteins and complement fragments. Thus, complement proteins, originally serum proteins, may turn into membrane proteins. Since various complement proteins have been demonstrated in different cell membranes, it is postu-

lated that incorporation of complement proteins in membrane proteins occurs *in vivo* as well.

The steps of both main pathways of complement activation are schematically presented in Fig. 1. Biologically, the cleavage of C3 and the activation of C5 are the most important steps of the activation process, which accordingly is divided into three main stages.

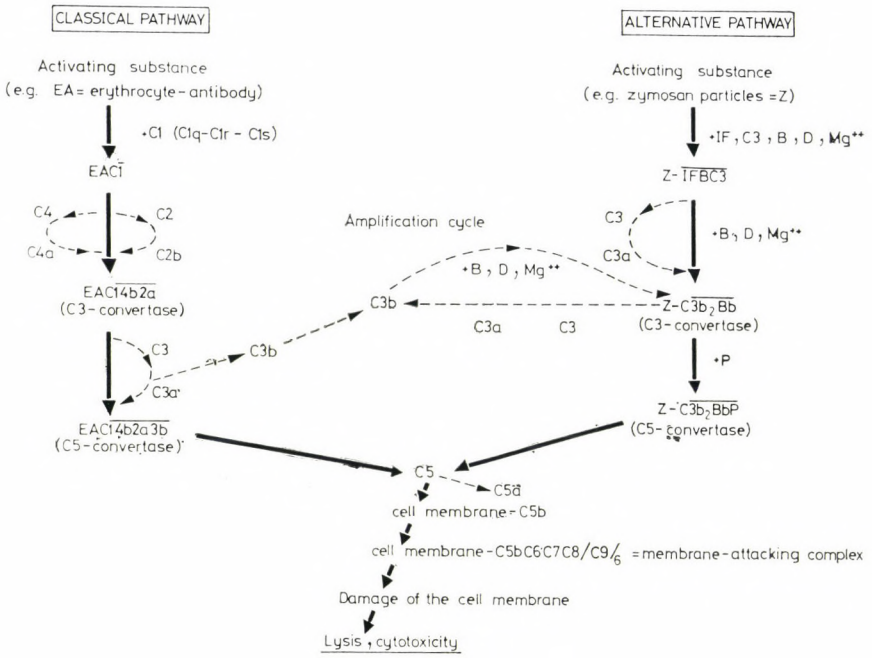

Fig. 1

a) First stage. Formation of C3-splitting enzyme

In CP, enzyme formation takes place in two steps; first the activators react with C1, then they start a so-called internal activation inside the complex macromolecule, which exposes an enzymatically active site on the C1s subcomponent. The internal activation of C1 also includes an enzymatic process, the course of which has been clarified only partially. According to our own investigations [14], the close fixation of C1 to the activator may not result in an activation of the complement component, though even a short-lasting fixation is sufficient to initiate the internal activation of C1. $\overline{C1}$ is a proteolytic enzyme, a serine esterase, and C4 and C2 are its substrates. After cleavage, the larger split products of C4 and C2, i.e. C4b and C2a, will form the C3-splitting enzyme, called also C3 convertase. C4b may attach to the cell membrane at the moment of its formation

or may become fixed to a polypeptide chain of an immunoglobulin molecule. The enzymatically active site of C3 convertase is located on C2a.

As the first step of AP, an enzyme is formed on the surface of the activator as a result of interactions between several complement proteins (IF, B, C3 and D). The enzyme contains C3 in intact form. The process requires Mg^{++} and factor D. The enzyme is labile and weak but is capable of splitting C3 and thus initiating the formation of a new, more efficient, C3 convertase. The process needs two C3b molecules fixed on the activator very close to each other and a subsequent "double" interaction with the larger fragment of the D-cleaved B component (Bb). The efficiently functioning C3 convertase, $\overline{C3bBb}$, is formed from these different fragments. This enzyme subsequently causes C3b fragments to arise again, and two of these will attach to the activator substance in close vicinity, bind factor B and in this way a new enzyme is formed. Thus, the process is self-amplifying. This step of the AP is called amplification cycle or positive feedback amplification loop. It is easy to understand that this cycle may be switched on not only by C3b fragments formed in the course of the AP; alternatively, C3b molecules formed *via* the CP or under the effect of tissue proteolytic enzymes may react similarly. Therefore, the activation pathways converge in this step.

Both C3-splitting enzymes, $\overline{C4b2a}$ and $\overline{C3bBb}$, are labile, their half-lives being several minutes at 37°C. For this reason, new complement proteins must be bound to the enzyme to enable it to initiate the late steps of complement activation. This takes place in the second stage. The most important role of the first stage is the preparation of further stages; yet, according to recent data, the processes of the first stage have also their biological importance. Thus, receptors reacting with C1q have been described on the surface of B lymphocytes [35] and it has been demonstrated that the presence of C1q on the surface of target cells may considerably increase the cytotoxicity of lymphocytes and lymphoblastoid cells against B lymphocytes [16]. It has been shown that a kinin-like substance is released from C2 during activation and – this is the most important – C4b is capable of mediating, with lower efficiency, almost all the biological effects that are mediated by C3b.

b) Second stage. Cleavage of C3, and the subsequent biological events. Formation of C5-splitting enzyme

Biologically, this is the most important stage of complement activation because the events of this stage prepare the complement-dependent cell interactions (according to Dierich's illustrative designation, complement bridges). It will be shown below that complement bridges play a part in the induction of immune responses, in the elimination of immune complexes and in the control of bacterial infections alike. Bridge formation is made possible by the existence of two binding sites on the C3b molecule. The binding sites arise at the moment of formation of this fragment. One of the binding sites, a labile one (LBS), is located

at the site where C3a is separated from C3b, the other, a stable binding site (SBS), is found on the other end of the molecule. If the fragment does not become fixed, the LBS is irreversibly inactivated within a few hundredth of a second, whereas SBS remains active permanently, even after the C3b molecule has attached to cell membrane. There are two different structures on the cell membrane for fixing both binding sites of C3b. (It should be noted that C4b is able to imitate this function of C3b, owing to its two binding sites and the corresponding membrane structures.) The fragment binds through the LBS to the so-called C3 acceptors and through the SBS to the C3 receptors. The former is an ubiquitous structure, supposedly present on every cell; little is known of its chemical structure. Our own observations suggest that the C3 acceptors on the B lymphocyte membrane are in close steric connection, or even identical, with the Fc receptors [8]. C3 receptors occur only on certain cells (in man, erythrocytes, lymphocytes, neutrophils and macrophages are bearing C3 receptors).

As to the formation of intercellular complement bridges, it may be stressed again that the SBS of C3b remains active after attachment through the LBS. Therefore, a cell that has fixed C3b can closely bind to any cell bearing C3 receptor on its surface. Since numerous immunological processes including phagocytosis of bacteria and the interaction between T and B lymphocytes, require such a close attachment and also the presence of C3, it seems reasonable to state that a complement bridge formation is the most important consequence of complement activation [7].

Complement bridge formation has been known for a long time as immune adherence, and the same phenomenon is utilized in the so-called complement rosette formation test, a method commonly used in immunology. In this test sheep erythrocytes that have fixed C3b on the LBS react with lymphocytes. As a result, a rosette consisting of erythrocytes is formed around each cell bearing C3 receptor on its surface.

C3b can attach through the LBS not only to the cell membrane but also to immunoglobulin. The importance of the latter reaction in the elimination of immune complexes will be dealt with below.

C3a, the other degradation product of C3, is also active biologically; it is of anaphylatoxin activity, i.e. liberates histamine from mast cells and basophils, thus increasing vascular permeability and exerting a smooth muscle-contracting effect. Furthermore, it is a chemotactic factor, attracting neutrophils to the site of complement activation.

The subsequent step of complement activation is the formation of C5-splitting enzyme. The C3 convertase formed *via* the CP turns into C5 convertase when a C3b fragment binds to the cell membrane in the vicinity of the $\overline{C4b2a}$ complex through the LBS of C3b. Three protein molecules come into interaction and, consequently, the specificity of the enzyme is shifted in the direction of C5 splitting. The active site of this new enzyme, too, is on the C2a component. – The C5-splitting activity resulting $\overline{C3bBb}$ enzyme is weak, due first of all to its great lability. An efficient enzyme effect needs stabilization of the enzyme by pro-

perdin (P), which is another protein. [The so-called C3-nephritic factor (C3NeF) has the same effect as P, or it may be its synergist. C3NeF has been isolated from nephritides of a certain type.] In conclusion, two C5-splitting enzymes, namely C4b2a3b and C3bBbP, are formed in the second stage.

c) Third stage. C5-splitting and formation of the membrane-attacking complex

The C5-splitting enzyme cleaves C5 into two fragments of which C5a is smaller than C5b. C5a, like C3a, is an anaphylatoxin and exerts a chemotactic effect. Unlike C3a, it attracts, besides neutrophils, eosinophils and mononuclear cells.

C5 cleavage is the last enzymatic step of complement activation; the subsequent steps are mainly interactions between proteins. On C5b, two binding sites arise, *viz.* a labile site for fixing C5b to the cell membrane and another site which enables an interaction between C5b and the other late components and thus the formation of a membrane-attacking complex. According to recent investigations, the most effective, so-called dekamolar, complex consists of one molecule of each of C5b, C6, C7 and C8 and 6 molecules of C9. C5b, C6 and C7 may form a complex before the attachment of C5b to the cell. This trimolecular complex has two important properties. First, besides C3a and C5a, it is the third chemotactic factor of complement origin; second, this complex binds not only to cells bearing the C5-splitting enzyme, but also to other (by-stander) cells. Thus, a membrane-attacking effect may develop on a great variety of cells. This process (so-called reactive lysis) is of importance mainly in the case of activation *via* the AP.

Our knowledge of the mechanism of action of the membrane-attacking complex is still incomplete. According to biochemical and electron microscopic investigations [3, 24], two processes take place, *viz.* (1) the complex may sink into the cell membrane and thus form a thin channel between the cytoplasm and the environment and (2) it has been shown that the membrane is damaged in a large area around the penetration of the complex, consequently, the dynamic structure of membrane lipids inside the membrane will be disturbed. As a result of both processes, membrane permeability will increase and the cell will be lyzed osmotically.

It should, however, be noted that cytolysis is not an obligatory mechanism, except for mammalian erythrocytes (haemolysis) on which the last steps of complement activation have already ensued. The outcome of the process is much more complicated in nucleated cells. These cells are sometimes killed without having been lyzed (complement-dependent cytotoxicity) or the complement activation may be completed on the cell surface without causing any cell damage. Some nucleated cells proved to be sensitive to the cytotoxic effect of complement only in certain phases of the cell cycle.

D) The biological importance of CP and AP

It has been shown in the foregoing that the two main pathways cannot strictly be separated from each other; they are closely interlinked during the amplification cycle. Thus, the biological importance of each mechanism can be characterized, first of all, by the substances that activate the mechanism. The most important activators are listed in Table 2.

Table 2

The most important activators of the two main pathways

Classical pathway

Antigen—antibody complexes (IgM and IgG)
Aggregated immunoglobulins (IgG1 and IgG3 are strong activators, IgG2 is weak)
Staphylococcus protein A–IgG complex
C-reactive protein—pneumococcus C polysaccharide complex
Complexes of polyanions and polycations (*e.g.* heparin–protamine sulphate)
Endotoxins of some bacteria
Plasmin and trypsin
Type-C oncoviruses

Alternative pathway

Aggregated IgA and IgE
Inulin
Snake venom (a factor isolated from cobra venom is the most important one)
Gram-negative and -positive bacterium strains
Some bacterial endotoxins
Mammalian cells (*e.g.* human lymphoblastoid cell lines and, in heterologous serum, also normal cells)
Mg^{++} concentrations above the normal level

It has been shown that the most important activators of the CP are immunoglobulins aggregated non-specifically or by forming complex with the antigen, especially those of the IgM class and the three subclasses of IgG, furthermore, complexes of polyanions and polycations (e.g. heparin—protamine sulphate) and the complex of C-reactive protein and type C pneumococcus polysaccharide. It is of interest that some viruses may also activate the CP. Obviously, CP has its main biological role in eliminating immune complexes and inducing inflammation, which is the most important non-specific defense mechanism of the organism.

Most of the AP activators are polysaccharides, substances present in the microbial cell wall. Consequently, this mechanism has its greatest importance in the antimicrobial defense mechanism. Interestingly, some mammalian membrane structures can also activate the AP. The biological role of this process is still unclear.

Finally, both mechanisms can be activated by bacterial endotoxins, though the endotoxin preparations derived from different bacteria are different in this respect.

E) The control mechanisms of complement activation

Since the complement system is activated by agents continuously present under physiological conditions, among others, mechanisms of its adequate control had to be developed during evolution. Some regulative factors are, so to say, involved in the mechanisms of activation; e.g. the extreme lability of the C3-splitting enzymes, which lose their activity soon after the detachment, and irreversible inactivation of C2a or Bb. Also, end-product inhibition has been demonstrated, *viz.* the initial steps of the CP are inhibited by the late complexes of the complement activation process. The most important factors of complement functions are the natural inhibitor proteins of the serum. The Cl esterase inhibitor, which forms a complex with the $\overline{Cl}$ molecule, prevents $\overline{Cl}$ from cleaving C4 and C2. Whether, in fact, complement activation does or does not start, is determined by the kinetics of enzyme formation.

The final intensity of complement activation is determined by the function of the amplification cycle. This cycle is inhibited by two serum proteins, the C3b inactivator and the β_{1H} globulin, working in close correlation with each other. The basic importance of these factors has been stressed by a recent observation of Fearon and Austen [10]. Accordingly, the ability of cells and other corpuscles to activate the AP is determined by the protection afforded against the C3 inactivator and the β_{1H} globulin. The mammalian and bacterial cells on the surface of which AP enzymes develop in a protected state are effective activators of this mechanism, in contrast to the cells on the surface of which both inhibitors are functioning without any restriction.

Among the natural complement inhibitors the anaphylatoxin inactivator also deserves mentioning. This is an enzyme of carboxypeptidase type which attacks the C ends of the C3a and C5a peptide chains. A single amino acid is split off, whereby the anaphylatoxin effect of the fragments is inactivated while their chemotactic activity remains intact.

F) Interactions between the complement system and other enzyme systems of the blood plasma

The interactions between complement and other plasma enzymes are manifold. (1) Different plasma enzymes may be activated by the same factors, e.g. in endotoxin shock thrombus formation, fibrinolysis and kinin formation are equally demonstrable besides *in vivo* complement activation [4]. The same occurs in immune complex diseases; this process, however, contributes to the tissue damage. (2) Activation of different plasma enzymes are subjected to a co-ordinated mechanism; activation of one of these systems is usually followed by activation of others. The "conductor" of the plasma enzyme systems is the Hageman factor. Activation of this factor initiates *via* complicated interactions the processes involved in blood coagulation, fibrinolysis and kallikrein–kinin formation. Meanwhile enzymes (thrombin and plasmin) are activated, which, through the activation

of C1 or the cleavage of C3, start the activation of the complement system [21]. An inverse interaction is also possible; complement activation, acting on blood coagulation factors or platelets, may start the process of blood coagulation [1]. (3) The four plasma enzyme systems have common inhibitors. Thus, C1 esterase inhibitor inhibits the Hageman factor-dependent blood coagulation, fibrinolysis and kinin formation, at several different steps; the carboxylpeptidase, besides inactivating C3 and C5, inactivates bradykinin as well [19]. Recently it has been reported [30] that the C1s enzyme activity is inhibited by antithrombin III in the presence of heparin. Obviously, the complement system closely co-operates with the other plasma enzyme systems.

II. The biological role of the complement system

The complement system plays an essential role in a number of physiological processes participating in the defense mechanism of the organism. These processes are mostly favourable for the organism. Like other plasma enzyme systems, complement plays a dual role. Events taking place in physiological complement activation and biologically active substances formed from complement components may be incriminated in induction of pathological processes; e.g. complement plays an essential defensive role in the elimination of immune complexes (ICs) on the one hand, and it is one of the main factors responsible for tissue destructions in IC diseases, on the other. Similarly, some of the pathogenic effects of endotoxin are mediated *via* the complement system and the same system participates in the detoxification of endotoxins in blood.

In the following, four processes will be dealt with, those in which the role of the complement system seems to have been proved. The presumed role of complement in further events needs confirmation; e.g. activation of B lymphocytes by C3b [18] and mobilization of leucocytes from the bone marrow [34] have been reported and recent data are suggestive of an important role of the complement system in tumour immunity [28].

A) The role of the complement system in the initial phase of the immune response

That complement is one of the most important effector systems in the immune response has been known for a long time. More recent investigations, mainly reported by British authors, point to another role of complement, especially of C3. Its presence seems to be indispensable in (a) the localization of antigens in the germinative centre of lymph nodes; (b) in the initiation of the immune response against T-dependent antigens; and (c) in developing B memory cells. These statements are based on the following experimental evidences.

(a) Pepys et al. [31] have shown that in mice decomplemented by cobra venom factor (CoF) the antibody response to T-dependent antigens is considerably reduced. This is equally valid for IgA and IgE antibodies and, to a lesser extent,

for the IgM response; the response to T-independent antigens was uninfluenced by C3 depletion.

(b) An essential role of C3 in the response to T-dependent antigens has been proved *in vitro*. The antibody production to T-dependent antigens, but not of T-independent ones, was considerably suppressed by treatment of antibody-producing cell cultures with anti-C3 serum or C3b (the latter blocks the C3b receptors).

(c) Pepys et al. [31] and, more recently, Klaus and Humphrey [22] succeeded in blocking the localization of aggregated IgG and ICs in the germinative centre of lymph nodes by pretreating mice with CoF. This finding strongly supports the role of C3 in initiation of the immune response, since the evidence is ever growing that the antigen must have access to the dendritic cells of the germinative centre, in the form of IC, to initiate an immune response [22].

(d) It was shown by Klaus and Humphrey [22] that B memory cells are not produced after antigenic stimulus in C3-dependent or CoF-pretreated mice.

To facilitate an understanding of the role of C3, I again refer to Dierich's complement bridge theory [7]. Cell-to-cell interactions take place in several phases of immune response induction. Such interactions are necessary for migration of antigen to the dendritic cells of the germinative centre, and T – B cell interactions and macrophage – T – B interactions are required for initiating the immune response itself. Since these interactions need bridge formation, which is a characteristic complement function, it seems reasonable to attribute the immunosuppressive effect of complement depletion to a lack of cell-to-cell interactions. Taking into account that with T-independent antigens such interactions are not required it is easy to understand that the immune response to T-independent antigens is not affected by C3 deficiency.

B) The role of the complement system in the elimination of ICs

Several complement-mediated processes co-operate in IC elimination.

(a) The ICs bearing C3b on surface as a result of complement activation can bind to the C3b receptors of polymorphonuclear leucocytes and those of cells of the mononuclear phagocyte system. According to more recent data, phagocytosis itself needs a co-operation between C3b receptors and Fc receptors. In fact, this is a special case of complement bridge formation, in which the bridge is formed between IC and the cell. If, however, the IC to be phagocyted consists of, e.g., a bacterium with antibody and C3b on its surface, a true intercellular bridge may come about.

(b) Complement activation in turn reacts with the IC; C3b binds to antibody molecules of the complex and changes their conformation. As a result, large ICs are split into smaller ones, which are unable to be deposited in tissues. Thus, the IC is detoxified. This phenomenon was described by Miller and Nussenzweig [26] as complement-dependent release activity (CRA). It may come about with ICs circulating in the blood and with those already fixed to cell receptors.

C) Complement system and the antibacterial defense mechanism

The essential role of the complement system in antibacterial defense has been proved primarily by observations made in complement-deficient patients. Patients lacking circulating C3 (owing to a genetically-determined lack of the protein itself or of the C3b inactivator) show an increased susceptibility to pyogenic infections [2]. Recently, Soothill and Harvey [36] have shown that children predisposed to frequent bacterial diseases are often deficient in a still unidentified factor of the AP. The essential role of complement in the protection against bacterial infections has been proved in animal experiments as well. Crosson et al. [6] infected with *Haemophilus influenzae* mice that had been made C3-depleted by CoF treatment. More than half of the pretreated mice died, though the same inoculum failed to kill any of the control mice. Similar results were obtained by other investigators with staphylococcus- or pneumococcus-infected mice. Investigating the AP-activating effect of different *Str. pneumoniae* serotypes, Fine [11] found that the serotypes isolated from human infections usually failed to activate the AP in a direct way, while those capable of doing so were rarely isolated from man. These and other observations suggest that the ability of bacteria to activate the AP without any co-operation of antibodies is a considerable factor of bacterial virulence. This ability is influenced by two factors, *viz.* the presence or absence of certain polysaccharides in the bacterial wall and some properties of the cell surface, in other words, how the enzymes of the AP are protected against C3b and β_{1H} globulin.

All these observations suggest that the AP represents the first defense line against bacterial infections; it is capable of reacting with bacteria, opsonizing them and supporting their elimination before the specific antibody response would start. Similarly, in the course of phylogenesis AP had appeared in invertebrates before the first appearance of immunoglobulins.

D) The role of the complement system in antiviral defense

It has been known for long that the virus-neutralizing effect of some types of antibody, first of all IgM, is enhanced by the presence of complement. Subsequently, it has been shown that complement-mediated decay of virus-infected cells may be another considerable factor of the control of virus infections. Yet, it is not an essential factor as shown, among others, by the fact that complement-deficient patients do not show enhanced susceptibility to viral infections.

In persistent viral infections, on the other hand, e.g. in subacute sclerotizing panencephalitis and multiple sclerosis, complement activation seems to contribute to the development of local tissue destruction [20].

An interesting observation of Cooper et al. [5] deserves to be mentioned in this context. Type-C oncovirus virions are lyzed exclusively by human serum. The lysis is due to the human complement and needs no cooperation of anti-

body because the human C1q binds to oncovirus virions directly. Presumably, this process represents a special defense mechanism against the horizontal spreading of type-C oncoviruses and, perhaps, against tumorigenesis.

E) Genetic relations of the complement system

Recently a great number of papers have shown the genetical determination of complement components and of the allotypes of certain complement components. These publications are too recent to be surveyed here in detail. In this review, only the interrelations between the genetics of the complement system, on the one hand, and its physiological and pathological roles, on the other hand, are discussed.

Genetic determinedness of all complement components except C9 has already been reported. The deficiencies are inherited codominantly in an autosomal way; in heterozygotes, the serum concentration of the deficient component is about 50% of the normal level.

A number of papers have dealt with the interrelations between genes responsible for complement deficiencies and the HLA system [19]. In man, deficiencies of C2 and C4 and, presumably, of C8, are associated with HLA antigens, whereas the inheritance of the deficiencies of C1r, C6, C7, and C1 esterase seem to be independent of the HLA system. The HLA haplotype most frequently associated with C2 deficiency is HLA-A10, -B18, -Dw2.

Genetically determined polymorphisms, like those known for immunoglobulins, have been reported for the complement components C3, C4, C2, and B. The inheritance of factors B and C4 are HLA-associated, while that of C3 is independent of the HLA system. The allotypes can be differentiated on the basis of their electrophoretic mobility.

The interrelations between complement deficiencies and human diseases seem to be the most interesting features of the complement system. Homozygotic and heterozygotic deficiencies of C2 have been demonstrated in SLE, SLE-like syndrome, synovitis, membranoproliferative glomerulonephritis, anaphylactoid purpura, dermatomyositis, discoid lupus, haemolytic anaemia, Hodgkin disease, CLL, and dermatitis herpetiformis. C4 deficiency has been observed in association with SLE, C8 deficiency with disseminated gonococcus infection, C6 deficiency with Raynaud syndrome, gonococcal arthritis and meningitis. Some allotypes of complement components have been shown to occur with increased frequency in connection with certain diseases. Thus, rheumatoid arthritis, hepatitis and atherosclerosis show an increased association with the C3F allotype [19].

It is difficult to explain these observations. Most of them refer to a coinheritance of C2 deficiency and some HLA haplotypes on the one hand, and autoimmune diseases in general, on the other. Consequently, these interrelations seem to be most suitable for analysis. It has been proved that the correlations cannot be attributed to chance. Glass et al. [17] have shown that homo- and heterozygotic

C2 deficiency occurs more frequently among patients with autoimmune disease than in the general population.

The association might originate in an inheritable mutation on chromosome 6, but genetical considerations do not support this view. The assumption that CP deficiency co-operates in the development of autoimmune diseases seems to be more acceptable. Presumably, elimination of ICs is disturbed in CP deficiencies. This view has been confirmed by observations in animals suffering from spontaneous autoimmune disease.

III. Aims, methodology and clinical value of complement measurements

A) Possible applications of complement measurements in clinical medicine

The foregoing have shown that (a) complement plays an important role in physiological processes, and (b) that phenomena involved in complement activation may participate in the development of tissue damage and consequently in human and animal diseases. Anaphylatoxins may increase vascular permeability, chemotactic factors may cause local accumulation of leucocytes and activation of other plasma enzyme systems, supported by complement activation, may co-operate in the development of local inflammation initiated by various complement-activating substances. Since complement activation can also be induced by substances other than ICs, i.e. by substances that do not require immunoglobulin to activate complement, it is easy to understand that complement may participate even in inflammatory changes of non-immunological origin. Among the inflammatory processes, extensive vasculitis is regarded as the most severe; its persistence may lead to shock or disseminated intravascular coagulation.

Partially or totally complement-mediated processes (initiated by autoantibodies, ICs or other substances) taking place in renal glomeruli may lead to grave pathological phenomena. The limited space does not allow to discuss in detail the role of complement in IC diseases. Instead, I refer to [38].

It is evident from the foregoing that complement activation may be expected in all diseases characterized by extensive inflammations, vasculitis and/or glomerulonephritis of immunological origin, moreover, the complement activation will be in direct relation to the severity of the pathological process. In such diseases, laboratory investigations will be aimed at demonstrating whether complement activation has occurred at all, and if so, to determine the intensity of the activation. The results thus obtained will characterize the activity of the disease and, in an indirect way, the efficiency of the applied therapy. In certain diseases, demonstration of complement activation may be of diagnostic value. Serial assays may be informative as to the persistence of the activation and thus may be of prognostic value.

Finally, complement measurements may be indispensable in revealing abnormalities of the complement system itself and of primary, genetically determined inhibitors regulating the system.

B) Methods

A detailed description of the complement assays is impossible within the present limits. In addition to the principles of the methods, I will present the reasonable aims of such investigations. Two main questions should be answered, namely (1) does or does not exist congenital lack of a complement component or inhibitor? (2) Are there signs of complement activation demonstrable in the patient at the time of the examination?

1. The phenomena suggestive of complement deficiency may be of two types, *viz.* (a) severe bacterial infections recurring in children and young adults at short intervals; (b) a clinical picture suggestive of autoimmune disease, first of all SLE, often with familial cumulation. The commonest deficiency, that of C1 esterase-inhibitor manifests itself with a well-defined clinical picture, hereditary angioneurotic edema, i.e. Quincke edema. Complement deficiencies may be heterozygotic or homozygotic. The latter is easy to demonstrate because the patient's serum fails to lyze sensitized sheep erythrocytes even if a single component of the CP is lacking. In the case of homozygotic complement deficiency, the total complement titre of the patient's serum is O CH_{50}, CH_{50} being a 50% haemolytic unit, i.e. the complement quantity in the presence of which 50% of the sensitized erythrocytes are lyzed. The total complement titration is performed as follows. Serial dilutions of the serum to be tested are mixed with a constant quantity of sensitized sheep erythrocytes and the mixture is incubated at 37°C for 1 hr. After subsequent centrifugation, the haemoglobin concentration is measured in the supernatant. The limit of normal values should be determined by assaying total complement in a satisfactory number of normal sera.

In heterozygotic complement deficiencies, the deficient component is present in a concentration which corresponds to 50% of the normal value. Since some components are present in great excess a partial deficiency of a single component may not be reflected in the total complement titre of the serum. Fortunately, in the case of the commonest complement deficiency, i.e. C2 deficiency, the total complement titre generally follows the C2 concentration. Accordingly, if a heterozygotic C2 deficiency occurs, the total complement titre will be approximately equal to half of the mean normal value.

Therefore, total complement titration can be used as a screening test for complement deficiencies in general. If the titre is subnormal or there is no haemolysis, one should show which of the components is deficient. As the next step, the single components should be assayed in the serum. Principally, two kinds of methods are available for this purpose, immunochemical and immunohaemolytic methods. From among the immunochemical methods, Mancini's technique is used most widely. These procedures are suitable for determining the absolute concentration of each complement protein by means of the corresponding monospecific serum. By immunohaemolytical methods the functional (haemolytic) activity of the components can be measured.

In an immunohaemolytic assay the process of complement activation is

imitated *in vitro*. As the first step, great excesses of the component that are activated in the complement sequence before the component to be assayed (e.g. C1 and C4 when C2 is to be titrated) are added to sensitized sheep erythrocytes. Then serial, generally very high, dilutions of the serum under testing are added to the system to serve as the only source of C2 in the sample, and finally, the components acting after the component under testing are added (C3 and C5–C9 if C2 is assayed). Various techniques based on the properties of complement activation have been evolved to dispense with the purified preparations. Under such circumstances, the serum concentration of the component to be assayed is the only determinant of the degree of haemolysis. The concentration of that component can be calculated from the per cent haemolysis and the corresponding serum dilution. Owing to principal and computation-technical reasons, the titre of the component is usually given in CH_{63}/ml units, CH_{63} being the complement quantity causing lysis of 63% of the erythrocytes. The correlation between the immunochemical and immunohaemolytical assays is, in general, satisfactory. It should, however, be noted that complement components may be present in an inactive state. In such cases only the latter method is adequate for identifying the genetically deficient component.

After this step, the final diagnosis can be established if the complement deficiency is homozygotic in nature. Heterozygotic deficiencies need further investigations. These cannot be diagnosed unless the other complement components are present in their normal concentrations. Otherwise, the possibility that the observed deficiency resulted from complement activation *in vivo* cannot be excluded. The diagnosis may be confirmed by family investigations demonstrating whether the deficiency was inherited according to genetical rules.

Lack of C1 esterase inhibitor is a special kind of complement deficiency. Here, besides revealing the genetic background, complement measurements may be of decisive importance. Lack of the inhibitor can be demonstrated by immunochemical or enzymatological methods or in an indirect simple way, as in the absence of the inhibitor the titres of both substrates (C4 and C2) of the circulating $\overline{C1}$ are very low (especially during, but also between, attacks), while the titres of the other components do not appreciably deviate from the normal values.

2. There are several methods for proving *in vivo* complement activation. Principally, these may be divided into three groups: (a) measurement of titre fluctuations of complement components; (b) direct demonstration of complement activation products; (c) demonstration of deposition of complement components in biopsy specimens obtained from lesions.

(a) In the first approximation, titration of complement components seems to be the most useful method. If massive complement activation is taking place anywhere in the organism, complement components are consumed and the corresponding titres fall below the normal level. Unfortunately, this is not so simple in practice. The titre fall due to complement activation is soon compensated by rapid complement synthesis, even overcompensation may take place. Long-lasting hypocomplementaemia needs persistent complement activation while the

rate of consumption exceeds the rate of complement synthesis. In some diseases, reduced complement synthesis may contribute to the hypocomplementaemia. Depression of complement synthesis may be due to the activity of complement activation products or may appear as a consequence of pathological functions characteristic of the disease; sometimes primary genetical causes may also contribute. In other clinical pictures, demonstration of hypercomplementaemia may support the diagnosis of active disease. Hypercomplementaemia may be genetically determined or may be due to overcompensation. Hypercomplementaemia may occur in glomerulonephritis and, according to recent observations [33] in patients with cervix carcinoma.

Finally, determination of the complement profile, i.e. of the relative titre changes of complement components, may provide information on the dominance of either of the two main pathways of complement activation. Activation of CP is suggestive of circulating or deposited IC, that of AP supports the role of bacterial endotoxin. In certain diseases, activation may take place in both pathways. CP dominance is suggested by reduced C1, C4 and C2 titres, whereas in the case of AP dominance, the decrease in the C3 and B concentrations is not accompanied by low C1, C4 and C2 titres. In practice, titration of C4, C3 and B is satisfactory for orientation.

(b) In the last few years, direct methods for the demonstration of complement activation products have become popular. Laurell's two-dimensional electroimmunoelectrophoresis is the simplest and most reliable technique. In brief, it consists of two runnings. The first one separates intact C4 (or C3) from the split products of higher mobility. During the second running, which is performed perpendicularly to the first one, the serum proteins migrate into an agarose layer containing monospecific anti-C3 or anti-C4 serum. Thus, the intact proteins are separated from the split products, which form rocket-like precipitation lines in a different area of the plate. The concentrations can be calculated from the heights and widths of the rockets. A positive result indicates *in vivo* activation, whereas a negative result does not exclude *in vivo* activation because, owing to their rapid degradation (more rapid than the degradation of the intact complement proteins) the split products are soon eliminated from the circulation. Orientation is easier if the test is performed in body fluids other than blood, e.g. synovial fluid, because activation products may cumulate in these fluids.

I should like to call attention to the special requirements of submitting samples for complement assays. Only fresh serum samples or samples frozen within 2 to 4 hours after venipuncture are suitable. For assaying complement degradation products, the requirements are still more severe. The blood sample should be taken with EDTA, an anticoagulant, and the plasma tested immediately or stored below $-40°C$.

(c) Deposition of complement components in various parts of renal glomeruli is of special importance in the diagnostics of certain renal diseases. The deposits are made visible by immunofluorescence. The structure of the deposit allows to distinguish between autoantibodies and ICs as primary pathogenetical factors.

Differential diagnostics is supported by revealing the pathway of activation; e.g. in SLE C1q, C4 and C3, whereas in mesangiocapillary glomerulonephritis C3 and P are commonly found in the deposits.

C) Diagnostic value of complement assays in certain human diseases

Table 3 shows the conditions in which complement assay may support the correct diagnosis. Thus, the diagnosis of complement component and inhibitor deficiencies needs complement measurements. Deficiencies of C1r, C1s, C4, C2 or, sometimes of C5, may be complicated by autoimmune diseases; frequently occurring bacterial infections may point to C3, C5, C6, C8 or AP deficiency. Demonstration of these deficiencies may be of practical interest, for fresh plasma is often administered with success in these cases [36].

Table 3

Clinical value of complement assays in some diseases

Disease	Possible use of complement assays
Genetic deficiency of complement components	In diagnosis
Hereditary angioneurotic edema (C1 esterase inhibitor deficiency)	In diagnosis
Systemic lupus erythematosus (SLE)	A diagnostic tool in establishing disease activity and in estimating the efficiency of therapy
Rheumatoid arthritis	In differential diagnostics: differentiation from other arthropathies and SLE
Glomerulonephritis	In differential diagnostics; for establishing disease activity; in the prognostics of acute glomerulonephritis
Gram-negative bacteriaemia	Forecasting of septic shock, prognostics
Subacute bacterial endocarditis	Forecasting of complicating glomerulonephritis, control of the treatment of complication

Hereditary angioneurotic oedema is a common disease in many countries including Hungary (as shown by us). It would be of interest to screen out these cases from the mass of patients with chronic urticaria, for the usual antiallergic therapy is not favourable in hereditary angioneurotic oedema. On the other hand, some recent procedures seem to reduce the frequency of attacks in patients with hereditary angioneurotic oedema.

Complement assays, especially measuring of C4 decline and demonstration of C4 or C3 degradation products are valuable diagnostic tools in SLE, mainly in its first stage. A high antinuclear antibody level combined with hypocomplementaemia is usually sufficient for the diagnosis of SLE [25], while serial complement assays may be useful in demonstration of the activity of the disease and in

estimating the therapeutic effect. However, the correlation between disease activity and hypocomplementaemia diminishes during the late stage of SLE.

In rheumatoid arthritis (RA), demonstration of complement activation is a valuable differential diagnostic sign. In contrast to SLE, a decline of the serum complement is uncommon in RA except in vascular complications.

Complement measurements, especially determination of the complement profiles and characterization of the complement components deposited in glomeruli are very useful in the differential diagnosis of the different types of nephritis (e.g. acute and mesangiocapillary glomerulonephritis). The same procedures may be valuable in the prognostics of acute poststreptococcal glomerulonephritis. Early in the course of the disease, the C3 serum titre shows a striking drop. Normalization of the C3 serum concentration in some weeks is a favourable prognostic sign, otherwise the illness may turn into a subacute form. It was an interesting observation difficult to explain that hypercomplementaemia, in most cases characterized by an excess of total complement and C4, was demonstrated occasionally in chronic glomerulonephritis. There is probably a positive correlation between the degree of hypercomplementaemia and the activity, and thus the prognosis, of the disease [15].

Besides ICs, bacteria and bacterial products are the main activators of the complement system. In gram-negative bacteriaemia, especially during septic shock, the serum titres of complement components may suddenly fall while activation products are cumulating in the blood. According to our observations [13], clinical shock may be preceded by signs of complement activation. This may indicate that, principally, serial complement assay may be valuable in forecasting the shock. Observations of Fearon et al. [9] suggest that the more intensive the C3 decline the more unfavourable the prognosis.

Hypocomplementaemia during subacute bacterial endocarditis is often associated with glomerulonephritis. Successful therapeutic intervention is followed by the regression of nephritis and, at the same time, normalization of the complement titre.

It should be stressed that, except for primary complement deficiencies, complement measurement can only be used as an auxiliary investigation. Any conclusion needs an evaluation in accordance with other laboratory results and with clinical symptoms.

IV. Therapeutical conclusions

Two kinds of therapeutic intervention may be justified: (1) substitution of complement components or inhibitors in genetically determined deficiencies, and (2) therapeutic manipulation (suppression or sometimes stimulation) of the complement system to influence the pathologic process.

1. The possibilities of complement substitution are very limited. Since the majority of complement deficiencies occurs rarely and the turnover of complement

proteins is very rapid, substitution with isolated factors does not seem to be a reasonable intervention.

On the other hand, transfusion of fresh human plasma or plasma frozen immediately after venipuncture may be considered. Plasma was successfully administered by Soothill et al. [36] to patients suffering from frequently recurring bacterial infections associated with AP deficiency. Hereditary angioneurotic edema is the only disease in which, besides fresh plasma, purified preparation has been administered, *viz.* the genetically deficient C1 esterase inhibitor was substituted by an inhibitor preparation partially purified from the plasma. Although even in this case, a continuous substitution cannot be accomplished, the treatment can successfully be applied for preventing attacks, especially before interventions such as dental extraction, etc. liable to induce an attack [12].

2. It has been mentioned that complement plays a "dual" role in the organism. Consequently, therapeutic influence on the complement system may be a double-edged weapon the application of which would require special care and consideration even if adequate therapeutic procedures were available. Since complement is one of the most important mediators of inflammation, it seems reasonable to use suppressors of complement activation as antiphlogistics. Interestingly, this is an everyday intervention even today, for it has been shown that acetylsalicylic acid and several other antiphlogistics act *via* inhibition of complement [32]; furthermore, the antiphlogistic effect of steroids is due, at least partly, to an inhibition of complement activation [29]. It may be expected that natural inhibitors prepared from blood, e.g. C3b inactivator and β_{1H} globulin, will valuably contribute to the complement-suppressive therapy, as those substances are complement-specific, i.e. they do not affect other processes in the organism.

Stimulation (or substitution) of the complement system would be useful in IC diseases and, perhaps, in neoplastic diseases. In IC diseases, the low complement level can be not only a consequence, but also the cause of the local cumulation of IC, owing to the deficient IC elimination. In tumour diseases, on the other hand, the deficiency of cellular immunity may be compensated by complement stimulation [28]. In this field a recent paper by Japanese authors [23] is of special interest, *viz.* normalization of the complement level was associated with successful levamisole therapy in previously hypocomplementaemic patients. I am certain that therapeutic procedures favourably influencing the complement system in a more or less selective way will be evolved in the near future.

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Natural Killer and Tumor Recognizing Lymphocyte Activity in Tumor Patients *

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Several mechanisms can lead to the killing of a target cell by lymphocytes. In order to study immunologically specific phenomena it has to be ensured that the natural killer (NK) effect does not operate in the system. Using targets which are sensitive to NK, the effector populations have to be depleted of lymphocytes with such potential. The blood lymphocytes of tumor carrying patients often have reduced NK activity.

In two assay systems anti-tumor autoimmune reactivity has been demonstrated (the majority of tested patients had lung carcinomas or osteosarcomas). The tests were: 1. Induction of blastogenesis in blood lymphocytes by in vitro confrontation with autologous biopsy cells. 2. Lymphocyte mediated killing of autologous biopsy cells in short term in vitro assay.

Cross reactivity between patients was rare which indicates either that the putative tumor antigens are individual specific or their recognition is restricted by histocompatibility.

In vitro cytotoxicity tests aimed to demonstrate disease related selective recognition are performed with the awareness that at least three known effector mechanisms exist in which lymphocytes participate. 1. T cells equipped with antigen recognizing receptors [8]. 2. ADCC-antibody dependent cytotoxicity cells affect targets which have been selected out by the recognition of antibodies [15]. 3. NK – natural killing – cells destruct on seemingly indiscriminative basis by unknown mechanism [18].

Natural killing

The NK effect is a general, in experimental animals, age related phenomenon. Because of the lack of knowledge about its initiation it was designated with the attribute "natural" – natural killer – though it is not excluded that it might be generated by classical immunization.

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In spite of intensive studies in several laboratories the nature of surface structure recognized by the NK cells is still unknown. In studies with a panel of human cell lines and several blood donors, selectivity was indicated [22].

Since cells from long term cultures are sensitive, incorporation of some calf serum component to the cell membrane was proposed as at least one of the factors which determine sensitivity [20]. However, this is unlikely because cell lines cultured in human serum were as sensitive to the killing by lymphocytes as the ones carried in parallel in fetal calf serum [9].

In experimental animals and in man, the active lymphocytes are not the mature T or B cells and the effect is not histocompatibility restricted [6].

With human blood lymphocytes, separation of subsets with monitoring of surface markers in parallel with functional studies revealed that the so-called "null" fraction, i.e. the population non-adherent to nylon wool and depleted of SRBC rosette forming lymphocytes, is the most active [1, 2, 14]. This population is rich in FC receptor positive cells. Most authors agree that the active cells do not carry SIg and are Fc receptor positive.

With regard to T cell markers, part of the active cells could be rosetted with SRBC depending on the condition of the rosetting [2, 4, 29]. These T cells were shown to carry FC receptors, T cells with high avidity SRBC receptors and devoid of FC receptors were not active.

The activity of the "null" fraction – which is a heterogeneous population was found to be stronger than that of the total lymphocyte population. After elimination of rosettes formed with neuraminidase treated SRBC, i.e. T cells with low avid E receptors, the residual cells were still highly efficient. Also after elimination of Fc receptor positive cells, the residual cells were strongly active. Thus on the basis of lymphocyte markers, it was not possible to subdivide the "null" subset in populations with and without cytotoxicity [4].

It is conceivable that more than one lymphocyte type can exert the effect and they may even act in collaboration [28]. Peter et al. [16] supposed that one subset produces soluble factors upon contact with the target and another subset is induced by the factor(s) to exert the killing. Similarly, Takasugi et al. [22] and Troye et al. [24] assumed the collaboration of antibody producing cells, the effect being at least in part ADCC. They proposed that the effector cells either carry cytophilic antibodies on their surface and/or a small number of admixed antibody producing cells contribute during the in vitro incubation. The subsets active in NK thus far were shown to be efficient in ADCC systems also. Even the organ distribution of the two types of effect overlaps.

Another possibility is that cells do not operate on the basis of a conventional antigen recognition. During certain stages of differentiation or activation, thymus dependent lymphocytes may acquire cytotoxic potential. Cytotoxicity would be manifested against target cells towards which the lymphocytes carry recognition receptors. The efficiency of killing would be determined by the target cell depending on its inherent sensitivity. This assumption is strengthened by the finding that thymocytes and activated T cells recognize and attach to cells of the same species

[12]. This recognition may be an important factor also in cytotoxicity of activated T lymphocytes which act preferentially on cells of their own species [19].

In view of the question whether the NK phenomenon has a role in immune surveillance we have investigated the cytotoxic efficiency of blood, lymph node, and tumor infiltrating lymphocytes from tumor patients towards the highly NK sensitive K562 cell line [26].

Cancer patients (the majority with lung carcinoma) failed to exert cytotoxicity in 47%, while all the healthy donors tested in parallel had strong reactivity. The composition of the blood lymphocyte population did not differ with regard to the proportion of E rosetting and Fc receptor-bearing cells. Only rarely was activity exhibited by lymph node cells and tumor infiltrating lymphocytes although cells with Fc receptor were present in both preparations. Tumor infiltrating lymphocyte preparations had in fact a high proportion (22%) EA rosettes. Tumor infiltrating lymphocytes and lymph node cells had good reactivity in other functional tests (PHA and MLC assays).

Low response in tumor-bearing patients was reported also by Pross and Baines [17]. Studies in mice gave similar results [7]. In three systems of tumor-bearing mice (murine sarcoma virus and methylcholanthrene-induced tumors and nude mice carrying grafts from human lymphoblastoid cell lines) the natural killer activity of spleen and blood was reduced compared to age matched controls.

Reactivity of patients with their own tumor cells

Since specific cellular recognition resides in the T cell population and mature T cells have no NK effect, either in man or mice and rats, detection of disease related cytotoxicity on cell lines is expected to be possible by using characterized lymphocyte subsets.

The T cell mediated effects can be investigated either directly or after in vitro sensitization, achieved in culture when the effector population is exposed to antigen carrying cells for several days [11].

A new aspect of the T system has recently evoked considerable interest when it was discovered that effector cells directed against virally or chemically altered cells have to share the histocompatibility antigens of the target in order to kill [10]. The present view is that this restriction is not absolute and can be overridden to some extent especially in long term cytotoxic assays.

In view of this restriction it is questionable whether established cell lines can be used as prototype targets. Consequently it is likely that in search for tumor specific reactivities the experiments have to be performed in autologous systems. However, at least in one human disease – infectious mononucleosis – T cell mediated cytotoxicity was demonstrated in short term assay on allogeneic target cells [5, 21]. Experiments with breast cancer patients using similar lymphocyte subsets failed to reveal selective cytotoxicity towards breast cancer derived lines [3].

In an effort to eliminate at least some of the factors which hamper interpretation of results with cell lines, two tests have been designed in our laboratory

for measuring cell-mediated anti-tumor recognition in man: the autologous tumor stimulation (ATS) and autologous lymphocyte cytotoxicity (ALC). In both tests, tumor cells separated from biopsy specimens were allowed to react with autologous lymphocytes.

The autologous tumor stimulation (ATS) test registers DNA synthesis of lymphocytes following 6-day cultivation with mitomycin-treated biopsy cells [25]. At least part of the responding cells belong to the T subset. Lymphocytes attached to the tumor cells during the early period of cocultivation were shown to rosette with SRBC. Moreover when prefractionated populations were used, the T-enriched fraction reacted while the T-depleted fractions did not.

In our initial series ATS was obtained in 30% of 197 patients with solid tumors (the majority with lung carcinomas or osteosarcomas).

Improvement of the test conditions by using exclusively tumor cell enriched populations of good viability as stimulators, by incubating the tumor cells prior to the test (this step was introduced to allow resynthesis of putative cell surface antigens if removed by the procedure of preparation of tumor cell enriched suspensions) and the use of T cell enriched responder population has given considerably increased positive cases (60%). This would indicate that the majority of patients recognize immunologically their tumor cells.

Recent experiments involving criss-cross tests indicated that the lymphocytes are stimulated for DNA synthesis when confronted with autologous and only exceptionally when confronted with allogeneic tumor cells. Only two biopsies among 36 tested stimulated allogeneic lymphocytes.

The autologous lymphocytotoxicity (ALC) test was worked out recently. Short term ^{51}Cr-release microtest [27] was used to register the killing of biopsy cells. Blood lymphocytes were cytotoxic for autologous tumors in 16/29 cases. The ALC positive lymphocytes reacted with allogeneic tumor cells – susceptible for autologous killing – in only 2/25 combinations and were cytotoxic to 2/27 tumor cell preparations which were not damaged by their autologous lymphocytes. Lymphocytes from healthy donors were negative in 13 experiments.

Thus when patients have been cross tested in both assays, ATS and ALC, reactivity occurred only in autologous combinations. This fact is strongly suggestive for genuine tumor specificity of the reactions. Because biopsy cells are only exceptionally affected by NK cells such effect does not disturb the experiments.

Comparison of our results with the earlier extensive material on human tumors shows an important difference because the latter indicated tissue specific tumor specificity. With various tumor types using short term cultured cells as targets cross reactivities between tumors originating in the same tissue were reported [13]. These studies may have been devoid of NK effects because the target cells were cultured only during a short time. Apart from technical factors (48 h microcytotoxicity or colony inhibition in the experiments of the Hellströms and short term ^{51}Cr release test in our series) the nature of target cells is the main difference.

The preferential if not absolute autologous reactivity may be indicative of

individual antigens or reflects the influence of histocompatibility on T cell mediated cytotoxicity. The earlier cross reactivities, indicating tissue related specificities may be reconciled with the duration of the cytotoxic test. In animal tumor systems with strong antigenicity long term tests have overridden the histocompatibility restriction, however, there was still a stronger effect if the effector and target cells were histocompatible [23].

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Surface Markers and Functional Characteristics of Human Blood Lymphocytes Residing in the Operational "Null" Subset

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After removal of nylon wool-adherent B and E rosetting T cells from the human blood lymphocyte population, a small fraction remains, often denoted as "null" subset. This fraction contains lymphocytes which are highly efficient in antibody-dependent and non-selective lymphocyte cytotoxicity. These cells possess markers. About half of them carry Fc receptors, and the majority has low avidity E and C3 receptors. Removal of low avidity E receptor-carrying cells did not abolish cytotoxicity to K-562 cells. The majority of cells carry receptors for Epstein-Barr virus but cannot be infected. The cells in mixed lymphocyte culture have a low reactivity. They are stimulated by PHA and ConA to a similar extent and differ from the cells which readily sediment as E rosettes: these respond stronger to PHA. The "null" fraction may contain stem cells common for both T and B lineages.

Introduction

The majority of human blood lymphocytes are SRBC-binding T cells. Their number, as detected by rosette formation, is influenced by the conditions of the assay and by the relative proportion of the cells in the mixture [19, 50, 51]. T cells can also be characterized by heterologous antisera raised against thymus cells [2, 19, 49]. The E receptor and the T antigen determined by such sera are probably separate entities [20].

Initially, the C3 receptor was considered an exclusive marker of SIg-carrying B lymphocytes, while a subset of T cells designated tentatively as D lymphocytes were shown also to form EAC' rosettes [12, 33].

Besides the SIg, the receptor for Epstein-Barr virus and the capacity to be "immortalized" by this virus is a reliable marker for B cells [27].

The Fc receptor is not a discriminative marker since subsets of T and B cells were shown to carry it [6, 7, 13, 14, 17, 22, 24, 34, 45].

When B and T cells are removed from the blood lymphocyte population by combination of nylon wool passage and sedimentation of E rosettes, 10% of the cells remains. This fraction is functionally important since it contains cells strongly cytotoxic against human tissue culture cells. The nature of this cytotoxic effect is not known. It is postulated by Akira et al. [3] to be due to antibodies carried on the lymphocyte surface and thus having the mechanism of ADCC. In fact,

spontaneous cytotoxicity and the conventional ADCC test were in all experiments displayed by the same lymphocyte subfractions [40, 41].

On the basis of the fractionation results we previously designated this fraction as "non-T non-B" cells [4] and found that about 50% of these cells carried Fc receptors. The fraction was designated as "null" cells and claimed to contain B cell precursors by Chess et al. [11]. On the other hand, Hersey et al. [26] classified them as predominantly T cells with low affinity E receptors. In the present paper we follow the designation of Chess et al. [11] and refer to the fraction as "null". It must, however, be emphasized that the designation is operational and only a fraction of it is real "null", i.e. without an easily detectable marker.

Judged by cell size and surface markers, the "null" fraction is heterogeneous, as it contains immature cells [4, 11, 26] on which the differentiation markers are not exclusively expressed. We show in the present work that most of the cells express both E and C' receptors in low density.

Materials and Methods

Lymphocyte source. 200 ml heparinized blood, drawn from healthy blood donors randomly chosen from Karolinska Hospital Blood Bank Centre.

Target cells in cytotoxic experiments. K-562, derived from a patient with chronic myeloid leukaemia is of myeloid origin according to its marker properties [31, 39]. 1301, an undifferentiated line, presumably of T cell origin, a subline of CCRF-CEM [18], was obtained from B. Hampar, Bethesda, through Y. Becker, Jerusalem. Both were grown as stationary suspension cultures.

Fractionation of lymphocytes. The Ficoll-Isopaque method of Böyum [10] was used to isolate lymphocytes from the blood. The yield was about 1×10^6 ly/ml of blood. Macrophages were removed by carbonyl iron treatment. This suspension is designated as "unfractionated lymphocytes".

For fractionation, the cells were passed through a nylon wool column [30]. The adherent cells (B-enriched) were eluted by rinsing the wool with FCS at 37 °C.

From the nylon wool passed population, the E rosettes were separated directly on Ficoll [5]. The lymphocytes were adjusted to 8×10^6/ml and mixed with three times washed SRBC in 2.5% solution. The SRBC was added in one-fifth of the volume of lymphocytes, in order to obtain a 25 : 1 erythrocyte : lymphocyte ratio. 5 ml of this suspension was layered directly on 3 ml Ficoll and centrifuged at 2000 r.p.m. for 20 minutes (500 g). The E rosettes were treated with Tris-buffered isotonic 0.83% NH_4Cl for 10 min at room temperature [40] or in some experiments with osmotic shock [9] to burst the erythrocytes. T cells were divided into Fc receptor-depleted and enriched fractions by separating the EA rosettes. As calculated from eight experiments the cells recovered in these two fractions were 34% and 6.2%, respectively, of the initial lymphocyte number.

The cells remaining in the interface were collected; these were designated as "null" cells. The "null" fraction was further separated on the basis of rosetting capacity with various indicator cells.

Lymphocyte markers. E rosettes were formed according to the method of Jondal using overnight incubation at 4 °C [28].

E-neuraminidase (E_N) rosettes were formed with SRBC incubated with 5 units of enzyme (Neuraminidase, Behringwerke) per ml of E (1%) at 37 °C for 20 min and washed three times [23]. The lymphocyte–erythrocyte mixture was incubated overnight at 4 °C.

EA rosettes were formed according to the method of Hallberg et al. [24].

EAC' rosettes were formed using sheep and ox erythrocytes according to the method of Bianco et al. [8].

Surface Ig was detected by immunofluorescence. The cells were stained for 45 min at 4 °C with FITC-labelled goat anti-human Ig (Hyland Laboratories, Los Angeles, Calif., USA), used at 1 : 30 dilution. The proportion of cells with surface immunoglobulin was determined by scoring 200 cells.

Recovery and marker characteristics in the fractions. Table 1 contains the yields and marker characteristics of the lymphocyte populations summarizing all our experiments in which this fractionation procedure was used. 74.9% of the cells were recovered in three fractions: nylon-adherent, E-rosetting and "null". As indicated by the characteristics of the fractions, the 25.1% loss was not selective for E, E_N and EA-rosetting cells, since 75.5, 79.9 and 69.4%, respectively, of these cells were recovered. There was some selective loss of the C_3 receptor (17.2%) and SIg-carrying cells (31.5%) probably due to the adherent properties of the B cells. It is likely that part of the cells which scored as SIg-positive in the total population were Fc receptor-positive with passive adherence of immunoglobulin. These might have detached during the manipulation involved in the fractionation, hence the loss of cells scored as SIg-positive.

Cytotoxic tests. The ^{51}Cr assay was carried out according to Svedmyr et al. [47].

In all experiments, the lymphocyte fractions were incubated prior to the cytotoxic assay at 37 °C for 12 hr, at a concentration of 1×10^6/ml in RPMI medium (10% FCS). Thereafter, they were washed with medium, counted and adjusted to the desired concentration.

Four effector : target cell ratios (50 : 1, 16 : 1, 8 : 1, 4 : 1) were tested in triplicate. After 7 hr incubation at 37 °C in 5% CO_2, 0.1 ml of the supernatant of each culture was removed. Cytotoxicity was expressed as per cent specific release according to the formula: per cent sp.r. = 100 (T-S)/(Max-S), where T = release in test, S = spontaneous release, in medium, Max = maximal release, obtained by lysis in distilled water overnight. Maximal release was between 60 and 90% of the total isotope uptake.

The spontaneous ^{51}Cr release of K-562 and 1301 was under 25%. The standard error of triplicate determinations of isotope release did not exceed 5 units.

Within the range of effector target ratios used, the dose response is linear allowing the expression of activity in lytic units (L.U.). This is the cell number necessary to lyse 50% of target cells under our experimental conditions.

Table 1

Fractionation of human

Populations	Percent of cells recovered in the fractions, mean ± S. E.*	E-rosettes		E_N-rosettes	
		A**	B***	A	B
Unfractionated		67.8 ± 5.9 (9)		68.3 ± 3.2 (7)	
Fraction 1					
Nylon adherent	13.7 ± 1.6	42.2 ± 5.7 (8)	5.8	42.4 ± 5.5 (6)	5.8
Fraction 2					
E-rosetting	52.2 ± 1.6	83.4 ± 5.5 (8)	43.5	88.0 ± 2.4 (7)	45.9
Fraction 3					
"null"	9.0 ± 0.9	21.6 ± 4.0 (9)	1.9	33.3 ± 5.4 (7)	2.9
Recovery in the three fractions	74.9		51.2		54.6
Recovery of cells with the marker****			75.5		79.9

* Mean of 30 experiments.

** Observed percentage in the fractions, mean ± S.E.; in parentheses number of experiments.

*** Calculated as percentage of the total unfractionated population $\frac{\%\,\text{yield}}{100} \times A$

**** Recovery of cells with the indicated marker

$$\frac{\%\ \text{total recovery in the fractions}}{\%\ A\ \text{for the unfractionated population}} \times 100$$

Mitogen stimulation. 2×10^4 to 10^5 cells were incubated in flat bottom wells (Micro Test II, 3040 Falcon) in 0.2 ml RPMI-1640 supplemented with 10% heat-inactivated FCS, 5×10^{-5} M 2-mercaptoethanol, 5 mM Hepes, in 5% CO_2 at 37 °C.

One μCi of ^{3}H-thymidine was added in 0.02 ml for the final 24 hr of incubation. Cells were harvested in filter paper with a multi harvester (Skatron, Norway) and the incorporation was assessed in a Beckman scintillation counter. Three to five replicas were done.

Phytohaemagglutinin (Leucoagglutinin, Pharmacia) and Concanavalin A (Sigma, Grade IV) were added in a final concentration of 1 μg/ml and 5 μg/ml, respectively.

Mixed lymphocyte reaction (MLC). 10^5 lymphocytes were mixed with equal number of allogeneic, mitomycin C (Sigma, crystalline; 25 μg/ml, 30 min, 30 °C) treated lymphocytes.

Epstein-Barr virus (EBV) infection was assessed by measuring the incorporation of ^{3}H-thymidine on the seventh day following exposure to EBV [44].

blood lymphocytes

EA-rosettes		EAC'-rosettes		Surface Ig	
A	B	A	B	A	B
25.5 ± 2.3 (8)		16.8 ± 2.1 (12)		17.5 ± 2.0 (12)	
47.7 ± 3.2 (8)	6.5	36.7 ± 2.7 (9)	5.0	44.4 ± 2.6 (6)	6.1
12.3 ± 1.0 (8)	6.4	5.6 ± 1.5 (7)	2.9	0.6 ± 0.2 (9)	0.3
53.8 ± 5.5 (8)	4.8	20.1 ± 5.8 (10)	1.8	13.3 ± 3.5 (6)	1.2
	17.7		9.7		7.6
	69.4		57.7		43.4

The lymphocytes in micro wells (in 0.02 ml) were infected with 0.02 ml of EBV containing fluid supernatant of B95-8 cells [37]. After 60 min at 37 °C, 0.15 ml medium was added. Termination of incubation and measuring the uptake of thymidine were carried out as described above for mitogen stimulation.

The virus batches used induced EBV nuclear antigen (EBNA) [42] in 20% of Ramos cells [32] 48 hr after infection.

Results

Characterization and subsets of the operational "null" fraction. Judged from several experiments the fraction contained 53.8% of Fc-positive cells, 20.1% EAC' receptor-positive cells and 21.6% E receptor-positive cells when rosetted with SRBC (Table 1). When the rosettes had sedimented on Ficoll (Table 2), the recoveries on the pellet reflected the observed values in the case of neuraminidase-treated sheep E, ox EA but were somewhat higher in the case of sheep E and ox EAC' rosette separation.

On the other hand, when the EAC' rosettes had formed with SRBC, the cell yield in the pellet was considerably higher than the proportion of rosettes scored before centrifugation. Similar results were obtained in two further experiments. The proportion of EAC' rosettes was 5% when scored before centrifugation but 67% of the cells sedimented to the pellet after centrifugation on Ficoll. Visual observation confirmed that these cells had indeed attached erythrocytes (>80%).

Table 2

Subfractionation of the "null" population

Exp. No.	Rosettes sedimented on Ficoll	Fraction	Cell number in the fraction ($\times 10^6$)	Recovery, per cent "null" population	Markers*					Cytotoxicity L.U. ($\times 10^5$)+
					E^{++}	$E_N{}^{+++}$	EA°	EAC[1]	Slg[2]	
I		"null", input	8.5		8	40	33	—	—	0.20
	E sheep^{++}	interface	5.0	59	—	—	—	—	—	0.18
	E sheep	pellet	1.9	22	—	—	—	—	—	0.44
I		"null", input	8.5		8	*40*	33	—	—	0.20
	E sheep N^{+++}	interface	3.1	36	—	—	—	—	—	0.12
	E sheep N	pellet	3.0	35	—	—	—	—	—	0.48
II		"null", input	11.0		—	—	41	6	—	—
	EA ox°	interface	2.0	18	—	—	3	7	—	—
	EA ox	pellet	4.0	36	—	—	85	16	2	—
III		"null", input	15.0		—	—	56	*0.5*(ox)	—	—
	EAC'ox[1]	interface	12.0	80	—	—	—	0 (ox)	5	—
	EAC' ox	pellet	0.8	7	—	—	—	—	6	—
IV		"null", input	15.0		22	—	62	*5*	—	0.06
	EAC' sheep[1]	interface	3.0	20	—	—	—	0	6	—
	EAC' sheep	pellet	10.0	67	3	—	58	8	10	0.08

* Percentage of positive cells

+ Lytic units, number of cells necessary to lyse 50% of the target cells. The target was 1301, except in Exp. IV in which K562 was used. $^{++}$ SRBC rosettes. $^{+++}$Rosettes with neuraminidase-treated SRBC. °EA rosettes assayed with ox erythrocytes. [1]EAC' rosette forming cells assayed with ox or sheep erythrocytes. [2] Surface immunoglobulin positive cells, determined by immunofluorescence.

Part of these rosettes could represent pure SRBC rosettes but since 22% sedimented in the E rosette separation, the majority of cells were in the pellet due to the attachment through C3 receptors. We assume that by using sheep EAC' two binding affinities, each being too weak alone – low affinity E and C3 receptors – contribute and the rosettes are formed under the enhancing condition of centrifugation through Ficoll.

Cytotoxic activities. The cytotoxic activities of the fractions were assayed in some of the experiments (Table 2, Fig. 1). In accordance with previous experience [4], the activity of the "null" fraction was considerably stronger than that of the total lymphocyte population (Fig. 1).

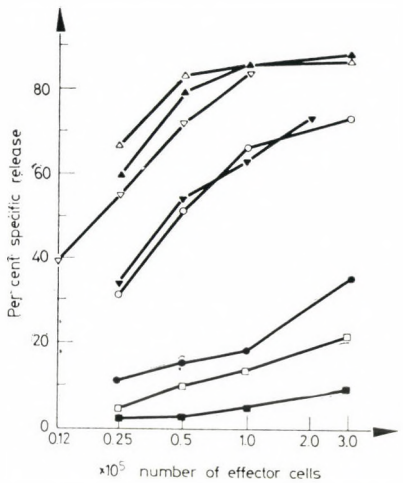

Fig. 1. Natural cytotoxicity of human peripheral lymphocyte fractions tested against 1301 lymphoblastoid cell line. Unfractionated (□ — □); E-rosetted cells depleted of Fc-positive cells (■ — ■); Fc-positive E-rosetted cells (• — •); "null" cells (▽ — ▽); "null" cells depleted of remaining T cells (▲ — ▲); recovered T cells from the "null" cell fraction (▼ — ▼); "null" cells depleted of neuraminidase E rosettes (△ — △); recovered E_N rosettes from the "null" fraction (○ — ○)

After eliminating residual E rosettes from the "null" fraction formed by renewed rosetting, the cells were still highly efficient (L.U. = 0.18×10^5). This was the case also when E_N rosettes were eliminated (L.U. = 0.12×10^5). The E-rosetted cells were also active though weaker (L.U. 0.44; 0.48 for E_N and E rosettes, respectively). These were, however, treated with NH_4Cl, a procedure known to impair cytotoxic activity. According to our previous experiments, about 50% of the activity returns during incubation overnight [5]. Consequently, there is no major difference in the efficiency of the E^+ and E^- subfractions of the "null" population.

In experiment IV, the L.U. of the "null" cell fraction was 0.06×10^5. The majority (67%) of the cells were pelleted with sheep EAC' and their activity was

similar (L.U. = 0.08×10^5). NH_4Cl was used to burst the erythrocytes and the cells were assayed after overnight incubation.

In accordance with our previous experience [4], the results in Fig. 1 show that the activity of the T cells pelleted after nylon wool passage with SRBC is mainly due to FC receptor-carrying cells.

Response in MCL to mitogens and EBV. In twelve experiments, the response of the fractions to PHA and ConA were assayed. We present here the results of two experiments (Table 3) performed each with the blood of different donors.

The results show that

1. during four days of incubation with PHA the E-rosetted T fraction and its two subfractions, EA rosette-enriched and EA-depleted ones, were stimulated. As expected, the peak of response to PHA subsided by the seventh day [25]. The response to ConA was regularly weaker;

2. the "null" fraction responded also to PHA but the response increased to day 7. The response to ConA was similarly high.

MLC reaction was also tested. Measured on the seventh day, the E-rosetted T fractions responded considerably stronger in MLC than the "null" fraction.

Table 3

Response of the various lymphocyte subsets to mitogens, EB virus and in MLC*

Lymphocyte population	Experiment 1					Experiment 2				
	Stim. index MLC	CPM control	Stimulation index			CPM control	Stimulation index			CPM EBV
			PHA**	ConA***	PHA/ConA		PHA**	ConA***	PHA/ConA	
B cells	8.4	3579	14.9	11.6		13092	1.5	1.8		34304
E-rosettes	42.6	1613	45.8	13.6	3.3	1549	20.4	1.3	15.7	5101
depleted of EA rosettes	22.1	1114	61.5	13.7	4.4	449	35.6	2.8	12.7	3947
enriched in EA rosettes	37.8	453	73.4	41.6	1.8	780	61.7	3.1	19.9	3342
"null" fraction	8.2	7230	10.3	10.7	1.0	179	108.0	66.5	1.62	9751
depleted of EA rosettes	4.0	10913	12.4	8.9	1.4	953	39.7	48.3	0.8	14698
enriched in EA rosettes	5.3	1503	26.6	23.2	1.2					

* The MLC and EBV infected cultures were assayed on day 7. The response to mitogens PHA and ConA was assayed on day 4 in Exp. 1, on day 7 in Exp. 2.

** 1 μg/ml final concentration

*** 5 μg/ml final concentration.

Thus, mitogen response of the E-rosetted cells and their two subsets (Fc-positive and Fc-negative) differed from that of the "null" fraction. When from the "null" fraction SRBC-rosetting cells were removed, reactivity did not change.

Since it is known that only B cells can be infected with EBV, ^{3}H-thymidine incorporation after addition of EBV was measured to detect B cells. Eight experiments were carried out, the results were similar. The nylon wool-adherent fraction (44% SIg-positive cells) had the highest incorporation. The "null' fraction was also stimulated, but to a lesser degree.

Already on the third day, large transformation centres were seen in the nylon-adherent and the "null" cell containing micro wells, though in the latter they were fewer. No such cell clumps were seen in the T cell (and their subfractions) containing micro wells. Lymphoblastoid cell lines were established from the "null" fraction. These results are in line with the detected few SIg-positive cells.

Discussion

Subdivision in the two main groups, B and T cells on the basis of their well defined markers such as SIg and affinity to SRBC, was the first step to characterize the human blood lymphocyte population. Other cell surface markers initially regarded to be exclusive for either the T or B population such as receptors for the Fc part of IgG and C3 are now known to be present on a proportion of cells belonging to both groups and also on cells designated as "null", i.e. remaining after removal of nylon wool-adherent and high avidity E receptor-carrying cells. The subsets carrying these markers within the B and T populations are probably functionally different and represent various differentiation steps.

We have focussed our attention on the characteristics of the operational "null" population. This small fraction of blood lymphocytes (10%) contains highly active cytotoxic cells [4, 26, 41]. Chess et al. [11] showed that more than 50% of these cells develop surface immunoglobulins after cultivation *in vitro*. Thus, at least part of the population may contain immature B cells. They also have low, with the fluorescence method undetectable, amounts of SIg. The fraction contains also cells with low avidity for SRBC.

Abo et al. [1] showed that about 50% of the population remaining in the interface after sedimenting E rosettes (adherent cells were removed previously) could be rerosetted after treatment of the lymphocytes with neuraminidase. A high proportion carried Fc and some cells also C3 receptors. In our hands also, the "null" population contained Fc (53.8%), E (after renewed rosetting) (21.6%) and EAC' (20.1) receptor-positive cells. 13.3% of the cells were SIg-positive. With sheep EAC' the majority of cells sedimented as rosettes on Ficoll, indicating low affinity E and C3 receptors.

Figure 2 gives a scheme of the constitution of human blood lymphocyte population. Seventy per cent of the cells are mature T with E receptor densities sufficient to rosette and sediment on Ficoll under standard conditions. B cells

with easily detectable SIg amount to about 15%. Cells with overlapping B and T characteristics are mainly found in the "null" fraction.

Characteristics of the "null" fractions and the distribution of markers are indicated by the following findings:

(a) 33.3% (average value of seven experiments) of cells in the "null" subset were E_N-positive. When instead of SRBC the lymphocytes were treated with neuraminidase, 58% rosetted with SRBC in the experiments of Abo et al. [1]. Thus, a considerable number of cells with low avidity E markers are present;

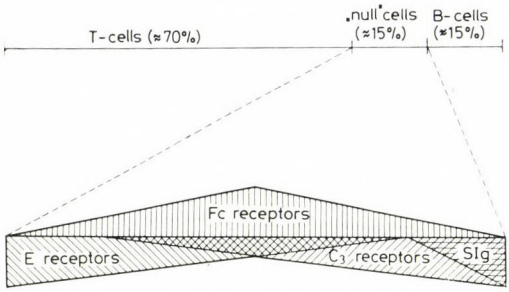

Fig. 2. Possible composition of the "null" cell fraction, indicating the quantities of cell surface receptors on individual cells

(b) we found that 12.3% (average of 31 experiments) of E-rosetting T cells have FC receptors. This is in accordance with previously published results [1, 7, 12, 14, 17, 24]. On the other hand, 42.5% of the cells which should be rerosetted with SRBC from the "null" fraction were found to have Fc receptors [4];

(c) 11 to 37% of mature SIg-positive B cells have Fc receptors [16, 22, 34];

(d) 0.5 to 8% of E-rosetting T cells carry C3 or Fc receptors [12]. We found 5.6% (average of five experiments) of EAC'-positive cells;

(e) the majority of cells in the "null" population could be pelleted by sheep EAC' but not with E sheep or EAC' ox alone (present results) indicative of low avidity E and C' receptors;

(f) studies using ^{125}I-labelled $(Fab)_2$ anti-Fab reagents indicated that the "null" population has significantly greater quantities of Ig than the T cells, but only 1% of the amount present on the surface of B cells [11];

(g) the majority of "null" cells have receptors for EBV [16] which is an exclusive B cell marker and probably identical with the C3 receptor [29];

(h) human leukaemic cases and lymphoblastoid cell lines exist in which the blasts rosette with SRBC or react with anti-T serum and carry C3 receptors [33, 38, 48];

i) there is some evidence suggesting that lymphocyte subpopulations differ in their relative avidity for the Fc part of IgG and "null" cells may have higher avidity than either B or T cells [15]. Revillard et al. [43] depleted the lym-

phocyte population of Fc- positive cells using EA indicators sensitized with low concentrations of antibody and yet inhibited ADCC activity.

It seems thus that in the "null" fraction stem cells in various degree of differentiation for both T and B cell lineages are present. Some of the characteristics of the "null" fraction can be found on early foetal thymus cells, over 70% of the cells possess complement receptors and less than 10% rosette with SRBC [21, 46].

The "null" fraction had low reactivity to mitogens if tested on the fourth day. Similar results were reported by MacDermott et al. [35]. They reported low mitogen response during four days, when the experiments were terminated. Our results show that the "null" fraction has a good capacity to respond but the kinetics are different from that of the E-rosetted T cells. They differed also by the relative response to PHA and ConA, the "null" fraction was stimulated equally, while the E rosettes responded stronger to PHA.

In accordance with the demonstration of C3 receptors, the majority of the "null" cells had EBV receptors [16] also detected by virus absorption. However, the EBV-determined nuclear antigen (EBNA) was not induced. Since the appearance of EBNA is an early event of EBV infection [36], virus absorption did not proceed into infection of the cells. This result too shows that a high number of cells had certain but not all B cell properties indicating that these were not fully differentiated B cells.

The stimulation of DNA synthesis by EBV detected the mature B cells. Their presence was also shown by the formation of a few characteristic transformed cell clusters. The T fractions were neither stimulated by the virus, nor did they absorb it.

The presence of surface markers on lymphoblastoid lines in a seemingly irregular way with regard to the cell lineages may indicate that these represent the phenotype of lymphocytes in various steps in the normal differentiation. E.g. the Molt-4 cell line which reacts with anti-T serum may be a typical cell for the "null" fraction. It was an E-rosetting line in its early passage [38]. It has C3 [20, 29, 38] and EBV receptors, but cannot be infected with EBV. A real "null" cell line, i.e. 1301, one without the known markers also exists [20, 29].

It was not possible to deplete the "null" fraction from cytotoxic cells. E, E_N depletion left population with increased cytotoxicity. Thus, cells other than those carrying T cell markers were also highly active.

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Acute Leukaemia: Cell Markers and Thymic Factor on E-Rosette Frequency*

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At least theoretically, the so-called "null cell" acute leukaemias (AL-s) could form clones from either the uncommitted stem cell, or the differently committed stem cells, including the T cell progenitor or the T_0 lymphocyte. An attempt to single out different forms among the miscellaneous groups of the above-mentioned AL-s, as well as to schematize both hemic and lymphatic cell ontogeny has been made either with personal experiments by incubating peripheral blood and bone marrow cells from AL-s with human serum thymic factor, or on the basis of the recent informations derived by membrane phenotyping, assaying of specific enzyme markers, testing cell reaction to antisera raised against given cell types, etc. Results of the personal assay showed that incubation of AL cells with human serum thymic factor (which induces maturation of committed precursors to T cells), caused a clear-cut increase of E^+ cells in 6 out of 36 patients for the peripheral blood mononuclears, and in 3 out of 11 patients for the bone marrow lymphoid cells. It results that significant percentages of cells interpreted as "null cells" were rather quite immature, not yet E-rosetting T cells. The latter have been shifted to a more mature (E-rosetting) stage by the thymic factor. These results seem to substantiate the interpretation that the T cell precursor (or the T_0 cell) may undergo leukaemic clonal expansion, or that AL cases may be accompanied by a contemporaneous growth, and peripheral blood traffic of quite immature T cells.

Introduction

Detection of immunologic cell markers is now largely integrating morphology, ultrastructure, cytochemistry, cytogenetics, cell and tissue culture in investigating the pattern and behaviour of cells in haematological malignancies. The differentiation stage of the lymphocyte precursors, the class or subclass, or even the immunological activation stage of the lymphocytes which underwent clonal expansion are the main criteria actually followed in classifying lymphomas.

On the other hand, cell membrane phenotyping, assaying of specific enzyme markers, testing cell reaction to antisera raised against given cell types associated with the search for malignant chromosomal translocation (Ph' marker) are the procedures which have recently provided important information to identify the

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cell target for the abnormal growth in the different categories of leukaemia. These studies may also bring to a better knowledge of the ontogeny of both lymphatic and hemic cells. We are mostly referring to the achievements by McCaffrey et al. [14, 15], Brown et al. [7], Greaves et al. [10, 11], Jánossy et al. [12] (and the numerous British collaborators who "have participated in various aspects" to their work), as well as to the achievements of Strominger et al. [19, 20], Billing et al. [5], Schlossman et al. [18].

The above-mentioned achievements include demonstration of: (a) The DNA-polymerizing enzyme terminal deoxynucleotidyl transferase (TdT); (b) the N-acetyl-beta-D-glucosaminidase or hexaminidase (in particular, the isoenzyme hexaminidase I: Hex 1); (c) the anti-stem cell serum raised against the leukaemia-uncommitted stem cells, and specific for the cells of that leukaemia (USCL marker), which Greaves et al. call ALL marker (marker for cells of acute lymphocytic leukaemia of non-T and non-B type, *viz.* "common" ALL); (d) the anti-p23,30 serum, which is an antiserum raised against a glycoprotein antigen complex of 23,000 and 30,000 dalton subunits isolated and purified from a human lymphoblastoid B cell line; it detects a unique antigen on B lymphocytes which is distinct from immunoglobulin surface antigen, beta-2-microglobulin surface antigen, F_c and complement receptors, and is proposed to be the human counterpart of murine IA (Ir-associated) antigens (HuBLA marker). Again: (e) The antisera-detecting antigen(s) carried by both immature (T_0, T_1) and mature (T_2) T cells, either normal or from lymphocytic leukaemia (HuTLA marker); (f) the antisera detecting antigen(s) carried by immature T cells either normal or from lymphocytic leukaemia, but lacking from the mature T cells (TLA'A marker); (g) the cholera toxin receptor (CT marker).

In addition, we have recently carried out some experiments by incubating *in vitro* with human serum thymic factor [1, 3] the peripheral blood cells from patients with "null cell leukaemia": We have found that few of those cases had rather high percentages of cells which were E-rosetting-negative (E^-) prior to, and E^+ after their incubation with thymic factor [4, 16]. In this paper we want to discuss classification of acute leukaemias, as well as hemic and lymphatic ontogeny according to the above-mentioned cell markers and the results of our personal study.

Material and Methods

Lymphoid cells were obtained from heparinized peripheral blood and from the bone marrow aspirates after separation procedure by means of density gradient centrifugation. Thirty-nine patients with acute leukaemia (AL) were studied, most of them being in childhood. Of them, 17 were at their initial presentation, 12 in relapse, and 10 in remission. Blood samples withdrawn on occasion of haemato-immunological routine investigations were used as control, and this donor group consisted of 32 healthy subjects of either sex, aged from 2 to 62 years.

The count of E^+ cells was made both before and after short (10 to 30 minutes) cell incubation with human serum thymic factor, a probably circulating thymic hormone prepared at the Central Laboratory of the Netherlands Red Cross

Table 1

Frequency (%) of E-rosettes before and after cell incubation with human serum thymic facto

Patient No.	Disease stage	Blood		Marrow		Differences	
		Before	After	Before	After	Blood	Marrow
1	RL	21.5	54.0			+32.5	
2	IP	18.3	17.2			− 1.1	
3	RL	11.8	14.5			+ 2.7	
4	RL	60.0	68.9			+ 8.9	
5	IP	70.0	68.9			− 1.1	
6	RM	47.7	39.7			− 8.0	
7	RL	21.2	48.3			+27.1	
8	RL	5.1	12.2			+ 7.1	
9	IP	69.8	75.8			+ 6.0	
10	IP	35.0	32.4			− 2.6	
11	IP	12.0	10.6			− 1.4	
12	RM	57.6	60.4			+ 2.8	
13	IP	8.9	8.1			− 0.8	
14	IP	63.8	65.2			+ 1.4	
15	IP	13.1	22.4			+ 9.3	
16	RL	18.2	15.8			− 2.4	
17	RM	42.0	56.4			+14.4	
18	IP	52.0	59.3			+ 7.3	
19	RL	18.2	18.1			+ 0.1	
20	RL	34.7	43.4			+ 8.7	
21	RL	53.9	66.0	28.8	61.2	+12.1	+32.4
22	IP	43.8	44.4			+ 0.6	
23	RM	45.8	41.7			− 4.1	
24	RL	45.0	41.9			− 3.1	
25	RL	16.05	21.23			+ 5.18	
26	IP			46.5	51.5		+ 5.0
27	RL	47.8	42.0	6.9	22.4	− 5.8	+15.5
28	IP	35.4	46.7	22.4	26.9	+11.3	+ 4.5
29	RM	37.7	36.9			− 0.8	
30	IP	0	4.5	5.4	8.9	+ 4.5	+ 3.5
31	RM	45.4	49.4	13.5	11.6	+ 4.0	− 1.9
32	RM	21.7	46.4			+24.7	
33	RM	37.3	45.7	16.2	42.7	+ 8.4	+26.5
34	IP			46.47	51.45		+ 4.98
35	IP	26.0	33.0	33.1	26.5	+ 7.0	− 6.6
36	IP	34.06	39.53			+ 5.47	
37	RM	34.29	41.08			+ 6.79	
38	IP			43.6	37.9		− 5.7
39	RM	8.6	8.3	17.6	19.5	− 0.3	+ 1.9

IP = Initial presentation; RM = Remission; RL = Relapse

Blood Transfusion Service, Amsterdam [1]. This is a soluble factor, rather unstable, with a molecular weight of about 1000. It has a target cell specificity for T cell precursors, inducing their maturation to T cells, both in humans and in animals.

The mentioned thymic factor is absent in human thymectomized donors, disappearing shortly after thymectomy. Again, it is absent in patients with thymic-dependent immune deficiency diseases. The human thymic serum factor was found to decline after the age of 30. There is also evidence that with age there is a relative increase in immature T cells at the expense of mature T cells. When patients lacking serum factor were injected with the thymic extract thymosin, the serum factor activity promptly appeared in their blood [2, 4].

Results

In 32 control subjects there were no significant differences between the counts of the E^+ cells from the non-incubated blood lymphocytes and the counts from the lymphocytes previously incubated with the human serum thymic factor (57.09 ± 6.11 versus 56.94 ± 7.26, respectively).

In contrast, several AL patients showed marked increased count of E^+ cells after incubation with the thymic factor. The results of the observations are quoted in Table 1. From the reported data, as far as peripheral blood mononuclears are concerned, it is evident that an increase of over 10 E^+ cells occurred in 6 out of 36 patients examined. Concerning the bone marrow lymphoid cells, an increase of over 10 E^+ cells was detected in 3 out of 11 patients examined.

The results, taken as a whole, show that cell incubation with human serum thymic factor raised the number of E^+ cells in several of the patients tested. That increase was apparently due to a maturation shift of E^- to E^+ cells, previously classified as null cells (E^-, SM-Ig^-). Indeed, a commensurate correlation between increase of E^+ cells and decrease of null cells was observed. Besides, no substantial difference prior to, and after human serum thymic factor incubation was observed as far as the count of the SM-Ig^+ cells is concerned.

Comments

The great deal of progress realized during the last two decades on lymphocytes and their functions has so much attracted the attention of immunologists and haematologists that all acute leukaemias (AL-s) are called "acute lymphocytic leukaemias" (ALL), with the exception of the more rare cases of myeloid or myelomonocytic acute leukaemias. That is true not only for leukaemias with surface Ig-bearing blasts (B-ALL) and leukaemias with E-rosette-forming blasts (T-ALL) [6, 17], but also for the case of growth of non-T, non-B cells ("null cell ALL"). Also the uncommitted stem cells (USC) specifically reacting to an antiserum raised

against them are called ALL$^+$ cells, though their USC nature has now been admitted also by Greaves et al. [11].

The so-called "null cell ALL" could probably comprise either a form with a clone derived from the USC, or AL types which formed clones growing from the differently committed stem cells. To single out and name different AL-s from the miscellaneous groups of the so-called "null cell ALL" is not a worthwhile semantic attempt. Possibly, concepts could be better defined, and even prognosis

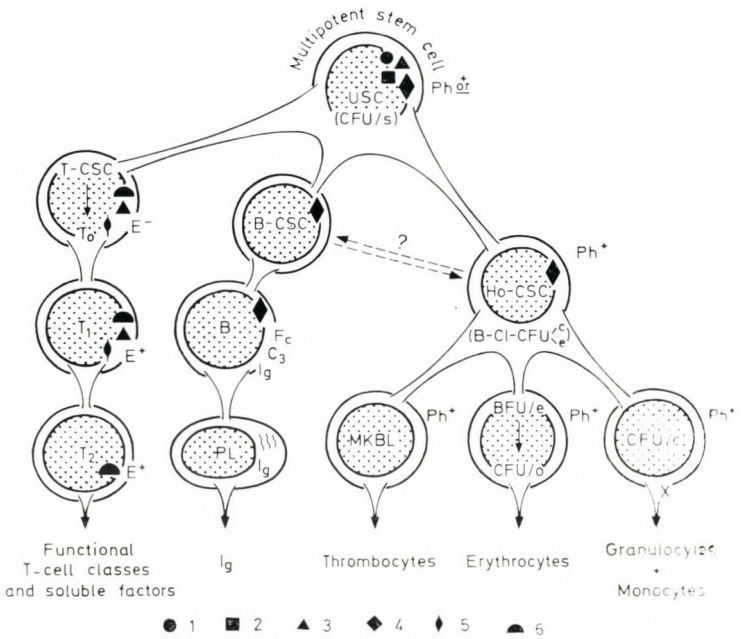

Fig. 1. Schematic illustration of hemic and lymphatic cell ontogeny: 1 = cell reacting to uncommitted stem cell antisera (USCA [ALL']); 2 = hexosaminidase I (Hex I); 3 = terminal deoxynucleotidyl transferase (TdT); 4= cell reacting to antisera to p23,30 antigen(s) (HuBLA); 5 = cell reacting to antisera to antigen(s) of immature T cells (TLA); 6 = cell reacting to antisera to antigen(s) of immature and mature T cells (HuTLA); X = cholera toxin receptor(s); E = E-rosette-positive (+) or negative (−) cells

and treatment might gain by more precise classification. Following the recent basic contributions mentioned in the introduction of this paper and summarized in our ontogeny scheme (Fig. 1), at least theoretically we propose the following tentative classification:

Stem cell (SC) leukaemia, characterized by a cell clone which actually is not "null" in nature, but does carry several markers being USCA$^+$ (ALL$^+$), TdT$^+$, Hex 1$^+$, HuBLA$^+$, whereas it is E$^-$, Sm-Ig$^-$, HuTLA$^-$. It is still questionable whether in chronic granulocytic leukaemia this cell is always Ph'$^+$ or some-

times Ph'$^-$. In normal ontogeny, it is the uncommitted multipotent self-maintaining stem cell (polyblast), *viz.* the CFU/s which transplanted into lethally irradiated animals proliferates and forms spleen macroscopic colonies containing new stem cells, as well as lympho-haemopoietic cell clones.

Hemic-committed stem cell (He-CSC) leukaemia, which is characterized by a clone of cells reacting to antisera to p23,30 antigen(s) (HuBLA$^+$), but being USCA$^-$, TdT$^-$, Hex l$^-$, E$^-$, Sm-Ig$^-$, HuTLA$^-$. Indeed, the pluripotent stem cell during both hemic or B cell line differentiation loses rapidly both TdT and Hex l, whereas p23,30 antigen(s) is retained. In normal ontogeny, the He-CSC is the Ferrata's haemocytoblast (HCBL), being the common precursor of the three unipotent hemic cell progenitors, *viz.*: (1) The granulocyte-monocyte progenitor, i.e. the colony- (and cluster-) forming unit (CFU/c), which in the agar culture system supplemented with the CSF ripens to granulocytes and monocytes; (2) the erythropoietin-sensitive cell(s), i.e. the burst- and the colony-forming unit (BFU/e → CFU/e), which in the methylcellulose culture system supplemented with erythropoietin ripen to Hb-positive cells; and (3) the megakaryocyte progenitor unit. Indeed, in chronic granulocytic leukaemia the He-CSC is always Ph'$^+$, as well as Ph'$^+$ are the granuloblast, erythroblast and megakaryoblast mitoses.

Finally, it is still an open question whether the same He-CSC in culture might directly differentiate to either B-CFU/e or CFU/c, and thus form clones of either erythroid or granulocyte-monocyte bursts, clusters and/or colonies according to the microenvironment and the type of the specific soluble factor supplemented. Our quite preliminary observation [8] does not seem to contradict this hypothesis.

B-committed stem cell (B-CSC) leukaemia. As mentioned above, B-CSC, likewise He-CSC, is HuBLA$^+$, and USCA$^-$, TdT$^-$, Hex l$^-$, E$^-$, Sm-Ig$^-$, HuTLA$^-$. Thus there are no specific phenotypic differences in individual cells between B-CSC and He-CSC. Is there any possible reversibility between them? Or are B-CSC and He-CSC unique cells being committed precursors to both B cells and hemic cells? This hypothesis might offer an explanation: (a) To the fact that B cells and monocytes carry some surface receptors which are similar; (b) to the controversial origin (monocytic or B lymphocytic) of the peculiar cells of "hairy cell leukaemia"; (c) to the fact that in chronic granulocytic leukaemia in blastic transformation there may be present – according to the patient, or perhaps to the blastic stage in the same patient – (1) uncommitted stem cells (USCA$^+$, TdT$^+$, Hex l$^+$, HuBLA$^+$, Ph'$^+$ or Ph'$^-$); or (2) cells which are only HuBLA$^+$ (and Ph'$^+$), but USCA$^-$, TdT$^-$, Hex l$^-$. These features are indeed peculiar to both the hemicommitted and the B-committed stem cells.

On the other hand, there are data supporting the possible existence of a common precursor of both B and T cells (the lymphoblast), carrying antigens of both B and T cells, which are then respectively repressed or de-repressed according to the further differentiation of the precursor cell (migration to the thymus or to the bursa equivalent, action of soluble agents such as thymic or bursal factor, EBV, etc.).

Possible existence of B-CSC leukaemia might be supported by the observation of Fu et al. [9] who provided evidence that cases of apparently "null cell ALL" were actually of B cell lineage. Indeed, via indirect immunofluorescence with alloantisera they detected the presence of Ia-related HL-B antigens on the cells of some cases of the so-called "nuil cell ALL". Also the leukaemic presentation of some cases of Burkitt lymphomas, as well as of poorly differentiated lymphocytic lymphomas – actually belonging to a B lymphocyte clone – might show the features of B-CSC leukaemia. The search of Ph' chromosomal translocation either in this leukaemia or in the B-CSC-s from chronic granulocytic leukaemia, as well as long-term cell culture might provide further necessary information for our knowledge on the differences between He-CSC-s and B-CSC-s, and consequently on this conjectured form of AL.

T-committed stem cell (T-CSC) leukaemia. The results of our study reported above seem to substantiate the interpretation that the T cell precursor (or the T_0 cell) may undergo leukaemic clonal expansion, or at least that AL cases may be accompanied by a contemporaneous growth and peripheral blood traffic of quite immature T cells. Our assay, consisting in the incubation of AL cells with human serum thymic factor (which induces maturation of committed precursors to T cells), showed indeed a clear-cut increase of E^+ cells in 6 out of 36 patients for the peripheral blood cells, and in 3 out of 11 patients for the bone marrow cells. It results that significant percentages of cells interpreted as "null cells" were rather quite immature, not yet E-rosetting T cells. The latter were shifted to a more mature (E-rosetting) stage by the thymic factor. These results also are in line with the recent work of Kaplan et al. [13] on identification of "null cell AL".

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T Lymphocyte Number and Functions in Paroxysmal Nocturnal Haemoglobinuria (PNH)

A Preliminary Report

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T lymphocyte counts in the peripheral blood, lymphocyte response to phytohaemagglutinin (PHA) and delayed hypersensitivity reactions were studied in ten patients with paroxysmal nocturnal haemoglobinuria (PNH).

Delayed hypersensitivity was abnormal in most patients and *in vitro* studies revealed impaired lymphocyte transformation to PHA in 50 per cent of the cases. These tests gave evidence of a functional alteration of lymphocytes in some PNH patients.

The hypothesis of a disorder originated in a pluripotent lymphohaematopoietic stem cell is suggested.

Paroxysmal nocturnal haemoglubinuria (PNH) is a remarkable and most interesting disease; it is considered rare, but it is clearly not as rare as thought at one time [6].

PNH has been associated with myeloproliferative disorders and with aplastic anaemia [18, 19]. Whether the inclusion of PNH among the myeloproliferative disorders [8] and its designation as a malignant disorder are justified is open to question [15].

Human lymphocytes are composed of at least two subpopulations. These subpopulations of lymphocytes can be identified on the basis of differences in their membrane properties. T lymphocytes can be distinguished by their ability to form non-immune rosettes with sheep erythrocytes (E rosettes). B lymphocytes can be identified by the presence of surface immunoglobulins [22], cell line-specific antigens [4, 23] and receptors for antigen-antibody complement complexes detected by using red cells coated with antibody and complement (EAC rosettes) [26].

A method for evaluating the immune system is to identify and enumerate the lymphoid cell subpopulations responsible for humoral and cell-mediated immunity by means of the markers on the surface of lymphocytes. Evaluation of the lymphocyte functions too are usually done.

The study of T and B cells based on the cell surface markers has been performed in several human diseases, including some myeloproliferative disorders [3, 13, 16, 17, 24].

Here we report the T lymphocyte counts in the peripheral blood, lymphocyte response to phytohaemagglutinin (PHA) and delayed hypersensitivity reactions in ten patients with PNH.

Materials and Methods

Ten patients with PNH, 3 males and 7 females within the age range 24 and 78 years (median 48 years), seen at the clinical service, were studied. Each patient consistently showed a positive hem test, thrombin test, sucrose lysis test, inulin test and their red cells had a low level of acetylcholine esterase activity and their neutrophils had low alkaline phosphatase activity [6, 7].

Venous blood from the test subjects was heparinized and lymphocytes were separated on a Ficoll-Telebrix (Laboratories André Guerbet, France) gradient [5]. Examination of the separated cells showed 90 to 95% mononuclear cells, of which less than 5% were monocytes morphologically.

T cell receptors were identified by spontaneous rosette formation with sheep erythrocytes (E rosette) [1]. At least 200 viable cells were counted in a cytometer chamber; a rosetted cell had three or more attached erythrocytes. Smears were prepared and May–Grünwald–Giemsa-stained to assess the morphology of the rosette-forming cell.

The white blood cell total and differential counts were simultaneously determined to permit calculation of the absolute number of circulating T lymphocytes.

PHA transformation at 72 hours was expressed as the percentage of transformed lymphocytes, after counting 1000 lymphocytes in each preparation on May–Grünwald–Giemsa-stained slides. Each sample was suspended in duplicate with PHA (Wellcome, Ha 15) and without PHA [12].

Skin tests used for evaluating T lymphocyte function were purified protein derivative (PPD), trichophyton and Candida antigens. An induration greater than 5 mm in diameter at 48 hours to at least one of the skin test antigens was considered a positive response.

The control group consisted of 20 healthy subjects.

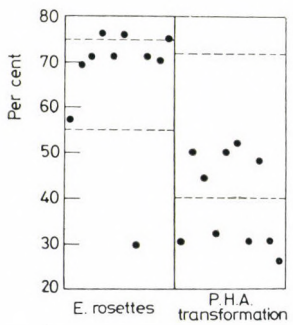

Fig. 1. E rosettes and PHA transformation in 10 cases of PNH. Normal range is indicated by the area between broken lines

Results

The white blood cell (WBC) total counts and the T lymphocyte number and function studies performed in the PNH patients are shown in Table 1. Only 3 cases presented low WBC counts. There was moderate lymphocytosis with a mean lymphocyte percentage of 49 and an absolute number of $3 \times 10^9/l$ as compared with 35% and $2.4 \times 10^9/l$ in the normal group.

In one patient a decrease in the percentage of T lymphocytes was found, but the absolute value was within the normal range. Increased absolute numbers of circulating T lymphocytes were observed in 40% of the patients.

PHA transformation was decreased in 50% of the patients (Fig. 1). In one patient (case 7) there were simultaneously decreased E rosettes and PHA transformation.

Skin-testing yielded a negative response in 80% of the patients: 60% anergic and 20% with a poor response.

Table 1

T lymphocyte number and functions in 10 cases of PNH

Cases	Leucocytes	Lymphocytes		E rosettes		PHA transformation	Skin tests		
	$\times 10^9/l$	per cent	$\times 10^9/l$	per cent	$\times 10^9/l$		PPD	Candida	Trichophyton
1	3.9	46	1.8	57	1.0	30	0	0	0
2	6.3	36	2.3	69	1.6	50	3	0	0
3	4.0	69	2.8	71	2.0	44	0	0	0
4	5.0	64	3.2	76	2.4	32	0	0	0
5	4.3	39	1.7	71	1.2	50	0	0	0
6	6.3	62	3.9	76	3.0	52	0	0	NT
7	7.5	66	4,9	29	1.5	30	0	0	NT
8	13.5	36	4.9	71	3.5	48	0	5	NT
9	5.6	25	1.4	70	1.0	30	14	0	NT
10	7.2	49	3.5	75	2.6	26	15	10	NT
Normal									
Mean	6.7	35	2.4	63	1.5	56	> 5	> 5	> 5
Range	5—10	16—46	1.2—3.7	55—75	0.7—2.3	40—72			

Comments

The total leucocyte count is usually but not invariably subnormal in PHA [6] and relative lymphocytosis is not uncommon [28].

It has been stated that PNH lymphocytes present normal immune reactions [14] but in fact, few studies have been published on the immune status in PNH [6, 11].

The results of our evaluation in 10 patients with PNH have shown that T lymphocytes were in most patients normal or elevated. Delayed hypersensitivity

was abnormal in 8 of the patients. They were unable to give a delayed hypersensitivity response to such agents as PPD, Candida or Trichophyton. *In vitro* studies of the cellular immune function revealed impaired lymphocyte transformation to non-specific mitogen such as PHA in 50% of the cases. Thus these tests gave evidence of a defect in cellular immune functions.

A number of experimental studies indicate that in the adult rat and mouse there are pluripotential stem cells whose progeny is capable of differentiating not only into myeloid, erythroid and megakaryocytic elements, but into immunocompetent lymphocytes as well [21].

There is general agreement that PNH is the result of the establishment of a clone of abnormal stem cells within the bone marrow, presumably following a somatic mutation [27].

There have been reports in the literature of genetic and acquired defects involving the haematopoietic and immune systems. Some of them have been hypothesized as stem cell disorders such as myelofibrosis [10, 20]. The occurrence of a lymphoblastic transformation of chronic granulocytic leukaemia and the finding of the Philadelphia chromosome in peripheral blood lymphocytes also lend support to the concept of a pluripotent stem cell for both myeloid and lymphoid cell lines [2, 25]. Dameshek suggested that PNH might be linked with leukaemia and other myeloproliferative diseases possibly by the abnormal clone leading to PNH in one patient and a myeloproliferative disorder in another [8, 9]. The functional alterations found in PNH lymphocytes strongly suggest the hypothesis of a disorder originating from a pluripotent lymphohaematopoietic stem cell. It is important to confirm these findings in a larger number of patients.

The clonal nature of PNH may provide a useful model for investigation. Further study of PNH as a postulated clonal disorder may advance our knowledge beyond the importance of this rare disorder in itself [15].

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Calculation of Disease Susceptibility Gene Frequency in Insulin-Dependent Diabetes Mellitus

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An analysis of HLA-linked genetical factors conferring susceptibility to IDDM is reported. On the basis of population and family studies a recessive mode of inheritance of disease susceptibility provided by an assumption of HLA-B8-linked DS gene was observed. The characteristic component of the immunogenetical background was the high frequency of HLA-B8 (0.208) and the HLA-A1, B8 haplotype (0.134) (linkage disequilibrium D = 0.1031), reminiscent of that found also in other disorders with autoimmune features, such as Graves disease, SLE, etc.

Considering the HLA-B8 and IDDM association, the DS gene frequency (pD = 0.25) was estimated and the gametic association between HLA-B8 andu DS gene was calculated.

The low value of penetrancy (4.8%) revealed the important role of non-HLA-linked genetical and environmental factors. The HLA-linked genetic factors in question might be responsible for an inclination to several kinds of autoimmune disorders.

On the basis of clinical, biochemical, immunological and pathological findings, the term diabetes mellitus is applied to a disorder consisting of at least five nosologically distinct disease entities. One of them is juvenile diabetes mellitus.

The pathological findings characteristic of insulin-dependent diabetes mellitus (IDDM) are a lymphocytic insulitis together with a selective disappearance of the cells of the islets of Langerhans [5], and a cell-mediated autoimmunity directed against antigenic determinants in the endocrine pancreas [11, 13].

Recently, evidence has been found to support the possible role in its pathogenesis of several viruses [2, 3, 7]. These facts and the well-known familial aggregation of the disease emphasize the particular importance of immunogenetic background in conferring susceptibility to IDDM.

Investigations aiming to reveal the genetic background have so far failed to create any pattern of inheritance that would properly fit reality. This is why the inheritance of IDDM is referred to as a "geneticist's nightmare" in the literature. Recessive, dominant, multifactorial and even X-linked inheritance has been suggested without any of them being confirmed by experience. Thus the stage was set to look for an association between HLA antigens and IIDDM.

There are a number of studies on the distribution of HLA antigens in patients with IDDM, and nearly all the authors found a significantly increased

frequency of HLA-B8 with a relative risk (RR) of about 2.4. Apart from B8, some other antigens too were reported to occur in patients, for example B15 by Nerup et al. [14], B18 in France by Seignalet et al. [17], B35 in Italy by Savi et al. [16], B8 and Cw3 by Mayr [12].

As for the different races, in the mongoloid population Bw22 seems to be associated with the disease, though B15, the possible role of which in Caucasians has just been mentioned, is frequent in Japanese.

On this experimental basis most authors propose the hypothesis for the aetiology of IDDM that one or more response genes are in linkage disequilibrium with B8 and/or any other gene having proved to be more frequent in patients might be responsible for an altered immune response unable to eliminate the infecting virus that triggers an autoimmune reaction. A recent paper by Rubinstein et al. [15] suggests a strong association between this disease susceptibility gene and HLA-Dw3 and Dw3 and Dw4.

From the data of population studies conclusions can be drawn as to the inheritance of IDDM, whether the pattern is one of recessivity or overdominance.

The RR indicates how many times more frequent the disease is in individuals carrying the antigens as compared to those lacking it. RR is four times higher in the B8/B15 genotype than in B8/x or B8/B8. This is the way RR can become informative, revealing the fact that the DS (disease susceptibility) gene(s) associated with B8 is (are) dominant – no higher the RR value is for B8/B8 genotype – and these two DS genes act in different ways.

This case demonstrates the overdominance, though this pattern of inheritance for IDDM has not been confirmed by others. Thus, Rubinstein et al. [15] found an increased RR value for Dw3 homozygous children compared to heterozygotes, thus disproving the dominance and consequently overdominance. Moreover, HLA-typing of affected sibs in diabetic families with two or more children showed that half of the HLA-identical sibs were diabetic against as few as 6% among HLA-non-identical ones.

Materials and Methods

Sixty-seven unrelated patients with IDDM, 23 males and 44 females aged 2 to 29 years (mean, 10.4 years) were HLA-typed for 25 antigens of the A and B segregant series using the NIH lymphocytotoxicity microtest. The family study comprised 55 diabetic families (220 haplotypes) involving 231 members. All diabetic patients were admitted to our Department with overt diabetes mellitus, i.e. severe ketoacidosis, hyperglycaemia, glycosuria, polyuria, polydipsia, polyphagia. The glucose tolerance test was carried out according to Gutherie et al. [9] and all diabetogenic conditions could be ruled out [6, 10]. A total of 450 healthy unrelated individuals living in the same area served as control. As a family study, the real haplotypes in 51 healthy families (204 haplotypes) with 306 members were determined by defining the HLA phenotypes of parents and their children for comparison.

Results

The occurrence of the two most frequent antigens is shown in Table 1. Only B8 displayed a significant deviation even after using a corrected p value ($p < 0.025$).

Table 1

Most frequently occurring HLA antigens in 67 patients with IDDM

Antigens	Patients			Controls			p <	Corr. p <	RR
	with the antigen		total	with the antigen		total			
	No.	per cent		No.	per cent				
HLA-A9	25	37.3	67	94	20.9	450	0.005	0.125	2.25
HLA-B8	25	37.3		85	18.9		0.001	0.025	2 38

The 134 real haplotypes of 67 patients with IDDM are seen in Table 2. Al, B8 was by far the most frequent haplotype. This could not be due only to the significantly increased B8 frequency but also to the higher gametic association of Al and B8 antigens (D = 0.1031), a sign characteristic of some immunological disorders, as in Graves disease, SLE, etc. [1, 8, 18, 19, 20]. Although there were some haplotypes, frequently occurring in our material but far less so than Al, B8, and since no significant deviations could be justified in these cases, they have not been taken into consideration.

Table 2

134 real haplotypes of 67 patients with IDDM

HLA antigens	B5	B7	B8	B12	B13	B14	B15	Bw16	B17	B18	Bw21	Bw22	B27	Bw35	B40	Bw41	B37	Y	Total
A1	0	0	18	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	20
A2	4	2	4	6	0	1	7	1	0	0	0	0	1	3	10	0	1	0	40
A3	1	2	1	0	0	1	0	0	0	0	0	0	0	3	1	0	0	0	9
A9	2	1	4	7	3	2	1	0	1	2	0	0	0	2	2	0	0	2	29
A10	0	2	0	2	0	5	1	1	0	0	0	0	0	2	1	0	0	1	15
A11	0	1	1	0	0	0	0	2	1	1	0	1	0	7	0	0	0	0	14
A26	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
A < 19	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	2
X	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	3
Total	7	8	28	17	3	9	10	5	3	3	0	1	2	19	14	0	1	4	134

Table 3

The distribution of HLA haplotypes in 55 diabetic and healthy control families

HLA antigens	B5	B7	B8	B12	B13	B14	B15	Bw16	B17	B18	Bw21	Bw22	B27	Bw35	B40	Bw41	B37	Y	Total
A1	1	0	21	0	0	0	0	0	1	0	0	0	0	3	0	0	0	1	27
	0	2	11	1	1	1	1	0	2	0	1	1	2	2	1	0	0	4	30
A2	5	6	8	10	1	4	10	2	0	0	0	0	1	6	7	0	1	4	65
	5	3	1	10	3	1	4	0	2	0	1	1	4	1	6	0	0	9	51
A3	2	7	2	1	0	3	0	0	0	0	0	0	0	5	1	0	0	1	22
	2	8	1	2	1	1	1	0	1	0	0	1	1	4	2	0	0	5	30
A9	2	3	1	6	5	3	0	1	1	2	0	0	2	1	2	0	0	3	32
	2	4	1	4	1	1	0	0	0	1	0	0	1	3	1	0	0	6	25
A10	0	1	1	2	0	4	2	1	1	0	0	0	1	2	3	0	0	3	21
	3	1	2	1	1	1	1	1	0	1	0		0	1	1	0	0	4	19
A11	1	4	1	2	0	2	0	0	0	0	0	0	1	5	0	0	0	1	17
	1	2	1	0	0	0	0	0	1	3	0	1	0	6	1	0	0	3	19
A28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	3	8
A < 19	0	0	0	0	0	0	0	1	0	1	0	0	0	2	0	0	0	1	5
	0	1	2	0	0	0	0	0	1	0	0	0	1	0	1	0	0	2	8
X	4	2	1	2	3	2	1	1	1	2	0	0	0	2	4	0	0	3	30
	2	1	1	3	1	1	0	0	0	0	0	3	2	0	2	0	0	0	14
Total	15	23	35	23	9	18	13	6	4	5	0	0	7	26	17	0	1	18	220
	16	23	20	21	8	7	7	0	9	5	2	8	9	17	16	0	0	36	204

Table 3 shows the haplotypes in 55 diabetic families where we had occasion to determine all the parental haplotypes. For comparison we quote the corresponding data of 51 healthy families. The number of haplotypes being nearly the same, the data are easy to compare.

Apart from the distribution of HLA antigens in patients to support the hypothesis that susceptibility to IDDM is inherited as a recessive trait linked to HLA, we selected 29 families each with two or more children of whom at least one was suffering from IDDM. They served as reference and were consequently excluded from Table 3, whereas their sibs were classifieed into three groups according to their HLA identity, haploidentity or non-identity, as shown in Table 4. Although only 27% of HLA-identical sibs seemed to be affected and this was less than the frequency of the association of HLA with IDDM in previous studies [14], it is remarkable that there were only two onset IDDM among 30 non-identical sibs including haploidentical ones. The difference was significant, $p < 0.005$.

Table 4

HLA identity and IDDM in 29 families in sibs of index cases

HLA identity	Sibs, No.	
	IDDM, %	Non-diabetic
Identical	4 (66.6)	10
Haploidentical	2 (33.3)	16
Non-identical	0 (0.0)	12
Total	6 (100.0)	38

Considering these data we have attempted to estimate the disease susceptibility gene frequency (pD) as described by Thomson and Bodmer [20]. By the inheritance of a recessive trait, if the assumed disease gene is HLA-linked as it is now and if penetrancy is ignored for the time being, the children with different HLA haplotypes have the chance of being affected

for HLA	identical	1
	haploidentical	2 pD
	non-identical	pD^2

So the proportion of HLA-identicals among diabetic sibs can be reckoned by the formula:

$$\text{the proportion of HLA-identicals} = \frac{1}{1 + 2\,pD + pD^2}$$

A similar formula is valid for HLA-haplo- and non-identical sibs. In our study these values were 0.66, 0.33, 0.00 for HLA-identicals, haploidenticals and non-identicals, respectively (see Table 4), suggesting a pD of 0.28 that would give the corresponding theoretical values of 0.64, 0.32 and 0.04.

Taking the population incidence of IDDM to be between 0.1 % and 0.5 %, we determined the penetrance factor X, using the equation,

$$FDPrec = X\, pD^2$$

(FDPrec = frequency of disease in the population, recessive mode of inheritance).

$$\text{By transformation } X = \frac{FDP\ rec}{pD^2} = \frac{0.003}{0.25^2} = 0.048$$

using the average value of 0.3 % for the population incidence of IDDM.

Knowing the DS gene frequency (pD) we determined the measure of linkage disequilibrium (D) with B8 as indicated by Thomson and Bodmer [20]:

$$D = (k - pA)\, pD$$

where k denotes the ratio of gametes with both B8 and DS genes, as compared to those with DS alone in patients

pA = B8 gene frequency in the population
pD = disease gene frequency in the population, that is:

$$D = (0.2101 - 0.1988)\ 0.25$$
$$D = 0.0028$$

Discussion

Examinations were carried out in order to establish the nature of the HLA linked genetical factors conferring susceptibility to IDDM. Despite our efforts to obtain evidence of a so-called disease susceptibility gene or genes mapped in the MHC region of chromosome No. 6, we should like to emphasize that many different non-HLA-linked genetical factors should be studied to approach the complicated genetical background of IDDM.

The population study including 67 diabetic unrelated patients showed an increased frequency of HLA-B8 ($p < 0.001$) but the significantly more frequent occurrence of other HLA antigens (B15, B18, B35) reported by different authors could not be demonstrated. In the same material the distribution of HLA haplotypes was determined by family studies. Although the HLA-A1 antigen was not more frequent in the patients than in the controls, a high frequency of HLA-A1, B8 (haplotype frequency = 0.1343) with a gametic association of D = 0.1031 could be observed.

Considering the distribution of HLA-identical, haplo- and non-identical types of the affected sibs in 29 diabetic families, we calculated the DS gene frequency to amount to about 0.25 in the population. There is some doubt about this value because working with data of patients' sibs ranging in age from 2 to 25 years there is a possibility of sibs found healthy for the time being to develop diabetes at later age.

Our calculations do not prove the existence of one DS gene for IDDM. Here the term DS gene just stands for the different HLA-linked genetical factors conferring susceptibility to the disease. Still, one may speak of DS gene, as experimental data such as crossing-over and HLA-typing for D locus indicate the existence of a gene or genes mapped between D and B loci and being thought to be responsible for susceptibility.

The value for DS gene frequency calculated by us might be altered by the fact that the HLA identity just as haplo- or non-identity means identity only for the antigens of the A and B segregant series, and not for the D locus, despite of its being at least as much important as the B locus antigens.

Considering the value of DS gene frequency it was possible to estimate the penetrance factor (4.8%) and the gametic association with B8 (D = 0.0028). Although this penetrance factor of 4.8 is not too high, it is in good agreement with that calculated by using Cudworth and Woodrow's data [4].

Perhaps the fact can generally be accepted that the more antigens in the MHC can be detected by tissue-typing as markers, the more exact answers can be given to all the questions concerning the DS gene frequency, its mapping and the role of the gene in the manifestation of IDDM.

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RNA Synthesis in Rabbit and Rat Kidney under Experimental Changes of Erythropoiesis

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RNA synthesis in rabbit and rat kidneys and erythropoietin level in plasma were studied under hypoxic stimuli (bleeding, phenylhydrazine and cobalt treatment, exposure to simulated altitude of 6000 m). An increase in renal RNA synthesis and in plasma erythropoietin activity was found. By using the "endocrine" kidney it was shown that 4-hr hypoxia produced a considerable increase in RNA synthesis both in the intact and in the "endocrine" kidney. The increase was correlated with the increase in the plasma titre of erythropoietin. The plasma titre of erythropoietin in rats having a single endocrine kidney also increased after 4-hr hypoxia. It is suggested that the renal biogenesis of erythropoietin under hypoxic stimuli involves, apparently, the mechanism of derepression of specific RNA synthesis.

The regulation of erythropoiesis is effected by erythropoietin the production of which increases under hypoxic conditions. There is a considerable body of indirect evidence showing that this hormone is produced by the kidneys. Jacobson et al. [13] failed to increase erythropoietin production in response to hypoxic stimuli in nephrectomized animals. Most other investigators [20, 21, 28, 29] also observed a fall in erythropoietin production after removal of the kidneys. To study the role of the kidneys in erythropoietin production, constriction of the renal artery or vein [6, 14, 18], injection of plastic microspheres into the renal artery [1], perfusion of the isolated kidney [10, 17], administration of kidney extracts [22, 27], homotransplantation of the kidney [2, 9], administration of nephrocytotoxic serum [23, 24] have been used. These investigations confirmed the important role of the kidney in erythropoietin production.

The mechanisms of erythropoietin production are not known. The appropriate approach to examine molecular mechanisms of the renal biogenesis of erythropoietin is a study of biochemical and biophysical processes in that organ under conditions of stimulation or depression of erythropoiesis. The present studies were undertaken to investigate the synthesis of RNA in the kidney of rabbits and rats under conditions of increased erythropoietin production (bleeding, phenylhydrazine and cobalt treatment, hypoxic hypoxia). In part of the studies we used the model of the "endocrine" kidney. Our data suggested that the mechanism of erythropoietin production probably involved a derepression of RNA synthesis specific to biogenesis of hormone.

Materials and Methods

Two hundred rabbits weighing 1.8 to 3.2 kg, 100 female rats weighing about 200 g, and 300 female rats weighing about 100 g (to yield the endocrine kidney) were used. Seventy female $F_1(CBA \times C_{57}B1)$ plethoric ex-hypoxic mice weighing 16 to 20 g were used for erythropoietin bioassay. Rabbits were bled from the carotid artery at 20 ml/kg; 0.6 ml/kg of a 2.5% solution of phenylhydrazine sulphate or 3 mg/kg of a 2.47% solution of cobalt nitrate were administered subcutaneously. Hypoxic hypoxia was induced by exposure in the hypobaric barocamera at a simulated altitude of 6000 m (349 mm Hg).

The endocrine kidney was prepared by the method of Selye [33]. The aorta of anaesthetized rats was ligated between the branchings of the renal arteries using a mandrin. This was then removed, and 6 days after the operation the rats were used for the study of RNA synthesis in the right (intact) and in the left (endocrine) kidney and for the estimation of erythropoietin levels in plasma under normal and hypoxic conditions. For the visual and histological control of the "endocrine" kidney, in one group the left ureter was ligated after removal of the mandrin. There were no signs of hydronephrosis and necrosis in the left (endocrine) kidney after 6 days constriction of the aorta. The changes were a cortical atrophy, a decrease in the diameter of glomerules and tubules, and a hyperplasia of the intertubular connective tissue. The tubules in the medulla were enlarged, the arterial and venous vessels were unchanged.

Blood samples were examined for the haemoglobin content, erythrocyte count, percentage of reticulocytes and the haematocrit. The erythropoietic activity in plasma filtrates was determined on basis of ^{59}Fe uptake into erythrocytes of ex-hypoxic polycythaemic mice. The plasma filtrates were prepared by the method of Borsook et al. [3]. Initially 70 fastened rats were used for testing [25, 26] by the method of Rosse and Waldmann [29], but later we used posthypoxic polycythaemic mice [4, 5].

RNA synthesis was studied by the thermal phenol method. By this method of Georgiev et al. [11, 12] cytoplasmic ribosomal (rRNA), nuclear ribosomal (RRNA) and DNA-like (DRNA) RNAs were isolated as described earlier [25, 26]. After exposure to the radioactive precursors the rabbits or rats were killed and their kidneys were removed. Aliquots of the cortex were homogenized in 10 vol. of 0.14 M NaCl (w/v). The homogenate was mixed with an equal volume of phenol (pH 6) and the mixture was shaken at 5°C for 15 min and then centrifuged at 6000 × *g* at 5°C for 20 min. The aqueous phase was removed and mixed with phenol, and the mixture was shaken and centrifuged. The nucleic acids from the aqueous phase were precipitated with 2 vol. of cold ethanol (96°) and dissolved in 2 M NaCl. The solution was kept at 5°C for about 16 hr to precipitate the rRNA which was separated from the supernatant by centrifugation. The intermediate phase (phenol nuclei) was shaken with 100 ml of phenol and 0.14 M NaCl (1 : 1) at 45°C for 15 min and then centrifuged at 8000 × *g* at 5°C for 15 min. The aqueous phase was shaken with phenol, and sodium lauryl sulfate was added

to 0.5% concentration. After centrifugation to separate the phases, 45°C RNA (RRNA) was precipitated with 2 vol. of ethanol. In the same manner, 65°C RNA (DRNA) was extracted from the intermediate phase after isolation of RRNA. The precipitates of 45°C RNA and 65°C RNA were dissolved in 2 M NaCl and kept at 5°C for 16 hr. After centrifugation, the precipitates of all types of RNA were dissolved in H_2O pH 7, and the absorbance was measured at 260 and 290 nm. By the method of Scherrer and Darnell [30] total high-polymer RNA was isolated. The kidneys were removed and homogenized in 10 vol. of 0.01 M sodium acetate pH 5.1. The homogenate was mixed with an equal volume of phenol pH 6, and sodium lauryl sulfate was added to 0.5% concentration. The mixture was then shaken at 60°C for 5 min and then centrifuged at 2500 × *g* for 30 min. The supernatant was removed and mixed with phenol, and the mixture was shaken and centrifuged. The total high-polymer RNA from the aqueous phase was precipitated with 2 vol. of cold ethanol and dissolved in 2.0 ml of H_2O. In the terminal stage, after measuring the absorbance at 260 and 290 nm, each fraction was precipitated by cold 50% trichloroacetic acid and then passed through Millipore filters "Rufs". The precipitates were washed twice with cold 5% trichloroacetic acid and then counted in a gas-flow counter.

For bioassay of erythropoietic activity, female $F_1(CBA \times C_{57}B1)$ mice weighing 16 to 20 g were subjected to intermittent hypoxia (0.4 atm. air) 18 hr daily for 3 weeks. Three days after the hypoxic exposures had been stopped the test plasma filtrates were given s.c. in a dosage of 0.5 ml for 2 days. Mice injected with isotonic saline or normal plasma were used as controls. Twenty-four hours following the last plasma filtrate injection, 1 mC ^{59}Fe ascorbate was injected i.p. in 0.5 ml of saline and 24 hr later 0.5 ml of blood was obtained by aortic puncture for measurement of iron uptake in a well-type scintillation counter and microhaematocrit. Data from mice with a haematocrit less than 60% were discarded. Results were expressed in per cent of the injected radioiron in the calculated blood volume, which was assumed to be 6% of body weight. Estimation of the erythropoietin units (EPO) was made from the ^{59}Fe uptakes by reference to a standard curve prepared using the International Reference Preparation (I.R.P.).

Student's *t* test was used for analysis of the significance of differences.

Results

Blood loss. Twelve and 24 hr after bleeding the erythrocyte count and the haemoglobin content decreased by 30 to 40%, while the percentage of reticulocytes increased by more than 200%. Erythropoietic activity of plasma increased considerably 4 hr after bleeding to reach the maximum at 12 hr; it increased to above the control value by 24 hr [25, 26]. Four hr after bleeding and simultaneous injection of ^{32}P the specific activities of all fractions of RNA, especially rRNA and RRNA, in the kidney were increased (Table 2). The specific activity of DRNA was the highest although the increase was only 66% as compared to the control.

Table 1

Specific activity of RNAs in rabbit kidney at various times after erythropoietic stimuli. Exposure to ^{32}P for one hour. Counts/min/mg RNA in M$\pm$m. In all cases $p < 0.01$

Fraction of RNA	Control (n = 14)	Bleeding		Phenylhydrazine		Cobalt	
		12 hr (n = 8)	24 hr (n = 12)	12 hr (n = 4)	72 hr (n = 8)	12 hr (n = 8)	4 weeks (n = 8)
rRNA	582$\pm$ 7	970$\pm$ 47	870$\pm$ 45	650$\pm$ 5	1 390$\pm$ 55	740$\pm$ 17	810$\pm$ 36
RRNA	4 332$\pm$ 32	7 886$\pm$531	9 885$\pm$998	4 800$\pm$ 72	6 875$\pm$ 305	4 531$\pm$108	5 065$\pm$ 93
DRNA	6 621$\pm$269	10 542$\pm$470	15 731$\pm$534	9 958$\pm$133	17 433$\pm$1660	10 822$\pm$539	8 871$\pm$929

* Rabbits were injected i.v. 0.540 mC/kg of $Na_2H^{32}PO_4$. They were killed by air embolism one hour after administration of the tracer.

Table 2

Specific activity of RNAs in rabbit kidneys 4 hours after erythropoietic stimulus and simultaneous administration of ^{32}P. M$\pm$m = counts/min/mg RNA. In all cases $p < 0.01$ as compared to the control

Fraction of RNA	Control (n = 10)	Bleeding (n = 6)	Phenylhydrazine (n = 6)	Cobalt (n = 8)	Hypoxia (n = 4)
rRNA	1 953$\pm$ 51	3 565$\pm$ 88	3 875$\pm$124	4 063$\pm$311	3 049$\pm$ 18
RRNA	5 254$\pm$247	14 494$\pm$540	8 299$\pm$238	9 633$\pm$132	12 266$\pm$ 385
DRNA	9 385$\pm$373	15 531$\pm$996	12 782$\pm$554	13 521$\pm$363	19 999$\pm$1 808

* Rabbits were injected i.v. 0.540 mC/kg of $Na_2H^{32}PO_4$ immediately after bleeding, injection of phenylhydrazine or cobalt and before exposure in the hypobaric barocamera. The animals were killed 4 hr after tracer administration.

Twelve and 24 hr after bleeding (exposure to ^{32}P for one hour) the specific activities of RNAs in the kidneys were also markedly increased (Table 1). The activity of DRNA was highest 4 hr after bleeding. Twenty-four hr after bleeding the specific activities of all RNA fractions were higher than at 12 hr. At that time the activity of nuclear RNAs increased to about the same value although the activity of DRNA was higher than that of RRNA (Table 1).

Phenylhydrazine. Four and 12 hr after a single injection of phenylhydrazine, the content of haemoglobin, the erythrocyte count and the haematocrit decreased by 10 to 15%, while the number of reticulocytes increased by 300% as compared to the control (100%). After 2 or 3 daily injections of phenylhydrazine the haemoglobin content, the erythrocyte count and the haematocrit decreased by 50%, while the number of reticulocytes increased by about 2100%. After phenylhydrazine treatment the erythropoietic activity of plasma increased by more than 100% in 3 to 4 days [25, 26].

Four hours after a single injection of phenylhydrazine and simultaneous administration of ^{32}P, the specific activity of rRNA increased by almost 100%, that of RRNA by 60% and that of DRNA by 40% (Table 2). At 12 hr the activities of rRNA and RRNA were increased by 10%, that of DRNA by 50% (Table 1). After repeated injections of phenylhydrazine the activities of all types of RNA in the kidneys increased to higher values than after a single injection (Table 1). At this time the activities of rRNA and DRNA were increased by about 150%, and the activity of RRNA by about 50%.

Cobalt. Four and 12 hr after a single injection of Co^{++} the haemoglobin content, the erythrocyte count and the haematocrit were unaffected but the percentage of reticulocytes increased by 50 to 100%. Four weeks after repeated injections of Co^{++} the same values increased by about 20%, the number of reticulocytes by almost 200%. The cobalt polycythaemia in rabbits was characterized by an increase in the amount of haemoglobin to 15.8 to 16.6 g per 100 ml, that of erythrocytes to 7.0 to 8.8 M/mm^3 and the haematocrit by 50%. The erythropoietic activity of plasma increased both after a single and repeated treatment of Co^{++} [25, 26].

Four hours after a simultaneous administration of Co^{++} and ^{32}P, the specific activities of rRNA and nuclear RNAs increased by about 100% in the kidneys (Table 2). Twelve hours after an injection of Co^{++} the activity of DRNA increased by 63%, that of rRNA by 26%, that of RRNA by 6% (Table 1). Four weeks after daily injections of Co^{++} the activity of all fractions of RNA persisted at the increased level (Table 1). At this period of polycythaemia the specific activity of rRNA and RRNA was somewhat higher, the activity of DRNA was lower than 12 hr after a single injection of Co^{++}.

Hypoxia. Under acute hypoxia, the synthesis of RNAs in the kidney of rabbits and rats increased sharply. Four hours after exposure in the barocamera, the specific activity of rRNA increased by more than 50%, the activities of DRNA and RRNA by more than 100% (Table 2). The specific activities of all RNA fractions in the rat kidney were increased by about 30% after a 2-hr period of hypoxia

Table 3

Specific activity of RNAs in rat kidneys after 2-hr hypoxia. Counts/min/mg RNA in M±m. In all cases $p < 0.001$

Fraction of RNA	Control (n = 8)	Hypoxia (n = 12)
rRNA	2 049± 9	3 425± 122
RRNA	17 567± 706	23 400±1500
DRNA	31 215±1425	39 543± 999

* Rats were injected i.p. 0.050 mC/100 g of 8-^{14}-adenine immediately before exposure to hypoxia, and killed 2 hr after exposure.

Table 4

Specific activity of RNAs in rat kidneys after 4-hr hypoxia. Counts/min/mg RNA in M±m. In all cases $p < 0.01$

Fraction of RNA	Control (n = 24)	Hypoxia (n = 33)
rRNA	618± 52	1114± 84
RRNA	5582±502	7018±406
DRNA	7538±412	9404±402

* Rats were injected i.p. 0.050 mC/100 g of $Na_2H^{32}PO_4$ one hour before the end of barocamera treatment.

Table 5

Incorporation of ^{32}P into RNA of intact and endocrine kidney of rats under normal and hypoxic conditions. Counts/min/mg RNA in M±m. In all cases $p < 0.001$

Control	Operation without hypoxia		Operation + hypoxia	
	intact	"endocrine"	intact	"endocrine"
8022±99 (n = 34)	6923±75 (n = 24)	2675±88 (n = 24)	9117±161 (n = 25)	4469±81 (n = 25)

* Rats were injected i.p. 0.050 mC/100 g $Na_2H^{32}PO_4$ and killed 2 hr thereafter.

(Table 3). After 4 hr the activity of nuclear RNAs increased by about 20% and the activity of rRNA by 80% (Table 4). The plasma titre of erythropoietin was increased after 2- and 4-hr hypoxia to about the same level (Table 6).

"Endocrine" kidney and hypoxia. Six days after surgery the erythrocyte count and the haemoglobin content decreased by 20%, while the number of reticulocytes increased by 600% as compared to the control. The specific activities of RNA in the intact (right) and in the endocrine (left) kidneys were lower than that in the control. The activity of RNA in the endocrine kidney was much lower than

that in the intact kidney (Table 5). Four-hour hypoxia produced a considerable increase in the activity of RNA both in the intact and in the endocrine kidney. The activity of RNA in the intact kidney increased by about 30%, while that in the endocrine kidney by 60% as compared with a group of operated rats without hypoxia (Table 4).

Table 6

Erythropoietic activity of plethoric ex-hypoxic mice injected with plasma filtrates from rats exposed to hypoxia six days after operation (to bring about "endocrine" kidney). $P < 0.001$

Treatment	No. of mice	24-h ^{59}Fe incorporation	I.R.P. units EPO/ml plasma
Intact rats	5	0.77±0.11	—
Intact rats + hypoxia 2 hr	7	2.43±0.10	0.10
Intact rats + hypoxia 4 hr	8	3.20±0.20	0.17
Rats after operation on day 7	8	1.92±0.21	0.08
Rats after operation on day 7 + hypoxia 4 hr	10	4.10±0.80	0.21
Rats after operation on day 7 + removal of intact kidney*	8	1.66±0.18	0.07
Rats after operation on day 7 + removal of intact kidney + hypoxia 4 hr**	14	3.34±0.40	0.18

* Blood was obtained 4 hr after removal of the intact kidney.
** Blood was obtained 8 hr after removal of the intact kidney (4 hr after hypoxia).

The plasma titre of erythropoietin of operated rats on day 7 was increased (1.92±0.21% of ^{59}Fe uptake, or 0.08 I.R.P.) as compared with the control (0.77± ±0.11% of ^{59}Fe uptake) (Table 6). Hypoxia produced a considerable increase in the plasma level of erythropoietin in the operated rats (4.10±0.8% of ^{59}Fe uptake, or 0.21 I.R.P.). The plasma titre of erythropoietin with the endocrine kidney was increased 4 hr after removal of the intact kidney (1.66±0.18% of ^{59}Fe uptake, or 0.07 I.R.P.). Hypoxia produced a considerable increase in the plasma titre of erythropoietin of rats with a single endocrine kidney (3.34±0.40% of ^{59}Fe uptake, or 0.18 I.R.P.).

Discussion

All erythropoietic stimuli used in our experiments (bleeding, phenylhydrazine, cobalt, hypoxia) induced a considerable increase in the synthesis of RNA, in the kidneys of rabbits and rats. The increase correlated with the increase in the plasma titre of erythropoietin. These findings suggest, in agreement with Schooley and Mahlmann [31, 32], that erythropoietic stimuli induce the biogenesis of erythropoietin through processes, one step at least, requiring a DNA-dependent renal RNA synthesis.

It is known that the biogenesis of erythropoietin in the kidney occurs side by side with other biosynthetic processes which, however, do not necessarily increase significantly under every erythropoietic stimulus. It has been established [15, 35] that functions so important as the regulation of osmotic pressure or of acid–base balance do not change considerably under pronounced hypoxia. It is unlikely that the increase in RNA synthesis in the kidneys after so short stimulus as a single injection of Co^{++} or 2 to 4 hr exposure to hypoxia should affect the control of isoosmia, isoionia and isohydria. It has been shown in our laboratory that alterations of the glomerular filtration rate and renal plasma flow are not correlated with the increase in RNA synthesis in the kidney of rats exposed to hypoxia or treated by Co^{++}.

Using the endocrine kidney we found that 4-hr hypoxia produced a considerable increase in RNA synthesis both in the intact and in the endocrine kidney. Consequently, the increase in RNA synthesis under hypoxia is related to such endocrine processes as the production of erythropoietin, renin, prostaglandins and kinins. We found that hypoxia produced a considerable increase in the plasma titre of erythropoietin in rats with a single endocrine kidney. It has been shown [7] that acute hypoxia for 8 hr or a single injection of Co^{++} cause the activity of erythropoietin to increase without a significant increase in the activity of renin in the plasma. Evidently, the hypoxic increase is related to the biogenesis of erythropoietin.

Our data suggest that the biogenesis of erythropoietin in the kidneys is a *de novo* synthesis of hormone and involves, apparently, the mechanism of derepression of RNAs specific to erythropoietin. The experiments of Schooley and Mahlmann [31, 32] with actinomycin D administered to rats before exposure to hypoxia offered indirect evidence of the DNA-dependent synthesis of erythropoietin. Its administration suppressed the increase in the serum titre of erythropoietin of rats exposed to hypoxia. Tentatively, derepression of the synthesis of RNAs specific to the biogenesis of erythropoietin occurs at a low pO_2, while its inhibition at a high pO_2. A possible repressor of erythropoietin synthesis might be the lipid inhibitor extracted from kidney homogenates of normal rabbits [8].

The increase in RNA and protein synthesis [19] in the kidneys under hypoxic stimuli suggests that the biogenesis of erythropoietin takes place throughout a whole nephrone and not only in the juxtaglomerular apparatus. Electron microscopy of rat kidney explants exposed to 0.4 atm. hypoxia for 20 hr showed distinct features of active protein synthesis in four different regions [34] with a hypertrophy of the Golgi complex, an increase in the content of polysomes and rough endoplasmic reticulum.

Our study showed that RNA and protein [19] synthesis increased at least during 12 to 24 hr after a single erythropoietic stimulus and for a long period after repeated stimuli (phenylhydrazine or cobalt treatment). These results do not agree with the observation of Katz et al. [16] that in rat kidneys protein synthesis was elevated for a single hour after a hypoxic stimulus.

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Aplastic Anaemia in Iraq
A Prospective Study*

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The problem of aplastic anaemia (AA) in Iraq has not been previously investigated. This paper describes 60 patients evaluated prospectively at the University of Baghdad Teaching Hospital during the period 1975—1978. Criteria for the diagnosis included pancytopenia and hypocellular or acellular bone marrow. A surprising finding at variance with published reports about the disease from other parts of the world was a 3 : 1 preponderance of males over females. A discernible aetiology of drug or chemical exposure was detected in half of the patients. Chloramphenicol, alone or in combination with other antibiotics, was thought to be responsible for marrow injury in 12 patients. The occurrence of AA seems to be on the rise in Iraq, probably as a result of the increased use of potentially toxic therapeutic agents and chemicals, and of the growing environmental pollution.

The occurrence of aplastic anaemia (AA) in certain people probably reflects the interaction of several genetic and environmental factors. Acquired AA may follow exposure to therapeutic agents, chemicals, ionizing radiation, and infection [3]. Besides, there must be some decisive individual susceptibility since only a small fraction of those at risk develop AA. The factor or factors which determine such individual susceptibility are not known and useful information may be obtained by studying the distribution and pattern of AA among different groups of people in different parts of the world. Reports including large number of patients from several medical centres continue to provide important and interesting findings concerning the aetiology, prognosis, and treatment of AA [2—10, 13].

Up to now no report has been published on the the problem of AA in Iraq, a developing nation of over 12 million inhabitants with a growing problem of environmental pollution and overuse of potentially toxic drugs. This paper reports studies on 60 patients with AA seen at a major centre over the last 35 months.

Materials and Methods

The diagnosis of AA was established in 60 patients admitted to the University of Baghdad Medical Centre or referred for consultation during the period June 1, 1975 to April 30, 1978. All patients were seen and investigated by the

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author. Criteria for the diagnosis were similar as those used by Williams et al. [13], and included

(1) pancytopenia: haemoglobin less than 10 g/dl, granulocyte count less than $1800 \times 10^6/1$, and platelet count less than $100 \times 10^9/1$;

(2) bone marrow biopsy showing hypocellular or acellular marrow, or two marrow aspirates showing hypocellular spicules. Patients with other primary haematologic diseases, malignancy, uraemia, chronic liver diseases, splenomegaly or lymphadenopathy, and those treated with cytotoxic drugs were excluded.

Bone marrow biopsies (32 patients) were obtained with the Westerman–Jensen or the Hamashidi–Swaim needles. Marrow aspirates (28 patients), and other laboratory procedures were performed by standard techniques. Marrow studies were reported as often as clinically indicated.

Following the establishment or confirmation of the diagnosis some patients were referred back to their physician for continued care. Intramuscular treatment with androgens, preferably nandrolone phenpropionate was recommended but a number of physicians used other androgens with or without corticosteroids (see Results). All patients were periodically examined by the author.

Results

The 60 patients included 45 males and 15 females. Seventeen patients were 10 years of age or younger, 15 were 11 to 20 years old, 23 were 21 to 50 years old, 5 were older than 50. The mean age was 20.9 years. Fourty-eight patients were Arabs, 5 Kurds, 2 Turkuman, and 5 from other minority groups.

Nearly all patients were referred to us because of symptoms of anaemia, bleeding, repeated infections, or any combination of such problems. Only one patient was relatively asymptomatic and was only diagnosed during the course of investigations.

The duration of symptoms prior to diagnosis ranged from a few months to more than 10 years; half of the patients had symptoms for less than one year.

Table 1

Haematological findings

	Range
Haemoglobin, g/dl	1.2–10.5
Packed cell volume PCV: vol. %	4.0–28.0
Neutrophils (absolute number) $\times 10^6/1$	50–1470
Platelets $\times 10^9/1$	2.5–97.0
Reticulocytes, per cent (corrected)	0–2.1

Table 1 shows the haematologic findings. All patients were pancytopenic and all but 5 were also reticulocytopenic.

A history of definite exposure to drugs or toxic chemicals was obtained in 18 patients, 16 of whom were over the age of 15. In 3 of these patients chloramphenicol alone seemed to be the offending agent. Nine other patients were exposed to multiple antibiotics including penicillin, ampicillin, tetracycline, rifampicine, cefalotin, sulpha compounds and chloramphenicol. Phenylbutazone was used by 2 patients a few months prior to the onset of bone marrow depression. One patient was given streptomycin, paraaminosalicylic acid (PAS) and isonicotinic acid hydrazine (INH) for the treatment of active pulmonary tuberculosis five months prior to hospitalization with aplastic anaemia. A young housewife and a 10-year-old child were heavily exposed to insecticides a few years before their referral with pancytopenia. The brands of insecticides available in Iraq contain gammabenzene hexochloride, chlordane, and chlorphenothane. Finally, a young man had a history of employment in a processing laboratory in an oil refinery for several years prior to the onset of his illness.

An additional 12 patients had a history of probable but not definite exposure to multiple antibiotics, antituberculotic drugs, antithyroid or anti-inflammatory agents.

Five patients from three different pedigrees including 3 siblings had classic Fanconi's congenital aplastic anaemia with pancytopenia, skin pigmentation, and skeletal malformation [14].

Twenty-five patients had no clue to the aetiology of their marrow aplasia and the condition was labelled "idiopathic". Of these, 2 young women developed signs and symptoms of pancytopenia six months following their first pregnancy. Both gave birth to healthy infants. One of these patients died with overwhelming

Table 2

Treatment and outcome

	Outcome* (Number of patients)				
	CR	PR	UC	Died	Total
Supportive care only	7	—	—	9	16
Corticosteroids	—	2	—	2	4
Corticosteroids and androgens	1	3	2	6	12
Androgens	6	5	7	5	23
Total	14	10	9	22	55**

* CR: Complete recovery
UC: Unchanged
PR: Partial recovery
** 5 patients lost to follow-up

sepsis and haemorrhage three days post-partum; the second made a full recovery 14 months after childbirth.

Table 2 gives details about treatment and outcome in 55 patients; 5 were lost to follow-up. Fourteen patients, of whom 6 had a history of definite or probable exposure to toxic agents made a full recovery with complete normalization of the blood count. In 9 patients, including 4 with Fanconi's congenital aplastic anaemia, the pancytopenia was unchanged after treatment for 4 to 26 months. Twenty-two patients, including 18 adults, died 10 days to 9 years after diagnosis. The cause of death in all instances was massive bleeding and/or severe infection. In 11 of these patients the condition was thought to be drug-induced.

The androgens used were methyltestosterone, 1 mg/kg b.w. (2 patients), oxymetholone, 3 mg/kg (8 patients) or nandrolone phenpropionate, 25 to 50 mg by intramuscular injection, bi-weekly (25 patients).

Two patients, a 28 years old woman and a 37 years old man, whose bone marrow failure was thought to be related to chloramphenicol ingestion, developed signs and symptoms and laboratory findings indicative of paroxysmal nocturnal haemoglobinuria (PNH) 30 and 37 months, respectively, following the diagnosis of AA. Both were treated with oxymetholone. The woman seems to have had mild disease and she no longer requires blood transfusion. The other patient who was seriously disabled by anaemia and thrombocytopenia died six months later of severe haemorrhage. Two young men whose aplastic anaemia was thought to be related to exposure to multiple antibiotics and a 55-year-old woman with idiopathic AA developed acute myelogenous leukaemia within one and nine years of the occurrence of AA. All 3 patients died shortly thereafter.

Discussion

Our patient material seems to be a roughly representative sample of the population of Iraq and, although there is a heavy concentration of patients from within or around the capital city of Baghdad, most patients have recent ancestral background from all over the country, fairly evenly. This study does not provide figures for the annual incidence of AA in the entire population, nor does it reflect the real frequency of the disease in Iraq.

The male preponderance with a male : female ratio of 3 : 1 is at variance with reports from other parts of the world where the sex distribution is about equal, or shows a slight female preponderance [4, 6, 7, 9, 13]. This is an interesting observation but the explanation is not clear. One possibility is that in Iraq, perhaps for social and economic reasons, men are exposed to toxic environmental agents more often than women and, probably for the same reasons, they seek medical help more often. It is noteworthy that the male preponderance is higher after the age of 10. Another possibility is a real difference between the two sexes in their susceptibility to bone marrow injury. Bias favouring males in admission to the hospital is unlikely. Hospital admissions during the last five years revealed no

significant disproportion in the male–female admission ratio. To support the view that the male preponderance in this series of 60 patients, admittedly a small sample, is not a chance occurrence, a retrospective review of the hospital medical records for the years 1972–175 was undertaken. During these three years 45 patients with AA were admitted to the hospital. Thirty-three of them were males, 12 were females. Thus, if we increase the sample size to 105, the male : female ratio will still be 2.88 : 1.

Exposure to drugs and toxic agents was implicated in the aetiology of AA in half of the patients, Chloramphenicol, alone or in combination with other antibiotics, was thought to be the responsible agent in 12 patients. Such findings are in agreement with many of the previously published reports [1, 9, 11, 13]. Antibiotics seemed to be the most common offending agents in this series. The overuse and misuse of these drugs, especially of chloramphenicol, and the unjustified combination of multiple antibiotics is a prevalent practice in Iraq as well as in many other parts of the world; this calls for a more effective control of antibiotic consumption. The other drugs and toxic agents implicated in the aetiology of AA have been adequately documented in other studies [13, 14].

Conversion of AA to a paroxysmal nocturnal haemoglobinuria-like disease in 2 patients, and the termination in acute leukaemia in 3, was observed; such changes in the course of AA are known to occur [13]. It is noteworthy that in all of these five patients the disease was probably drug-induced.

In 2 young women the symptoms of AA seem to have started during the first six months of their first pregnancy. Such an association is rare but has been observed in over 50 cases [12, 14]. One of these two patients died of fulminant post-partum septicaemia while the other made a full recovery 14 months after delivery and treatment with nandrolone phenpropionate.

The response to treatment and follow-up data shown in Table 2 depict the usual picture of aplastic anaemia. The high rate of mortality (40% in less than three years) was most likely due to the inclusion of patients with a poor prognosis, thus adults with severe thrombocytopenia and granulocytopenia complicated by bleedings and infection. Eleven patients died within 9 to 27 days of diagnosis. Of the patients who survived long enough to receive androgens about half showed a complete or partial response. Nearly similar results were reported by other workers [1, 2, 4], but the number of our patients treated with androgens is too small to allow an analysis of the response to the various hormonal preparations used.

It is hoped that the control of environmental pollution and a reduction of the exposure of people to drugs and chemicals will be a priority project for developing nations.

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The Effect of Basic Proteins on the Haemostatic System of the Dog

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Intravenous administration of histones or protamine to dogs resulted in a fall of the number of blood platelets, a lowered prothrombin consumption, a fall in the fibrinogen level, activation of the fibrinolytic system and deterioration of blood clot retraction.

Numerous experimental data indicate that basic proteins have a marked effect on some of the components of the haemostatic system. They precipitate fibrinogen and its degradation products in the form of insoluble sediments [5, 7], inhibit the enzymatic degradation of fibrin [4, 16], neutralize the anticoagulant action of heparin [3, 11] and aggregate blood platelets [1, 2].

Studies giving a complete picture of the changes induced by basic proteins in the blood clotting system and fibrinolysis are of a practical value since protamine is administered for the treatment of heparin overdosage and histones, on the other hand, they are released into the blood from the disintegrating cells of various tissues and organs.

Methods

Experiments were carried out on 8 mongrel dogs weighing 12 to 18 kg, divided into two groups of 4 dogs each. Total histone (produced by Polish Chemical Reagents, Gliwice) or protamine (produced by the Warsaw Sera and Vaccine Laboratories) were administered to the dogs intravenously in doses of 20 mg/kg body-weight. Blood samples were taken for the tests immediately before the experiment, then 10 and 30 minutes, 1, 12 and 24 hours after administration of the basic proteins.

In each case, blood was taken from the tibial vein through a polystyrene cannula into 0.1 M sodium citrate at a ratio 9 : 1. The citrated blood was centrifuged at 1500 *g* for 20 minutes in order to obtain platelet-poor plasma.

The parameters determined were: Recalcination and thrombin time [9], prothrombin time [12], prothrombin consumption [13], fibrinogen level [12], platelet count [14], euglobulin fibrinolysis time [6] and clot retraction [10]. In the method employed for clot retraction measurement, the blood clot is located in the upper part of a glass tube and the liberated serum collects in the lower calibrated part of the tube. It is thus possible to study the process in time.

Table 1

Haemostasis after intravenous administration of basic proteins

Test		Control	Time after administration of basic proteins				
			10 min	30 min	1 h	12 h	24 h
Platelet count in $mm^3 \times 10^3$	H	144.0 (113–170)	109.0 (88–122)	131.0 (111–150)	143.0 (126–176)	133.0 (115–146)	142.0 (122–156)
	P	145.0 (113–233)	41.0 (33–48)	76.0 (62–88)	94.0 (86–99)	123.0 (122–124)	135.0 (125–140)
Prothrombin consumption, sec	H	24.5 (20–28)	16.0 (14–20)	18.5 (15–22)	17.5 (16–19)	22.5 (20–25)	24.0 (20–26)
	P	27.7 (27–28)	16.5 (14–19)	16.5 (15–18)	18.0 (17–20)	25.2 (24–27)	25.2 (23–27)
Fibrinogen, mg/dl	H	430.0 (420–440)	400.0 (370–410)	350.0 (320–380)	310.0 (290–330)	400.0 (360–440)	420.0 (400–430)
	P	340.0 (328–350)	320.0 (305–330)	295.0 (273–307)	262.0 (230–290)	310.0 (296–334)	335.0 (324–346)
Euglobulin fibrinolysis time, min	H	40.7 (35–45)	15.5 (14–18)	15.2 (12–18)	17.7 (16–19)	37.7 (28–48)	43.7 (38–49)
	P	44.2 (38–52)	25.0 (15–38)	32.5 (16–42)	34.0 (21–39)	45.0 (40–50)	44.0 (40–54)

H — histones; P — protamine. Numbers in brackets denote variation range.

Results and Discussion

After intravenous administration of histones or protamine a transient fall in the blood platelet count, a lowered prothrombin consumption, a fall in the fibrinogen level and an increase in the euglobulin fibrinolytic activity occurred (Table 1). Neither of the proteins investigated induced changes in recalcination time, prothrombin time or thrombin time. Retraction of the blood clot considerably deteriorated after administration of either histones or protamine (Fig. 1).

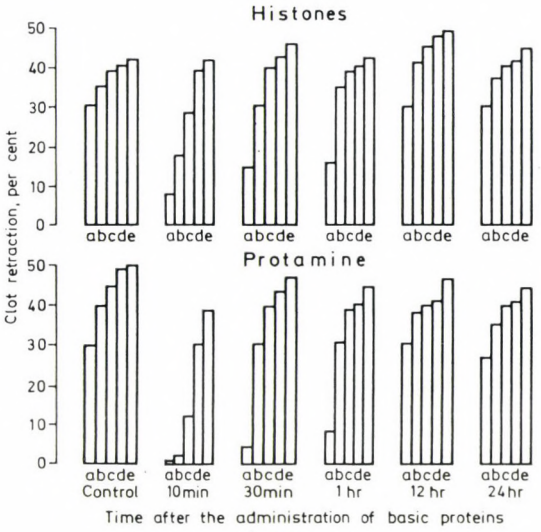

Fig. 1. Clot retraction after intravenous administration of basic proteins in dogs. Clot retraction was evaluated after: a — 1 hr, b — 2 hr, c — 3 hr, d — 4 hr, e — 12 hr

A characteristic of histones and protamine is their high basic amino acid content. As a result, their molecules are polycations of a high chemical reactivity and hence their multidirectional effect on the haemostatic system.

The ~~fall~~ in the number of blood platelets is probably due to the aggregation of these cells by histones and protamine similarly as with other basic proteins [2]. The fall in the plasma fibrinogen level is due to the formation of insoluble complexes with basic proteins. The activation of the fibrinolytic system, on the other hand, is in all probability the result of an increase in the blood plasminogen activator content released from the vascular endothelia by the action of the basic proteins. The effect of the basic proteins on the fibrinolytic system *in vivo* is therefore different from their effect in isolated systems in which they inhibit degradation of the fibrin clot [4]. The reason for this is that the complexes of insoluble fibrinogen and basic proteins, resistant to the action of plasmin, are eliminated from the circulation by the reticulo-endothelial system. Thus, they were not present in

the blood used for the experiments while the plasminogen activator content rises. The lowered prothrombin consumption and the unfavourable effect of the basic proteins on blood clot retraction are due to a fall in the number of blood platelets and inhibition of the synthesis of high-energy compounds in these cells [8, 15].

One may assume that the formation of haemostatic platelet and fibrin plugs at the site of damage to the vascular wall is facilitated by the presence of basic proteins. The reduced retraction of the blood clot, however, delays recanalization of the vessels.

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Role of the Kidney in the Inactivation Rate Constant of Urokinase Activity*

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The role of the kidney in the inactivation rate constant of UK activity (ke) was examined experimentally. The results obtained were as follows. There was a remarkable divergence in FA between the abdominal aorta and renal vein. When the bilateral renal arteries and veins were ligated, ke was 0.38, in contrast to 0.79 in the normal state. When UK was infused into rabbits with ligated renal vessels, the FA lasted for an increased period after the termination of UK infusion. These findings suggest that the kidney functions as an inactivator of UK.

Introduction

When urokinase (UK) was injected into the circulatory system, the resultant fibrinolytic activity (FA) disappeared rapidly. The decay in such FA was defined in terms of an inactivation rate constant of UK activity (ke) [5, 6, 8, 9]. UK is known to be secreted into the urine from the kidney [2, 11, 15], although the origin of the intrinsic FA in the plasma remains unclear. UK, as an extrinsic plasminogen activator, has been widely used for the treatment of thromboembolism [9, 14, 15]. In the present study, the role of the kidney in relation to ke was investigated.

Materials and Methods

UK (Uronase, Mochida Pharm.) was dissolved in physiological saline and administered *via* the marginal vein of the ear of rabbits by a single shot or infused using an infusion pump (Harvard 975).

Rabbits. Healthy adult rabbits weighing 2.5 to 3 kg were used.

Measurement of FA. (1) Fibrin plates prepared from bovine plasminogen-rich fibrinogen (Miles Lab.) and bovine thrombin (Mochida Pharm.) according to the method of Astrup and Müllertz [1] were used. (2) Synthetic chromogenic substrate, H-D-valyl-L-leucyl-L-lysine-p-nitroanilide (S-2251, KABI), was used as described in the instruction manual [4].

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I. *Effect of renal circulation on plasma FA*

Under pentobarbital sodium anaesthesia, laparotomy was carried out and the left renal vein was prepared for catheterization: connective and fat tissues were gently separated from the renal vein. In order to obtain blood samples from the renal artery, a catheter (ID 1 mm) was inserted into the femoral artery and positioned at the branching point of the renal artery in the abdominal aorta. On ligating the left renal vein on the proximal side, an incision was made transversely in the renal vein and a silicon tube (ID 5 mm) was inserted into it. These procedures were completed within a few seconds, without significant disturbance of blood flow. Since blood from the renal vein was collected over a period of 1 min every minute, blood from the abdominal aorta was also collected over a 1-min period, every minute. Both blood samples were subjected to measurement of the haematocrit (using a microcapillary, Kokusan) and of the FA in plasma.

II. *Effect of kidney isolation on ke*

Under pentobarbital sodium anaesthesia, the bilateral renal arteries and veins were simultaneously ligated, and blood samples were obtained from a catheter inserted into the femoral vein. UK was injected at a single shot (20,000 ploug units) or at various speeds (3158 to 6000 units/min). The lysis area measured on fibrin plates was converted to units using a calibration curve for a standard UK preparation (Lot No. MM-002, Mochida Pharm.). The number of units was then plotted as the ordinate on semilogarithmic paper against time as the abscissa, and the half life ($t\ \frac{1}{2}$) for FA decay was obtained from the best straight line over the given time course of the plasma FA. The inactivation rate constant of UK (ke) was calculated as reported previously [6, 8, 9].

Results and Discussion

I. *Effect of renal circulation on plasma FA*

Before UK administration, no plasma FA was observed in blood samples obtained from either the abdominal aorta or renal vein. During the first 1 min after UK injection, remarkable FA was observed in the abdominal aorta (Fig. 1). On the other hand, the plasma FA in the renal vein was slight. During the next 1 min, a similar relationship was obtained. Since the haematocrit values in the renal vein were always 10 to 30% higher than those of the abdominal aorta, it can be said that the plasma FA in the abdominal aorta would be much higher if the haematocrit values were averaged. The samples from the abdominal aorta and the renal vein were unable to split the synthetic chromogenic substrate, S-2251, which is the plasmin-specific substrate [3, 4]. The plasma FA observed on the fibrin plates thus seems to represent the activity of the administered plasminogen activator, UK. It is concluded therefore that the considerable divergence

in fibrinolytic activity between the abdominal aorta and renal vein is due to a difference in UK amounts between them, and not to plasmin activity; and that the kidney functions as an inactivator of UK, although it is not yet clear whether inactivated UK is actually excreted into the urine [7].

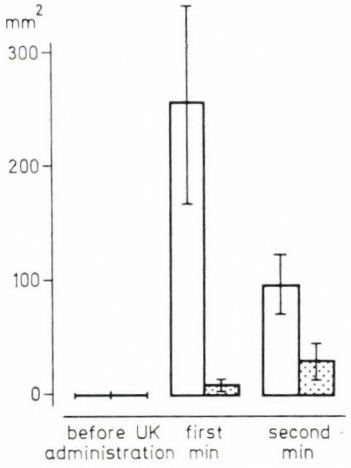

Fig. 1. Fibrinolytic activity of plasma obtained from the abdominal aorta (open columns) and renal vein (stippled columns). The vertical bars indicate the standard deviations

II. *Effect of kidney isolation on ke*

After isolation of the renal and general circulation, 20,000 ploug units of UK were injected into the marginal vein of the ear. As shown in Fig. 2 (solid line),

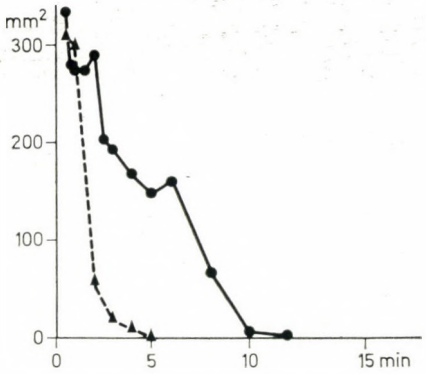

Fig. 2. Plasma fibrinolytic activity after a single administration of UK. ●— ● kidney-isolated rabbit; ▲— — —▲ normal rabbit. UK (20,000 ploug units) was injected. Ordinate: lysis area (mm²) on fibrin plates. Abscissa: time (min) after UK injection

clear FA was observed just after the injection, it was moderately decreased at 5–6 min after injection (dotted line in Fig. 2). The value of $t\frac{1}{2}$ in normal rabbits for FA decay was 0.88 ± 0.38 (min) (mean ± standard deviation) and ke was 0.79 (min^{-1}) [8]. On the other hand, the $t\frac{1}{2}$ value in kidney-isolated rabbits was 2.05 ± 1.58 (min) and ke was 0.38 (min^{-1}). This halving of the value of ke in kidney-isolated rabbits clearly supports the idea that the kidney functions as an inactivator of UK.

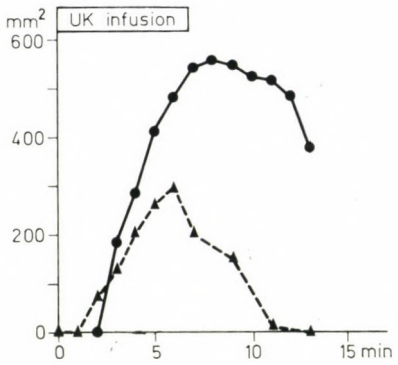

Fig. 3. Plasma fibrinolytic activity induced by infusion of UK. Symbols as in Fig. 2. In the kidney-isolated rabbits, UK was infused at a rate of 5000 ploug units/min, whereas in normal rabbits, UK was 6000 ploug units/min

When the liver, spleen and kidneys were isolated simultaneously from the circulation, the ke value was 0.19 [9]. This suggested that the kidney plays an important role in relation to ke as well as the reticuloendothelial system [12].

When UK solution was infused into normal rabbits the pattern of appearance of FA showed three stages: A latent phase, the FA-increasing phase, and an FA-decreasing phase (dotted line in Fig. 3). Urokinase activity (ke) was large in the latent phase, small in the FA-increasing phase, but large again in the FA-decreasing phase [6]. In kidney-isolated rabbits, the latent phase and FA-increasing phase resembled those of normal rabbits. However, the FA-decreasing phase differed from that observed in normal rabbits (Fig. 3). In normal rabbits, the FA decreased as after a single injection; in kidney-isolated rabbits, the FA did not, however, decrease rapidly but fluctuated at a higher value, suggesting that ke remained small. These results suggest that when the kidneys are isolated from the general circulation, the switch in ke from a small value during UK infusion to a large value on termination of the infusion may be impaired, so that a small value of ke is maintained during the FA-decreasing phase.

Our results demonstrate that the kidney serves as an inactivator of UK and plays an important role in the regulation of the fibrinolytic system in the blood. In other words, the kidney secretes UK into the urine, and inactivates UK in the blood. Some regulatory system is thus apparently present in the kidney.

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Fruchtwasser und Blutgerinnung

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(Eingegangen am 2. Februar, 1978)

Methode und Resultate einer systematischen Untersuchung von Fruchtwasserproben aus normalen Schwangerschaften zur Ermittlung des Vorkommens und der Aktivität von Gerinnungselementen werden beschrieben. Anhand der Resultate wird normales Fruchtwasser in zwei Gruppen unterteilt: A. klar-durchsichtig und B. trübmilchig. Beide Gruppen haben eine Russell-Viper-Venom-ähnliche Aktivität (wie früher schon beschrieben). Gruppe B scheint aber zusätzlich Faktor VIII als Substrat zu brauchen, um ihre maximale gerinnungsaktivierende Aktivität ausüben zu können. Die klinischen Konsequenzen werden kurz diskutiert.

Einleitung

Weiner und Mitarb. [12, 13] haben die gerinnungsaktivierende Wirkung des Fruchtwassers festgestellt. Diese Beobachtungen wurden von Randelstein und Mitarb. [8] und Verstraete [10] bestätigt. Aufgrund eigener Resultate, vermuteten Kundu und Chatterjea [4], daß im normalen Fruchtwasser Faktor-VIII-Aktivität vorhanden ist. Courtney und Allington [3] haben Fruchtwasser von normalen Graviden und solchen mit intrauterinem Fruchttod analysiert; dabei fanden sie, daß normales Fruchtwasser eine Russel-Viper-Venom-ähnliche Aktivierung von Faktor X bewirkt, während Fruchtwasser bei intrauterinem Fruchttod Faktor VII braucht, um den Gerinnungsprozeß aktivieren zu können. Phillipps und Davidson [7] bestätigten diese Befunde, kamen aber zum Schluß, daß Fruchtwasser eine zu niedrige Aktivität besitzt, um Gerinnungsstörungen oder sogar intravasale Gerinnung verursachen zu können, auch wenn in großen Mengen injiziert. Ziel dieser Arbeit ist die systematische Untersuchung von Fruchtwasserproben zur Ermittlung der Präsenz und Aktivität von Gerinnungselementen.

Material und Methode

Fruchtwasser

Fruchtwasser wurde durch transabdominale Amniocentese gewonnen. Vom gynäkologischem Gesichtspunkt aus handelt es sich bei allen Proben um normales Fruchtwasser.

Das gewonnene Material wurde in zwei Gruppen eingeteilt: Durchsichtiges klares Fruchtwasser mit wenig Niederschlag nach der Zentrifugation und milchigtrübes Fruchtwasser mit viel Niederschlag nach der Zentrifugation. Sofort nach der Entnahme wurde das Fruchtwasser in Plastikröhrchen gegossen, 4mal gekippt und die Hälfte als Fruchtwassernativ (= FWnat) bezeichnet und weiterbearbeitet. Die andere Hälfte wurde bei 16,000 *g* bei 4°C während 10 Minuten zentrifugiert. Der Überstand (= FWüb) wurde abpipettiert und der Niederschlag (= FWnied) in der gleichen Menge physiologischer Kochsalzlösung resuspendiert. Die Fruchtwasserproben wurden bei 4°C gelagert. Alle Aktivitätsbestimmungen wurden innerhalb von 24 Stunden nach Amniocentese durchgeführt.

Aktivitätsbestimmungen

Alle Gerinnungszeiten wurden mit dem Depex-Coagulometer (Depex NV de Bilt, Holland) durchgeführt.

Ansätze Serie A

Material. Normal-Plasma, thrombozytenarm; Faktor II, Faktor V, Faktor VII-X (diese Substrate wurden nach Duckert [11] hergestellt, und zwar F II- sowie F VII-X-Mangel-Plasma aus bovinem Plasma, F V-Mangel-Plasma aus Humanplasma), Faktor VIII- und Faktor IX-Mangel-Plasma (natürlichem Substrat aus Hämophilie A- bzw. Hämophilie B-Patienten); Gewebsthrombokinase aus Menschenhirn; Fruchtwasser (FWnat, FWüb, FWnied); $CaCl_2$ 1/40 mol.

Methode. Jeder der unten angegebenen Ansätze A_1 bis A_6 wurde 4mal durchgeführt, und zwar 1mal mit Gewebsthrombokinase, 1mal mit FWnat, 1mal mit FWüb, 1mal mit FWnied als aktivierende Substanz.

Ansatz A_1 (Ermittlung der Thrombokinase-ähnlichen Aktivität im Fruchtwasser).

Zu 0,1 ml Normal-Plasma werden 0,1 ml aktivierende Substanz zugegeben und nach 20 Sekunden Inkubation mit 0,1 ml $CaCl_2$ bei 37°C rekalzifiziert. Die Rekalzifizierungszeit wird mit dem Koagulometer gemessen und in Sekunden angegeben.

Ansatz A_2 (Ermittlung von Faktor II im Fruchtwasser).

Wie A_1, wobei statt 0,1 ml Normal-Plasma, 0,1 ml Faktor II-Mangel-Plasma angewendet wird.

Ansatz A_3 (Ermittlung von Faktor V im Fruchtwasser).

Wie A_1, wobei statt Normal-Plasma, Faktor V-Mangel-Plasma angesetzt wird.

Ansatz A_4 (Ermittlung von Faktor VII-X im Fruchtwasser).

Wie A_1, mit Faktor VII-X-Mangel-Plasma statt Normal-Plasma.

Ansatz A_5 (Ermittlung von Faktor VIII im Fruchtwasser).

Wie A_1, mit Faktor VIII-Mangel-Plasma statt Normal-Plasma.

Ansatz A_6 (Ermittlung von Faktor IX im Fruchtwasser).

Wie A_1, mit Faktor IX-Mangel-Plasma statt Normal-Plasma.

Ansätze Serie B

(Zur Erfassung der fibrinolytischen Aktivität vom Fruchtwasser)

Material. Fibrinplatten für die Messung der fibrinolytischen Aktivität [2]; Fruchtwasser (FWnat, FWüb, FWnied).

Methode. Auf jede Platte werden je 30 µl Fruchtwasser neben physiologischer Kochsalzlösung als Kontrolle aufgetragen. Nach 24stündiger Inkubation bei 37°C werden die Resultate abgelesen [6].

Ansätze Serie C

Material. Humanfibrinogen Grade L (Firma Kabi, Stockholm) in physiologischer Kochsalzlösung aufgelöst (Endkonzentration: 200 mg/dl); Thrombin (Firma Roche, Basel; 50 NIH/mg Trockensubstanz) in Veronal-Azetat-Puffer pH 7,39 aufgelöst (Endkonzentration: 5 NIH/ml Puffer).

Methode

Ansatz C_1 (Nachweis von Thrombinaktivität im Fruchtwasser).

Zu 0,1 ml Fibrinogenlösung werden 0,1 ml Fruchtwasser (FWnat, FWüb, FWnied) zugegeben und die Gerinnungszeit gemessen. Als Leerwert verwendet man den gleichen Ansatz, wobei Fruchtwasser durch physiologische Kochsalzlösung ersetzt wird.

Ansatz C_2 (Nachweis von Fibrinogen im Fruchtwasser).

Zu 0,2 ml Fruchtwasser (FWnat, FWüb, FWnied) wird 0,1 ml Thrombinlösung zugegeben und die Gerinnungszeit gemessen.

Immunologische Bestimmungen von Fibrinogen, Fibrinogen-Spaltprodukten D und E, Faktor VIII (antihämophiles Globulin), Plasminogen, Antithrombin III, alpha$_2$-Makroglobulin.

Material. Es wurden folgende Antiseren verwendet: Anti-Fibrinogen, Anti-Fibrinogen-Spaltprodukt D und E, Anti-Faktor VIII (Firma Behring, D Marburg), und Partigen®-Platten (Firma Behring, D Marburg) mit folgenden Antiseren: Anti-Plasminogen, Anti-Antithrombin III, Anti-alpha$_2$-Makroglobulin.

Methode. Immunelektrophorese nach Laurell [5] mit Anti-F VIII-Antiserum.

Immunelektrophorese mit den übrigen Antiseren.

Die Partigen®-Platten wurden nach Angabe der Herstellerfirma angewendet und die Resultate in Prozenten angegeben.

Resultate

Die von uns untersuchten Fruchtwasserproben wurden während einer Periode von mehreren Monaten gewonnen. Die Substrate für die verschiedenen Bestimmungen stammten also aus verschiedenen Chargen, so daß keine absoluten

Vergleichswerte zwischen Bestimmungen mit Gewebsthrombokinase oder mit Fruchtwasser ermittelt werden konnten.

Nach Zugabe von Fruchtwasser führte die Rekalzifizierung in den Ansätzen A_2 (mit Faktor II-Mangel-Plasma), A_3 (mit Faktor V-Mangel-Plasma), A_4 (mit Faktor VII-X-Komplex-Mangel-Plasma) zu keiner Gerinnung. Das schließt praktisch das Vorhandensein von Faktor II (A_2), Faktor V (A_3), Faktor VII-X-Komplex (A_4) aus, wie dies schon früher beschrieben wurde [3].

FW Nr.	Ansatz A_1 (normal Plasma)				Ansatz A_5 (F VIII-Mangel)			Ansatz A_6 (F IX-Mangel)		
	FW nat	FW üb.	FW nied	Thrombokinase	FW nat	FW üb.	FW nied	FW nat	FW üb.	FW nied
1	68	76	83	17	102	115	119	6	58	10
2	86	85	86	17	16	123	118	21	64	10
3	9	64	9	17	10	102	86	10	55	21
4	13	70	16	17	10	112	16	33	64	14
5	101	132	124	17	93	115	84	97	106	81
6	113	162	101	16	111	157	78	95	132	74
7	155	220	118	17	116	136	116	129	145	110
8	274	370	176	15	150	182	118	102	121	95
9	177	277	140	15	132	152	96	88	105	80
10	250	280	177	16	154	183	102	96	118	82
11	198	230	191	16	169	220	131	113	131	96
12	169	224	123	16	204	225	163	114	176	135
13	134	160	125	16	166	204	156	117	144	127

Abb. 1. Gerinnungszeit in Sekunden der Ansätze A_1 (mit normal Plasma), A_5 (mit F VIII-Mangel-Plasma) und A_6 (mit F IX-Mangel-Plasma) mit trüb-milchigem Fruchtwasser (Nr. 1—4) und klar-durchsichtigem Fruchtwasser (Nr. 5—13). Im Ansatz A_5, insbesondere bei FWüb, sind keine eindeutige Unterschiede zwischen trüb-milchigem und klar-durchsichtigem Fruchtwasser vorhanden. In den Ansätzen A_1 und A_6 (ab Faktor VIII intakte Gerinnungskaskade) sind die GZ bei trüb-milchigem Fruchtwasser kürzer als GZ bei klar-durchsichtigem Fruchtwasser

Nach Zugabe von Thrombokinase war die Gerinnungszeit (GZ) verzögert, entsprechend dem niedrigen Faktor II-, V- bzw. VII-X-Komplex-Gehalt (0%/1%) des Mangelsubstrates.

Die Resultate der Ansätze A_1 (mit Normal-Plasma), A_5 (mit Faktor VIII-Mangel-Plasma), A_6 (mit Faktor IX-Mangel-Plasma) bzw. die entsprechenden GZ sind in Abb. 1 zusammengefaßt.

Im Ansatz A_5 (mit Faktor VIII-Mangel-Plasma) kann kein eindeutiger Unterschied zwischen GZ für klar-durchsichtige Fruchtwässer und milch-trübe Fruchtwässer festgestellt werden (insbesondere bei FWüb und FWnied), im Gegensatz dazu zeigen die Ansätze A_1 (mit Normal-Plasma) und A_6 (mit Faktor IX-Mangel-Plasma), daß milchig-trübe Fruchtwässer kürzere GZ als klar-durchsichtige Fruchtwässer aufweisen (Abb. 1).

In den Ansätzen der Serie B wurde die fibrinolytische Aktivität des Fruchtwassers untersucht. Alle Resultate fielen negativ aus, d.h. keine Probe führte zur Bildung eines Lysenhofes auf der Platte. Da unsere Fibrinogenplatten Plasminogen enthalten (es wurde keine Hitze-Denaturierung des Plasminogens vorgenommen) sprechen die Resultate dafür, daß im Fruchtwasser weder eigene fibrinolytische Aktivität (Plasmin) noch Plasminogen-Aktivator vorhanden sind.

Mit den Ansätzen der Serie C hat man Fruchtwasser auf Thrombin (C_1) und auf Fibrinogen (C_2) untersucht. Da weder C_1 noch C_2 zur Bildung eines Gerinnsels geführt haben, kann man ableiten, daß im Fruchtwasser keine mit dieser Versuchsanordnung erfaßbaren Mengen von Fibrinogen und Thrombin vorhanden sind.

Die immunologischen Bestimmungen zeigten, daß im Fruchtwasser keine mit unseren Methoden erfaßbaren Mengen folgenden Antigene vorhanden sind: Fibrinogen, Fibrinogen-Spaltprodukte D und E, Faktor VIII, Plasminogen, Antithrombin III, alpha$_2$-Makroglobulin.

Besprechung

Unsere Resultate zeigten, daß milchig-trübes und auch klar-durchsichtiges Fruchtwasser den Gerinnungsmechanismus aktivieren kann (Ansätze A_1 mit Normal-Plasma, A_5 mit Faktor VIII-Mangel-Plasma und A_6 mit Faktor IX-Mangel-Plasma).

Die Aktivierung erfolgt im Gerinnungsschema (Abb. 2) oberhalb der Faktoren VII-X, weil beim Mangel eines in der "Gerinnungskaskade" tiefer liegenden Faktoren (VII-X-Komplex, V, II, I) (Ansätze A_4, A_3, A_2, C_2) die Fibrinbildung nicht stattfindet. Wie frühere Untersuchungen gezeigt haben [3], wird die "Ge-

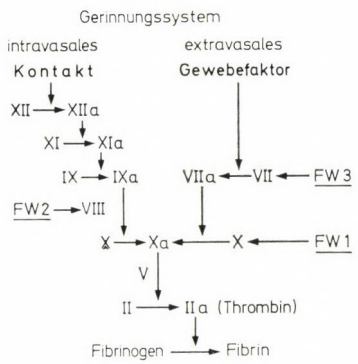

Abb. 2. Vorgeschlagener Aktionsmechanismus des Fruchtwassers: FW 1: normales Fruchtwasser (klar-durchsichtig und trüb-milchig). FW 2: normales trüb-milchiges Fruchtwasser: erst in Anwesenheit von Faktor VIII kann das Gerinnungssystem durch trüb-milchiges Fruchtwasser maximal aktiviert werden. FW 3: Fruchtwasser nach intrauterinem Fruchttod (nach Courtney und Allington [3])

rinnungskaskade" aktiviert, sobald im Ansatz Faktor VII-X-Komplex vorhanden ist. Ob die Aktivierung auf Höhe von Faktor VII oder X stattfindet, konnten wir nicht unterscheiden, da uns kein Plasma mit selektivem Faktor X- bzw. Faktor VII-Mangel zu Verfügung stand. Im Ansatz A_5 (Faktor VIII-Mangel-Plasma), wo die "Gerinnungskaskade" ab Faktor VII-X intakt vorhanden ist, sind keine eindeutige Unterschiede zwischen trüb-milchigem und klar-durchsichtigem Fruchtwasser zu objektivieren (Abb. 1). Das gilt insbesondere für die Ansätze mit FW-Überstand, da keine feste Partikeln enthalten sind und damit können mögliche mechanische Störungen der Messung mit dem Koagulometer durch die Partikeln ausgeschlossen werden.

Wenn man als Substrat Plasma mit ab Faktor VIII intakter "Gerinnungskaskade" verwendet (Ansätze A_1 mit Normal-Plasma und A_6 mit Faktor IX-Mangel-Plasma) merkt man, daß trüb-milchige Fruchtwässer den Gerinnungsmechanismus viel stärker als klar-durchsichtige Fruchtwässer aktivieren können. Man bekommt also den Eindruck, daß in Anwesenheit von F VIII trüb-milchige Fruchtwässer die "Gerinnungskaskade" viel stärker als klar-durchsichtige Fruchtwässer aktivieren können. Es scheint also, daß trüb-milchige Fruchtwässer F VIII als Substrat brauchen, um ihre gerinnungsaktivierende Eigenschaft maximal entfalten zu können. Über die Art des Aktivators erlaubt unsere Studie praktisch keine Aussage. In Abb. 2 wird der vorgeschlagene Aktivierungsmodus graphisch dargestellt. Die klinische Bedeutung dieser Eigenschaften kann nicht präzisiert werden; möglicherweise ist eine Aktivierung des intravasalen Gerinnungsprozesses bei massiver Fruchtwasserembolie pathogenetisch damit verbunden.

Die Resultate der immunologischen Faktor VIII-AG-Bestimmung (Laurell-Immunelektrophorese) zeigen leider, daß die theoretische Möglichkeit der Hämophilie-A-Diagnose in utero durch Faktor VIII-AG-Bestimmung praktisch nicht anwendbar ist, da auch normales Fruchtwasser kein Faktor VIII-AG enthält.

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Amniotic Fluid and Blood Coagulation

A study was made of the incidence and activity of coagulation factors in normal amniotic fluids. According to the results, two groups of amniotic fluid can be distinguished. To group A belong those that are transparent, showing an activity similar to Russell's viper venom. To group B belong the fluids that are milky and translucent. In this second category, factor VIII seems to be a necessary component in order to obtain maximal coagulation activity. The clinical consequences of these facts are discussed.

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Correction by Factor VIII of Decreased Neutrophil Alkaline Phosphatase Activity in Classic Haemophilia and von Willebrand's Disease

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Neutrophil alkaline phosphatase (NAP) score was estimated in ten patients with hereditary disorders in which antihaemophilic factor (factor VIII) is functionally deficient. NAP scores were estimated immediately before cryoprecipitate was administered, and following correction of patients' clinical condition and factor VIII activity in their plasma. While lower than normal NAP scores were observed before the treatment has been started, normal NAP score estimates were obtained following the successful correction of decreased procoagulant factor VIII ($F_{VIII}C$) activity in plasma of patients with classic haemophilia and von Willebrand's disease (vWd).

Introduction

Both classic haemophilia and vWd are hereditary disorders in which factor VIII is functionally deficient [22]. In patients with vWd the deficiency of factor VIII is usually associated with a decrease in antigenic material related to this substance ($F_{VIII}RAg$) to a degree similar to the reduction in $F_{VIII}C$. In all patients with classic haemophilia, the plasma contains $F_{VIII}RAg$ in amounts at least equivalent to those present in normal plasma. Thus, patients with vWd, rather than those with classic haemophilia, have a true deficiency of factor VIII.

Marginated, non-circulating leucocytes adhere to the endothelial cells of blood vessels [23]. $F_{VIII}RAg$ has been identified on the endothelial cells of normal persons [3, 11, 12] but not in patients with vWd [10]. Despite extensive studies, the exact cells synthetizing $F_{VIII}C$, which solely exhibits procoagulant activity, remain unknown [4, 24]. Organ transplantation experiments involving normal and haemophilic dogs [18, 19, 20, 28] and organ perfusion studies [7, 8] provide somewhat equivocal evidence suggesting that $F_{VIII}C$ might be synthetized in the liver and/or spleen. $F_{VIII}C$ has been reported to be elevated in leukaemia [22]. It has been demonstrated experimentally that blood leucocytes can synthetize $F_{VIII}C$ even in haemophiliacs [2]. The same cells are well known to be a source of a tissue-factor-like procoagulant [23, 24].

As it appears that leucocyte-endothelial cell juxtaposition might be of more than purely spatial character, we decided to test NAP scores in factor VIII deficiency disorders. We hoped that NAP scores might, albeit indirectly, yield certain intimations concerning leucocyte-endothelial cell cooperation.

Patients and Methods

Blood for plasma was taken into 0.129 M trisodium citrate (1 part to 9 parts of blood) in a glass tube. Platelet-poor plasma was obtained by centrifuging whole blood (3000 × *g* at 4 °C for 10 min).

$F_{VIII}C$ was determined by the two-stage assay described by Pool and Robinson [21], performed within one hour of collecting the sample. Three consecutive determinations of $F_{VIII}C$ activity were done. The mean value is expressed against fresh pooled normal adult plasma from 22 donors (mean age 34.5 years, ratio women/men 1 : 1, women taking oral contraceptives being excluded) which was defined as containing $F_{VIII}C$ of 100%.

All patients with haemophilia had severe joint and/or muscle bleeding. The following factor VIII concentrates were used for the treatment of patients: Kryobulin (Immuno AG, Vienna); Krioprecipitat (Imunološki Zavod, Zagreb); Krioprecipitat (Zavod za transfuziju krvi SR Srbije, Belgrade).

In the patient with vWd, the diagnosis was arrived at following the adopted procedure defined by Weiss et al. [29] based on defective platelet aggregation on contact with ristocetin. The patient had a severe form of the disease with spontaneous joint and muscle bleeding and no family history.

NAP activity was determined using the cytochemical method described by Hayhoe and Quaglino [9, 13] which makes use of Kaplow's original azo-dye coupling technique [16]. Thin blood films were freshly prepared without anticoagulants, allowed to dry in the open air for about 30 min, and stained immediately or stored in the dry state at −15 °C. Control films previously stored in the same way showed no detectable change in NAP score over three months. NAP activity was recorded as a weighted score, determined by examining 100 consecutive neutrophils under oil immersion and grading each cell from 0 to 4 according to criteria laid down by Kaplow [15]. The sum of the grades represent NAP score; the normal range is 25 to 140 [5], the maximum score, 400. All films were scored by G.M.J. and most were scored independently by G.M.J. and by a skilled technician. The difference of between-observer variation was less than 5%.

The mean (± SD) absolute neutrophil count before treatment was 4700 ± 1200 per μl, and following the treatment it was 4400 ± 800 per μl.

Results

The results were yielded by clinical studies for three months. Over this time ten patients reported here were admitted to the Clinic, where they received adequate treatment for symptoms due to functional factor VIII deficiency.

Plasma from nine patients with clinically severe haemophilia and from one patient with vWd were assayed for $F_{VIII}C$ and their respective NAP scores were evaluated before the treatment with factor VIII concentrate has been started. $F_{VIII}C$ and NAP scores were measured again (6.4 ± 1.11 days following $F_{VIII}C$

substitution) at the time the patients left the Clinic, with no subjective symptoms. NAP score evaluation was performed on the days when $F_{VIII}C$ was assayed. The average (mean ± SD) time span of the measurements was 37 ± 7 days. The results of the measurements done before and after treatment are summarized in Table 1.

Table 1

Factor VIII activity in plasma samples and NAP scores in patients studied before and after cryoprecipitate therapy

No. of patients	Age (years)	Sex	Factor VIII(%)*		NAP score	
			before(x_1)	after(x_2)	before(x_3)	after(x_4)
Haemophilia						
1	20	m	0.5	20.0	8	45
2	19	m	1.0	15.0	11	44
3	42	m	1.0	22.0	10	60
4	20	m	0.5	30.0	12	42
5	33	m	3.0	20.0	6	48
6	29	m	1.0	15.0	4	35
7	31	m	0.5	15.0	15	45
8	27	m	2.0	15.0	10	56
9	20	m	1.5	10.0	8	32
von Willebrand's disease						
10	23	f	1.0	15.0	8	60
Mean			1.25	17.7	9.2	46.7
SD			0.75	5.25	2.96	9.11

* All factor VIII assays against fresh plasma pooled from 22 normal adults. Normal values for NAP scores: 25—140.

Note: Factor VIII measurements were done 6.4 ± 1.11 days following its substitution with cryoprecipitate.

At admission, three patients with classic haemophilia had 0.5% $F_{VIII}C$, two had 1.0%, another two 1.5%, one had 2.0% and another patient had 3.0%. The patient with vWd had 1.0% $F_{VIII}C$ before the tratment had started. All patients had severe joint and/or muscle bleeding. NAP scores showed consistently decreased values and all patients had pathological NAP scores before the treatment had begun.

37 ± 7 days following admission, the patients were dismissed from the ward with corrected $F_{VIII}C$ activity and improved clinical condition. After this period, their $F_{VIII}C$ rose to 17.7 ± 5.25% whereas their NAP scores reached normal values of 46.7 ± 9.11 (Table 1).

$F_{VIII}C$ values were corrected in all patients and, concomitantly, normal NAP scores were now observed in all the patients studied.

Table 2

t test values and significance calculated for means and $\bar{d}$ values

n: 10	*t*	p
$\bar{x}_1 - \bar{x}_2$	− 53.94	$\ll 0.01$
$\bar{x}_3 - \bar{x}_4$	2.35	< 0.05
$\bar{d}_1$	2.26	< 0.02
$\bar{d}_2$	2.92	< 0.02

$$\bar{d}_1 = \frac{\Sigma (x_1 - x_2)}{n}$$

$$\bar{d}_2 = \frac{\Sigma (x_3 - x_4)}{n}$$

The difference in $F_{VIII}C$ values before and after the treatment was highly significant ($p \ll 0.01$). Similarly, there was a significant difference ($p < 0.05$) in NAP scores before and after therapy (Table 2). Student's *t* test for $\bar{d}$ values revealed that the difference was significant ($\bar{d}_1$, $p < 0.02$; $\bar{d}_2$, $p < 0.02$) (Table 2).

Discussion

We were surprised by the peculiar finding of a consistently low NAP content in circulating neutrophils of severely bleeding patients with hereditary functionally deficient $F_{VIII}C$. Reduced levels of NAP were first described by Wachstein [27] in chronic granulocytic leukaemia. A significant decrease in NAP activity was observed also in paroxysmal nocturnal haemoglobinuria [17] and infectious mononucleosis [26], and associated with pernicious anaemia, aplastic anaemia, thrombocytopenic purpura, sarcoidosis and hypophosphatasia [6]. To our knowledge, decreased NAP activity in untreated, severely bleeding patients with classic haemophilia and vWd has not been reported previously.

The patients investigated in this study were subjected to considerable stress when the first measurements were done. Therefore, we expected raised NAP values as previously recorded in "stressful" conditions and during the administration of ACTH and adrenocorticoids to normal individuals [25]. Strikingly, we observed subnormal NAP estimates in our patients before the therapy with cryoprecipitate. As demonstrated by $F_{VIII}C$ assay, correction of $F_{VIII}C$ activity by the administration of cryoprecipitate restored NAP scores to normal values.

It is difficult to interprete the results adequately. We did not expect that a therapy with cryoprecipitate would normalize the NAP levels. We feel that $F_{VIII}C$ or some unknown metabolic product, lacking in our patients' plasma before the commencement of therapy but directly or indirectly replaced by cryopre-

cipitate, might be related to the apparent restoration to normal NAP scores. Juxtaposition between endothelial cells of blood vessels and marginated neutrophils might, perhaps, add another tempting clue to the interpretation of the surprising phenomenon described above.

One possible explanation might be that, in untreated, bleeding $F_{VIII}C$-deficient haemophiliacs, the decrease in NAP content may be due to repression of a regulator (modifier) gene for NAP as has been suggested previously [1]. In some way, cryoprecipitate therapy leads to de-repression, allowing normal synthesis of NAP to recommence.

*

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Serum Thrombocytopoietic Activity in Thrombocytopenias

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By means of a bioassay performed in rats utilizing ^{75}Se-selenomethionine as a platelet marker *in vivo*, serum thrombocytopoietic activity (STA) was determined in 9 healthy volunteers and in 82 patients with thrombocytopenia, viz. 27 cases of ITP (9 acute and 18 chronic), 19 of aplastic anaemia, 19 of acute leukaemia and 17 of chronic proliferative disorders.

STA was the highest in the ITP group ($p < 0.01$), particularly in the cases of short duration ($p < 0.001$). In acute leukaemia, the STA was remarkably elevated ($p < 0.02$) and a negative correlation between STA and blood platelet count could be demonstrated. A negative correlation between STA and the duration of thrombocytopenia was also found in the patients with ITP. No significant increase of STA was observed in patients with ITP after corticosteroid administration. In 5 out of 7 cases who responded satisfactorily to splenectomy a significant fall of STA was demonstrated after surgery.

A slight, but statistically significant, rise of the STA value was found in patients with aplastic anaemia ($p < 0.05$). No STA elevation was observed in the cases of chronic proliferative disorders with thrombocytopenia.

New isotope methods for determination [8, 32] of the substance stimulating the production of blood platelets, termed thrombopoietin (TP) by Kelemen et al. [18] allowed a significant advance in the knowledge of thrombocytopoiesis. Most of the pertinent papers have been focussed on the mechanism and the site of TP production, its biochemical characteristics and relation to erythropoietin. Clinical investigations, however, were scarce and serum thrombocytopoietic activity (STA) was evaluated in few patients [6, 17, 19, 20, 33, 38].

In view of this, the purpose of the present work was to determine the STA level in patients with some haematological disorders associated with thrombocytopenia of various severity and duration. The effects of splenectomy and corticosteroid treatment on STA level have also been studied.

Abbreviations:

ITP	= idiopathic thrombocytopenic purpura
STA	= serum thrombocytopoietic activity
TP	= thrombopoietin
EP	= erythropoietin
c/mg PP	= counts/mg of platelet protein
G/l	= Giga/litre (10^9/1000 cc)

Materials and Methods

STA was determined according to the method of de Gabriele and Penington [8, 32] in our modification [37]. Male Wistar rats weighing 202±32 g were used. Their PCV was 50.2±0.54% and the platelet count determined by the method of Nygaard [31], 685,000±61,800/μl (685±61.8 G/l). The rats were injected intraperitoneally with rabbit antiplatelet serum. After five days, at the time of rebound thrombocytosis, they were given intravenously 2 μC of ^{75}Se-selenomethionine per 100 g body-weight and intraperitoneally 5 ml/100 g body-weight of the tested human serum. The following day the serum injection was repeated and on the next two days the incorporation of isotope into the platelets was assayed.

Under general ether anaesthesia blood was obtained from the abdominal aorta, added in a 9 : 1 ratio to the 0.054 M EDTA solution in 0.12 M NaCl, and centrifuged at 500 *g* at room temperature for 10 minutes. The pellet was resuspended in 1% solution of ammonium oxalate and centrifuged at 1200 *g* for 12 minutes. Then the platelets were washed twice in phosphate-buffered saline (PBS), resuspended in 0.1 N sodium hydroxide and radioactivity was estimated with a well-type scintillation counter. Subsequently, after two-hour hydrolysis in a water-bath of 100°C the protein content was determined in a hydrolysate by a modification of the method of Lowry et al. [24]. The measurement of isotope incorporation in counts per mg of platelet protein (c/mg PP) allowed to avoid considerable errors in the platelet count.

STA determinations were performed in siliconized tubes on serum samples stored at −30°C for three weeks at most. Normal STA values were determined in 9 healthy volunteers.

STA determination was performed in 82 patients with haematological disorders associated with thrombocytopenia including 27 cases of idiopathic thrombocytopenic purpura (ITP), 19 cases of aplastic anaemia, 19 of acute leukaemia and 17 of chronic leukaemia and lymphoma.

The patients with ITP were divided into two groups: chronic (18 cases) and acute (9 cases) according to the duration of the disease for more or less than six months. Since the borderline duration of 6 months was admitted according to Williams et al. [41], there is no immediate way to differentiate acute and chronic ITP in its early phase. Follow-up of patients with acute ITP revealed that all but one (Case 5, Table 1) did not recover during the first six months. Therefore, the time shown in Table 1 is related to the moment of STA determination and not to the recovery from the illness.

In 7 cases of chronic ITP subjected to splenectomy the determinations were performed twice, before and five to ten days after surgery. In patients with ITP the effect of corticosteroid treatment on STA was also evaluated.

In the patients with symptomatic thrombocytopenia in the course of acute leukaemias or chronic proliferative disorder the correlation between the platelet count below or over 50,000/μl (50 G/l) and STA was observed. In other patients

Table 1

STA in patients with ITP

No.	ITP acute					ITP chronic				
	1	2	3	4	5	1	2	3	4	5
1	P. T. 24 F	6.6	213	5 m	30 mg 5 m	G. R. 23 F	26.4	219	20 y	15 mg
2	S. O. 69 F	30.8	196	3 m	40 mg 2 m	D. S. 69 F	17.6	192	20 y	30 mg
3	I. U. 28 F	6.6	195	2 m	60 mg 1 m	J. B. 53 F	48.2	178	10 y	(—)
4	M. T. 45 F	11	190	2 m	60 mg 1 m	P. R. 27 M	10	172	9 y	40 mg
5	B. K. 20 F	13.2	190	6 w	50 mg 2 m	K. S. 22 F	13.2	170	10 m	40 mg
6	C. T. 71 F	30	185	3 m	20 mg 1 m	R. B. 42 F	44	170	4 y	(—)
7	S. T. 17 F	41.8	184	4 m	20 mg 3 m	A. K. 15 F	19.5	169	2 y	15 mg
8	Z. A. 56 F	22.2	176	2 m	60 mg 1 m	K. M. 30 F	29.6	164	10 y	(—)
9	J. Z. 28 F	17.3	130	4 w	(—)	C. W. 39 F	17.8	164	18 m	(—)
10						T. B. 26 F	17.6	160	4 y	40 mg
11						W. J. 55 F	41	155	2.5 y	20 mg
12						S. L. 41 F	17.6	152	20 y	50 mg
13						B. H. 24 F	44	150	18 m	80 mg
14						W. M. 42 F	22	147	3 y	(—)
15						P. W. 38 M	24.2	143	18 y	(—)
16						P. S. 74 M	28.6	139	11 m	40 mg
17						T. B. 78 M	28.6	133	7 m	(—)
18						B. T. 52 F	15.4	128	30 y	100 mg

1 — patients, age, sex, 2 — platelet count (Giga/l), 3 — STA in counts per mg of platelet protein, 4 — duration of the disease (y — years, m — months, w — weeks), 5 — daily dose and duration of prednisone administration at the time of STA assay.

such a relationship could not be established since most of them displayed low platelet counts.

Results

The mean STA value in normal volunteers was 136 ± 11.4 counts/mg PP (Fig. 1).

The STA level was the highest in patients with ITP (Table 1). The mean STA value in this group (169 ± 26.2 c/mg PP) was significantly higher than in the control group ($p < 0.01$). STA was very high in the acute group, 184 ± 26.2 c/mg PP ($p < 0.001$) while in chronic cases it was remarkably lower, 161 ± 22.8 c/mg PP, although still higher than in the controls ($p < 0.01$). The difference between he two subgroups was not significant ($p < 0.1$) (Fig. 1). A slight, but significan

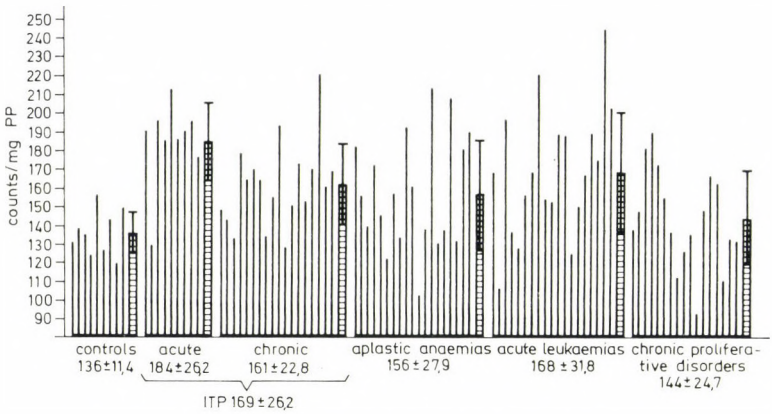

Fig. 1. Serum thrombocytopoietic activity

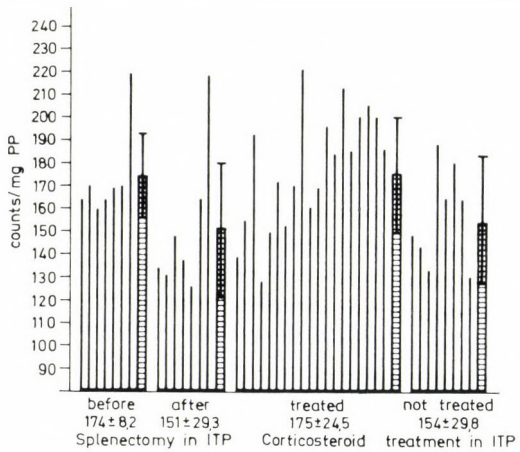

Fig. 2. Effect of splenectomy and corticosteroid treatment on STA value in patients with ITP

elevation of STA compared to the control group was found in patients with aplastic anaemia: 156 ± 27.9 c/mg PP ($p < 0.05$) (Fig. 1).

In 7 patients with chronic ITP subjected to splenectomy the STA value fell after the surgery from a mean level of 174 ± 8.2 to 151 ± 29.3 c/mg PP (Fig. 2). The difference was not significant ($p < 0.2$). In 2 patients splenectomy failed to cause a rise of the platelet count, the thrombocytopenia persisted and the STA remained as high as before surgery. If these cases are neglected, the difference in STA before and after splenectomy becomes significant: 165 ± 8.2 and 135 ± 16.9 c/mg PP, respectively ($p < 0.01$) (Table 2).

In the group of 27 patients with ITP, 8 were not given corticosteroids for various reasons. Their mean STA was 154 ± 29.8 as compared with 175 ± 24.5

Table 2

STA determination in splenectomized patients with ITP

No.	Patients, age, sex	Duration of ITP m — months y — years	Platelet survival time T 1/2	Spleen : liver activity ratio	Platelet count G/l		STA c/mgPP	
					before	after	before	after
1	C. W. 39, F	2 y	1.5 days	2 : 1	17.8	178.6	164	138
2	R. B. 42, F	4 y	1 day	2 : 1	44	330	170	130
3	T. B. 26, F	4 y		—	17.6	386	160	148
4	K. M. 30, F	10 y	1 day	2.25 : 1	29.6	312	164	134
5	A. K. 15, F	2 y		—	19.5	286	169	128
6	K. S. 22, F	10 m	1 day	2 : 1	21.6	13.2	170	164
7	G. R. 23, F	20 y	2.5 days	2.9 : 1	26.4	32.8	219	218

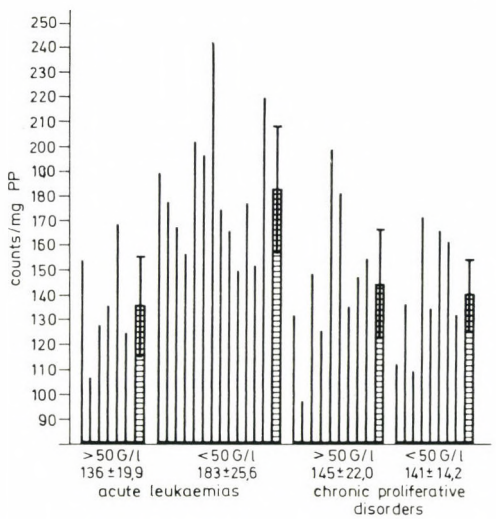

Fig. 3. Correlation between platelet count and STA in patients with proliferative disorders

c/mg PP in 19 patients who had corticosteroid treatment. The difference was not significant ($p < 0.1$) (Fig. 2).

In patients with acute leukaemia and thrombocytopenia, the mean STA level was 168 ± 31.8 c/mg PP, significantly higher than in the control group ($p < 0.02$), while almost no STA elevation was found in thrombocytopenic patients with chronic proliferative disorders: 144 ± 24.7 c/mg PP ($p < 0.4$) (Fig. 1).

In acute leukaemia, a correlation was found between STA and platelet count below and over 50,000/μl (50 G/l): STA values were 183 ± 25.6 and 136 ± 19.9 c/mg PP, respectively ($p < 0.01$). Such a correlation could not be observed in chronic proliferative disorders where the corresponding STA values were 141 ± 14.2 and 145 ± 22.0 ($p < 0.8$) (Fig. 3).

Discussion

The high STA value in patients with ITP is consistent with the observations of other authors [4, 26, 33]. Kelemen et al. failed to find a STA elevation in chronic cases [17, 20] and in the present material the STA level was also lower in chronic than in acute ITP. The difference was not significant ($p < 0.1$) which may have been due to the small number of cases. It is assumed that with the longer duration of the disease, STA tends to fall gradually, in spite of the low platelet count. This hypothesis is confirmed by the finding of McDonald et al. [28] who in persisting thrombocytopenia observed a fall of the TP level as early as 16 hours following the administration of allogeneic antiplatelet serum. In the 5 patients with chronic ITP STA was within normal limits in spite of a remarkable thrombocytopenia (Cases 15–18, Table 1). The fact that aged males predominated among them suggests that sex and age may be of some importance in TP production.

The role of the spleen in the pathogenesis of thrombocytopenia has not been sufficiently elucidated. Its function of storage and destruction of circulating platelets and as a site of antibody production seems to be proved [15, 21, 30, 34]. Nevertheless the hypothesis of Dameshek, that the spleen can produce an inhibitor of thrombopoiesis [7], despite many doubts [1, 11, 12], has not yet been rejected. For example, the hyperthrombocytosis persisting sometimes for many years after splenectomy can hardly be explained by the removal of the splenic platelet pool only [5, 39, 40]. The problem was somewhat clarified by the observations of Kelemen et al. [19] and Buonanno et al. [3] who observed a rise of STA after splenectomy.

In our material, however, a fall of STA was observed in all the 5 patients who responded satisfactorily to splenectomy. It seems that removal of the splenic platelet pool in conditions of increased thrombocytopoiesis caused an elevation of the platelet count which then reciprocally blocked TP secretion. Similar conclusions were drawn by Krizsa et al. from experiments in mice subjected to splenectomy [21, 22]. These observations provide evidence against the thrombocytopoiesis inhibitory role of the spleen.

In 2 cases of ITP, splenectomy had no result. This type of thrombocytopenia might correspond to the TP deficiency syndrome [23, 29, 36]. In our patients, however, the STA values were high before as well as after the splenectomy. Therefore, TP deficiency could play no role in the pathogenesis of thrombocytopenia in these patients, although more cases are necessary for such conclusions.

The therapeutic role of corticosteroids in ITP is not fully clear. Branehög and Weinfeld [2] observed a prolongation of thrombocyte survival in ITP after prednisolone administration. Shulman et al. [35] found that corticosteroids had no effect on platelet survival or production and *in vitro* the drug did not change the sensitization rate of platelets by alloantibodies. They demonstrated that corticosteroids inhibit the sequestration of platelets by the reticuloendothelial system. On the other hand Kelemen et al. [19] observed in 2 patients with "toxico-allergic" thrombocytopenia a rapid rise of STA after prednisone administration.

In our material 8 out of 27 patients with ITP were not given corticosteroids at the time of STA determination. Their STA compared to the treated group was lower, but the difference was not significant statistically ($p < 0.1$). This suggests that corticosteroids do not permanently affect TP secretion, while a transient rise of STA at the beginning of recovery from thrombocytopenia cannot be ruled out.

STA was significantly higher in patients with aplastic anaemia than in the control group ($p < 0.05$). Earlier, we failed to find a significant STA rise in patients with aplastic syndrome, which was probably due to the small material (5 cases) [38]. It should, however, be stressed that the STA elevation was less expressed here than in the other types of thrombocytopenia.

The occurrence of increased levels of erythropoietin (EP) in these syndromes [14] might suggest a similar physiological mechanism of synthesis and release of both these poietins. Large amounts of partially purified EP were found to have a thrombocytopoietic activity [10, 13], but this is probably due to a non-specific EP stimulation of other cell systems [16].

Many differences have been noted between TP and EP [8, 25, 27, 42]. Depletion of erythrocytes or platelets usually results in an appropriate elevation of EP or TP. But when in long-standing anaemias high EP can persist for a long time [14, 16], in chronic thrombocytopenia STA showed a decreasing tendency. It seems therefore that the STA regulating system is more effective in the early, acute, phase while EP remains efficient also in the chronic phase of the illness. This may suggest that either the platelet count is not the only factor determining TP synthesis or that the prolonged stimulation leads to a reduced sensitivity of the perceptive system, or to the partial exhaustion of a TP source.

In the cases of acute leukaemia, the STA-regulating system seems to be efficient. In this group STA was significantly elevated and in the patients with severe thrombocytopenia (below 50000/μl, 50 G/l) it was close to that observed in acute ITP. In these cases, however, the thrombocytopenia was of short duration, not exceeding five months, and this suggests that in ITP as well as in thrombocytopenia in the course of acute leukaemia the way and intensity of STA

stimulation are similar and related to the grade of platelet depletion. This is consistent with the hypothesis that the most important STA-increasing factor is a reduction of the circulating platelet pool irrespective of its cause [1, 9, 11].

In the patients with chronic proliferative disorders, STA was low and did not correspond to the platelet count. This probably resulted from the long-lasting, distressing disease associated with prolonged cytostatic treatment and the possible involvement of the organs affecting the thrombopoiesis-regulating mechanisms.

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Investigation of the Chemiluminescence Associated with Phagocytotic and Intracellular Killing Activity of Human Polymorphonuclear Leucocytes and Monocytes

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The chemiluminescence associated with phagocytotic and intracellular killing activity of human polymorphonuclear leucocytes and monocytes was investigated. A significant, opsonization-dependent increase in photon emission of polymorphonuclear leucocytes and monocytes was observed induced by engulfment of opsonized, heat-killed and living fungi *(Saccharomyces cerevisiae)*, aggregated gamma globulin, latex and India ink particles. Mononuclear cells displayed only minimal enhancement of photon emission after incubation of various particles. It is suggested that the method can be used in clinical practice as a test of phagocytotic and intracellular killing function.

Introduction

The phenomenon of chemiluminescence (CL) has been known for a long time. The light of lantern fishes *(Myctophidae)* [1] or fireflies [2] is well observable at night. The great majority of living cells and organisms are, however, capable of very weak photon emission, not observable to the eye. Nevertheless, as supersensitive photomultipliers can measure extremely low light intensities, it was possible to prove that photon emission is one of the basic properties of every living cell [3]. The rate of photon emission depends on metabolic processes, and so it increases with the increase in metabolism.

The present paper discusses the CL associated with phagocytotic and intracellular killing activity of polymorphonuclear leucocytes (PMNLs) and monocytes separated from human peripheral venous blood. CL values are compared with the phagocytosis index (PHI) and the intracellular killing index (IKI) obtained after traditional microscopic evaluation [4, 5, 6].

Materials and Methods

Examinations were made on 46 healthy persons, 32 females and 14 males, who had no dysphagocytosis and did not take drugs. The age of the persons varied between 15 and 82 years, the majority was 45 years old. We compared the phagocytosis index and the intracellular killing index obtained from microscopic

evaluation and the CL values. The method used for mathematical analysis was correlation calculation.

Separation of PMNLs and monocytes. 10 ml of peripheral venous blood was obtained from each person and 10 U/ml of heparin was added. After spontaneous sedimentation at 37 °C during 60 minutes, the buffy coat rich in PMNLs and monocytes was obtained with a Pasteur pipette, washed three times in phosphate buffer (PBS) pH 7.4, and the residual cell button was resuspended in PBS. The final concentration of leucocytes was 1.0×10^6 cells/ml.

Preparation of particles for phagocytosis. Particles of five different kinds were used:

(a) heat-killed *Saccharomyces cerevisiae* fungi;
(b) living *Saccharomyces cerevisiae* fungi;
(c) aggregated gamma globulin (aggregation at 63 °C for 30 min);
(d) 1% latex solution;
(e) 1% black India ink solution.

Opsonization was done with autoserum at 37 °C for 30 min.

Determination of PHI. 20 µl of the leucocyte suspension was dropped on a sterile slide; 20 µl of a suspension of opsonized *Saccharomyces cerevisiae* (1.0×10^7 fungi/ml) was added and the slide was incubated in a moist chamber at 37 °C for 60 minutes. After incubation the samples were smeared carefully and dried at room temperature. The dry smears were stained according to Pappenheim and the number of phagocyted fungi was determined by microscopical examination. Each set of cultures was run in duplicate. Lymphoid cells were not taken into consideration. PHI was determined by the formula

$$\text{Phagocytosis index (PHI)} = \frac{\text{Number of phagocyted fungi}}{\text{Number of PMNLs + monocytes}}$$

Thus, PHI designates the average number of fungus particles phagocyted by one phagocyte.

Evaluation of intracellular killing of living Saccharomyces cerevisiae by PMNLs and monocytes. After incubation with living opsonized *Saccharomyces cerevisiae* particles at 37 °C for 60 min, the samples were stained with 1% methylene blue. The number of stained, killed phagocyted particles found intracellularly in the PMNLs and monocytes was estimated within 5 to 10 min.

$$\text{Intracellular killing index (IKI)} = \frac{\text{Number of killed fungi}}{\text{Number of PMNLs + monocytes}}$$

Measurement of CL. Photon emission was measured in a Nuclear Chicago Isocap/300 Liquid Scintillation System, in the full visible light spectrum at a temperature of 27 ± 1 °C. The vials used in the experiments had been prescreened for background luminescence, and vials with low and nearly equal values were

chosen to keep background readings as low as possible. The cell cultures were prepared as follows.

Sample A. 2.5 ml of PBS + 0.5 ml of leucocyte suspension + 0.5 ml of fungus suspension. The leucocyte : fungus ratio was 1 : 10.

Sample B. 3.0 ml of PBS + 0.5 ml of leucocyte suspension.

The vials containing the cultures were placed into the counter chamber of the photomultiplier. The measurements were automatic and continuous during 120 minutes. The instrument recorded the number of photon counts per minute. On quantification the background counts of living leucocytes had to be taken into consideration (counts of sample B). Thus the rate of phagocytosis and intracellular killing was indicated by the difference (A – B). The index of the area under the curve of the function $f(x)$ obtained from the difference of sample A and B – between 0 and 60 minutes – was considered to show the rate of increase in photon emission associated with phagocytosis and intracellular killing.

Results

Human PMNLs and monocytes gave low photon emission values under normal conditions. On continuous examination over several hours a slow decrease of the emission values can be observed indicating the decline of metabolism and the decay of cells (Fig. 1).

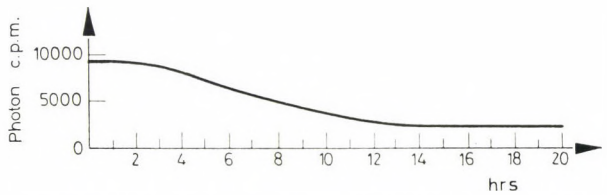

Fig. 1. Chemiluminescence of PMNLs and monocytes of healthy persons, in the normal state. The cells were separated from peripheral venous blood, and suspended in phosphate buffer pH 7.4. Number of cells 5×10^5 cells/culture. Total volume 3.5 ml. Temperature $27\pm1\,^{\circ}C$

The background values ("self-light") of heat-killed, opsonized *Saccharomyces cerevisiae* particles, aggregated gamma globulin, latex and black India ink particles can be neglected. Immediately after preparation of the phagocytotic sample, an extremely fast increase of photon emission, many times exceeding the background value of the cells, can be observed. Photon emission reached its maximum within the first 30 minutes, to be followed in the second 30 minutes by a slow, and finally a rapid decrease. The initial state is usually restored within the first 120 minutes, depending on the different cell cultures (Fig. 2).

In the case of mononuclear cells (lymphocytes, monocytes) separated on Ficoll-Uromiro gradient, CL increased only slightly on addition of particles.

The reaction was strongest when particles of aggregated gamma globulin or latex were added (Fig. 3).

In the 46 persons examined in the experiment, mathematical analysis of the PHI and CL readings proved a strong correlation between these values. Correlation coefficient (r) = 0.99 ± 0.001 (Table 1).

Table 1

Correlation between phagocytosis index (PHI) and chemiluminescence (CL) values of 46 healthy persons taking no drugs. Phagocyted particles: heat-killed, opsonized *Saccharomyces cerevisiae*

No.	PHI	CL values	No.	PHI	CL values
1	2.2	3.2	24	2.8	4.0
2	2.7	3.9	25	1.9	3.1
3	3.8	5.2	26	2.6	3.8
4	2.2	3.0	27	3.0	4.6
5	3.1	4.6	28	2.4	3.8
6	2.9	3.7	29	2.8	4.1
7	3.5	4.8	30	2.3	3.6
8	4.0	6.1	31	2.8	3.9
9	2.8	3.9	32	2.2.	3.4
10	2.7	3.3	33	3.5	4.5
11	3.1	4.2	34	3.2	4.2
12	3.5	4.4	35	3.6	5.2
13	3.2	3.9	36	3.2	4.4
14	2.8	3.9	37	2.6	3.8
15	2.0	3.5	38	3.4	4.2
16	2.8	3.9	39	3.0	4.1
17	2.6	3.4	40	2.9	3.8
18	3.9	5.4	41	2.8	3.8
19	3.1	4.0	42	3.0	4.2
20	3.1	4.5	43	3.2	4.7
21	2.4	3.9	44	3.1	4.8
22	2.7	3.8	45	2.6	3.7
23	2.8	4.1	46	2.4	3.8

Mean ± SEM: 2.89 ± 0.47 (PHI), 4.08 ± 0.61 (CL values).
Correlation coefficient (r) = 0.99 ± 0.001.

In the 15 persons examined in the experiment, mathematical analysis of the IKI and CL readings proved a strong correlation also between these values. Correlation coefficient (r) = 0.99 ± 0.02 (Table 2).

Opsonization was done with different dilutions of serum (undiluted and diluted to 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32). Following opsonization the intensity of CL changed in the phagocytotic sample. Photon emission decreases with the rate

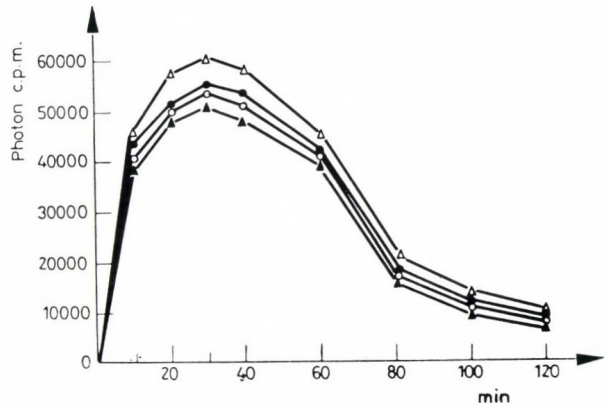

Fig. 2. Chemiluminescence of PMNLs and monocytes induced by phagocytosis. 5×10^5 cells were cultured with 5×10^6 heat-killed, opsonized *Saccharomyces cerevisiae* (△); 1% of aggregated gamma globulin (●); 1% of latex (○) and 1% of black India ink particles (▲). The $f_i(x)$ functions were obtained from the difference of samples A and B

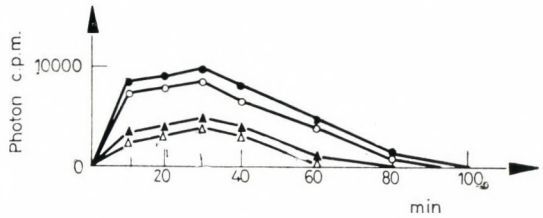

Fig. 3. Chemiluminescence of mononuclear cells of peripheral blood in the presence of heat-killed, opsonized *Saccharomyces cerevisiae* (△); 1% of aggregated gamma globulin (●); 1% of latex (○); and 1% of black India ink particles (▲). The $f_i(x)$ functions were obtained from the difference of samples A and B

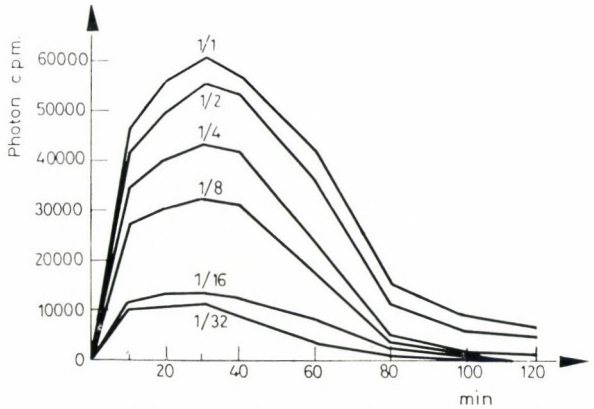

Fig. 4. Effect of opsonization on chemiluminescence of PMNLs and monocytes induced by phagocytosis of yeast-fungi. Opsonization was carried out after preincubation with different serum concentrations (undiluted, diluted to 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32). The serum was diluted by PBS, pH 7.4. The $f_i(x)$ functions were obtained from the difference of samples A and B

Table 2

Correlation between intracellular killing index (IKI) and chemiluminescence (CL) values of 15 healthy persons taking no drugs. Phagocyted particles: living, opsonized *Saccharomyces cerevisiae*

No.	IKI	CL values
1	0.9	3.4
2	1.8	5.1
3	2.1	5.8
4	1.1	3.9
5	1.5	4.4
6	1.2	3.8
7	1.6	5.0
8	1.7	4.8
9	2.0	5.3
10	1.1	3.7
11	1.3	3.7
12	1.2	3.8
13	1.9	5.4
14	1.2	4.0
15	1.6	4.6

Mean $\pm$ SEM: 1.48 $\pm$ 0.09 (IKI), 4.44 $\pm$ 0.19 (CL values)
Correlation coefficient (r) = 0.99 $\pm$ 0.002

of the serum (Fig. 4). At all serum dilutions, a strong correlation was found between PHI and CL values (Table 3).

Table 3

Correlation between phagocytosis index (PHI) and chemiluminescence (CL) values of 10 (undiluted, diluted to 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32). Phagocyted particles: heat-killed,

	Dilutions of serum		
	1 : 1	1 : 2	1 : 4
PHI Mean $\pm$ SEM	3.14 $\pm$ 0.15	2.96 $\pm$ 0.15	2.68 $\pm$ 0.10
CL values Mean $\pm$ SEM	4.31 $\pm$ 0.24	4.15 $\pm$ 0.22	3.48 $\pm$ 0.14
Correlation coefficient (r)	0.99 $\pm$ 0.001	0.99 $\pm$ 0.001	0.99 $\pm$ 0.006

The results are averages of ten experiments.

Discussion

Investigation of phagocytotic and intracellular killing activity of PMNLs and monocytes has a diagnostic significance [7]. Several methods are used for the purpose but most of them are time-consuming. For the diagnosis of dysphagocytotic states it is necessary promptly to obtain reliable data on the phagocytotic and intracellular killing functions. Thus, clinical practice requires a simple, cheap and accurate solution of the problem.

An analysis of phagocytosis and intracellular killing reveals several phenomena accompanying these processes (increasing O_2 and glucose consumption, increasing hexose-monophosphate shunt and myeloperoxidase system activity, increased CL and heat generation, etc.). Of these phenomena, in the present investigation, CL was studied, the exact mechanism of which is not known in detail, but the main phases have already been defined. Photon emission is due to several metabolic processes. At the beginning of phagocytosis increased hexose-monophosphate shunt activity occurs and leads to an increase in NADPH concentration. Simultaneously, the NADPH-dehydrogenase present in the cells [8, 9] generates a superoxide anion (O_2^-) which in turn either reacts directly with phagocyted particles in the cells (oxidation) or affects them through the myeloperoxidase system in the form of singlet oxygen ($^1O_2$), causing further oxidation and photon emission [10]. The basic function of PMNLs and monocytes is the bactericidal and fungicidal effect. Allen et al. [8] were the first to demonstrate the fact that through bactericidal activity the light emitted by these cells increases to many times the initial value. They suggested that CL intensity was in direct proportion to hexose-monophosphate shunt activity and to the rate of glucolysis and that the weak light emitted can be measured with a suitable photomultiplier.

Our investigations have shown that living PMNLs and monocytes give off a very weak but measurable light even in the resting state. On addition of particles

healthy persons taking no drugs. Opsonization was done with different dilutions of serum opsonized *Saccharomyces.cerevisiae*

Dilutions of serum			PBS
1 : 8	1 : 16	1 : 32	
1.96 ± 0.08	1.28 ± 0.11	1.26 ± 0.08	1.22 ± 0.10
2.27 ± 0.12	1.39 ± 0.16	1.27 ± 0.17	1.24 ± 0.16
0.97 ± 0.016	0.99 ± 0.046	0.95 ± 0.026	0.94 ± 0.036

photon emission increases sharply in the first 30 minutes, to reach a value many times exceeding the initial readings, then it gradually decreases and in two hours the initial state is restored.

Mononuclear cell cultures separated on Ficoll-Uromiro gradient, containing predominantly lymphocytes, also show a slight increase of photon emission, especially when the particles added were aggregated by gamma globulin or latex. In the separation process it is impossible to remove all the monocytes capable of phagocytosis and so the phenomenon described above may partly be due to monocyte contamination. Increasing purity of the lymphocyte sample results in a decrease of photon emission.

In agreement with other authors [11] the investigations showed that the intensity of photon emission associated with phagocytosis depends also on the quality of opsonization. Better opsonization leads to more intensive CL and conversely. The opsonic activity of human serum is known to promote phagocytosis and with the uptake of phagocyted particles the metabolism of PMLs and monocytes is activated and this results in an increased CL [12, 13].

Our PHI and CL values displayed a 0.99 correlation; this remarkable agreement is explained by the fact that the studies were made on healthy persons who did not take drugs and had no dysphagocytosis.

In certain conditions, such as chronic granulomatous disease of children, leucocyte glucose-6-phosphate-dehydrogenase deficiency, or familial lipochrome histiocytosis, although phagocytosis occurs, intracellular killing and, as a result, chemiluminescence, decrease or are absent. This is especially marked in homozygotes, but in a less severe form it appears in heterozygotes as well [7]. Presumably, besides genetic factors also others (hormones, metabolic products, drugs [4, 5], etc.) are responsible for the discrepancy between phagocytosis and CL, which is mainly the product of intracellular killing.

Summarizing, the measurement of leucocyte chemiluminescence seems to be a very suitable method for judging the phagocytotic function of PMNLs. The advantages of the method are simplicity, sensitivity and reproducibility. A number of examinations can be made in a short time with the exclusion of subjective judgement and the result gives a satisfactory account of the metabolic processes accompanying and following phagocytosis. The method requires a sensitive photomultiplier, an expensive instrument that can be utilized satisfactorily only if it is used for other purposes as well.

As the method gives a summation of cell activity, it appears especially suitable for screening tests. Abnormal CL values call for a more detailed examination of the phagocytotic intracellular killing system by the nitroblue-tetrazolium test, examination of fungicidal and bactericidal function after supravital staining, microbiological methods, etc.

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The Uptake of ^{3}H-Dexamethasone During Phagocytosis of *Staphylococcus aureus* by Human Neutrophils

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In the normal human neutrophil under basal *resting* conditions, cell-associated levels of ^{3}H-dexamethasone (^{3}H-DXM) increased in a linear fashion following addition of the drug. However, during phagocytosis of ^{14}C-labelled *Staphylococcus aureus (Staph. aureus)*, the intracellular ^{3}H-DXM levels rose above basal concentrations in direct relation to the quantity of ingested organisms. The observation that the cell-associated activity of ^{3}H-DXM rises in a parallel fashion to the quantity of ingested *Staph. aureus* must be recognized and taken into consideration as a variable, when assessing the results between *resting* and *activated* phases of neutrophil function in the presence of glucocorticoids.

The influence of glucocorticoids in general [4], and dexamethasone (DXM) in particular [2], on the various physiological functions of animal and human neutrophils has been extensively investigated. There is, however, no general agreement as to the effect of natural or synthetic glucocorticoids, given in conventional pharmacological dosage, on adhesion and phagocytic or bactericidal capacity of neutrophils, when tested under *in vitro* or *in vivo* conditions [4]. Glucocorticoids in high concentrations (>10 mmol $\times$ l^{-1}) will impair neutrophil function, but for pharmacological dosages the issue remains unresolved. One aspect of this problem worth considering is the actual kinetic uptake of glucocorticoids by neutrophils under normal *resting* conditions, and situations in which these cells are *activated* to phagocytize particles. It may be that intracellular glucocorticoid concentrations vary considerably under these experimental conditions. I have endeavoured to investigate this situation by incubating normal human neutrophils with ^{3}H-labelled DXM (^{3}H-DXM) under *resting* conditions, and in situations where these cells are phagocytizing ^{14}C-labelled *Staphylococcus aureus* (^{14}C-*Staph. aureus*).

Materials and Methods

Technique

The phagocytosis of ^{14}C-*Staph. aureus* (Oxford strain) *in vitro* by normal human neutrophils was assessed by utilizing a micro-modification of the technique described by Root et al. [7]. Each reaction mixture (1 ml) contained 2.5×10^{6}

neutrophils, 1×10^{8} ^{14}C-*Staph. aureus* and serum opsonins (20 $g \times dl^{-1}$ pooled normal human serum) suspended in Basal Medium (Eagle) with Earle's salts (with 25 $mmol \times l^{-1}$ HEPES buffer) (BME, Gibco-Biocult Ltd., Scotland) containing gelatin (100 $mg \times dl^{-1}$) and heparin (5 $IU \times ml^{-1}$). BME containing foetal calf serum (10 $g \times dl^{-1}$) and sodium fluoride (10 $mmol \times l^{-1}$) was used as the washing solution following incubation of the reaction mixtures. ^{3}H-DXM, Na_2PO_4 (Merck Sharp & Dohme Research Laboratories, USA), hydroxyethylated amylopectin (HES, $\bar{M}_w$ 450,000 daltons, McGaw Laboratoris, USA) and Na_3-citrate (British Drug House, U.K.) were suspended in distilled-H_2O and appropriate aliquots added to the reaction mixtures. The ^{14}C-radioactivity in washed neutrophil pellets taken immediately (time 0) and after incubation for 5, 10, 15, 25 and 35 minutes at 37 °C were used as a measure of the extent of phagocytosis. Radioactivity contributed by ^{3}H-DXM in the double-labelled experiments (^{3}H + ^{14}C) was calculated by the formula:

$$A - \left[\frac{B}{C} \times D\right] = \text{total adjusted cpm of } {}^{3}\text{H}$$

where: A = total cpm of ^{3}H in double-labelled experiment
B = total cpm contributed by ^{3}H in ^{14}C-only experiment at time 0
C = total cpm in ^{14}C-only experiment at time 0
D = total cpm of ^{14}C in double-labelled experiment

Experimental design

Neutrophils harvested from a total of 16 normal healthy subjects were divided into 4 groups. Each of these 4 groups contained cells pooled from four subjects, all possessing the same blood group type. The neutrophils from Groups I and II were further divided according to whether the cells were incubated with either ^{3}H-DXM alone, ^{3}H-DXM with ^{14}C-*Staph. aureus* or ^{3}H-DXM with ^{14}C-*Staph. aureus* plus HES and Na_3-citrate (final concentrations of each substance added to the reaction mixture are given in Fig. 1). Cells from subjects in Groups III and IV were incubated under identical conditions as stated above, with the exception that the concentration of ^{3}H-DXM in the reaction mixture was *increased* by a factor of 10 (Fig. 2).

Results

Under normal *resting* conditions, the intracellular concentration of ^{3}H-DXM increased in a linear fashion with time of incubation [upper portion (A) of Figs 1 and 2]. The addition of ^{14}C-*Staph. aureus* to the reaction mixture (neutrophils + opsonins), in the absence of ^{3}H-DXM, led to cytoplasmic internalisation as witnessed by the rise in radioactivity observed in washed neutrophil pellets [lower portion (B) of Figs 1 and 2]. The addition of both ^{3}H-DXM and ^{14}C-*Staph.*

aureus to the reaction mixture resulted in a noticeable increase in intracellular ^{3}H-DXM concentration, above that observed under normal basal *resting* conditions. In situations where the binding of bacteria to neutrophil membrane receptors can be enhanced, as has been previously demonstrated by the addition of large molecular weight colloids (HES [5] or dextran [1]) to the reaction mixture, the cell-associated level of ^{3}H-DXM rose in a parallel manner to that of the ingested ^{14}C-*Staph. aureus*.

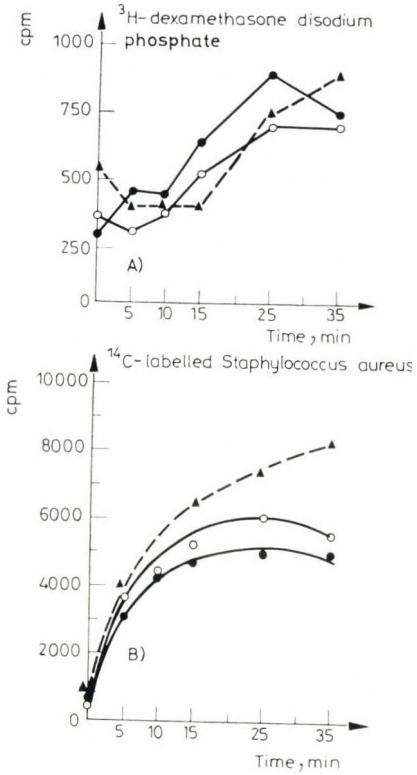

Fig. 1. Neutrophils from four healthy female subjects in Group I (all blood group type A) were pooled after sedimentation of whole blood with Dextran-110 and subsequent washing with BME (see Materials and Methods). Each point represents the mean of a duplicate determination. ^{3}H-DXM was used in a final concentration of 0.1 mmol $\times$ l^{-1}, HES at 1.5 g $\times$ dl^{-1} and Na_3-citrate at 5 mmol $\times$ l^{-1}. The upper portion (A) of the figure represents only counts per minute (cpm) of ^{3}H, while the lower portion (B) only cpm of ^{14}C. (A) ○: Neutrophils + ^{3}H-DXM; ●: Neutrophils + ^{3}H-DXM + ^{14}C-*Staph. aureus;* ▲: Neutrophils + ^{3}H-DXM + ^{14}C-*Staph. aureus* + HES + Na_3-citrate. (B) ○: Neutrophils + ^{14}C-*Staph. aureus;* ●: Neutrophils + ^{14}C-*Staph. aureus* + ^{3}H-DXM; ▲: Neutrophils + ^{14}C-*Staph. aureus* + ^{4}H-DXM + HES + Na_3-citrate. Cells from subjects in Group II, ncubated under identical conditions as those in Group I, had similar patterns of radioactive uptake of both ^{3}H-DXM and ^{14}C-*Staph. aureus*

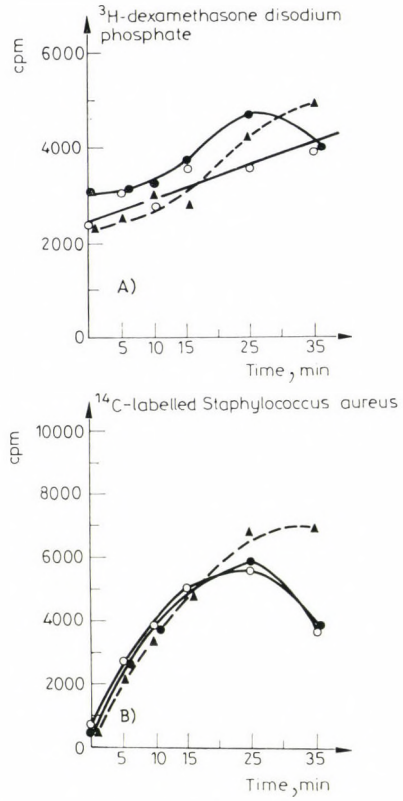

Fig. 2. Neutrophils from four healthy male subjects in Group III (all blood group type O) were again pooled after preparation from whole blood (see Fig. 1). Each point is the average of a duplicate determination. ^{3}H-DXM was used in a final concentration of 1 mmol × × l^{-1}, HES at 1.5 g × dl^{-1} and Na_3-citrate at 5 mmol × l^{-1}. For explanation of test conditions in portions A and B see Fig. 1. Neutrophils from Group IV subjects tested under identical conditions as those in Group III, behaved in a similar manner in regard to uptake of both ^{3}H-DXM and ^{14}C-*Staph. aureus*

Discussion

Being lipophilic, DXM has no difficulty in entering cells under normal physiological conditions; this is facilitated by the observation that plasma proteins do not bind synthetic steroids [3]. In general, glucocorticoids enter the cell and combine noncovalently with a limited number of specific receptor proteins in the cytoplasm [3]. The cytoplasmic steroid-receptor complex is then transported to the nucleus, thence attaches to chromatin. Whether a similar sequence of events is followed in the neutrophil under basal resting conditions is unknown, even though it appears in most cases that the steroid enters the cytoplasm rather than remaining associated with the cellular membrane. In the present study it was

shown that under *resting* conditions at 37 °C, the cell-associated concentration of ^{3}H-DXM rose in a linear fashion (Figs 1 and 2) and I therefore believe, referring to the scheme of steroid transport outlined above [3], that the radioactivity attributed to ^{3}H-DXM was located in the cytoplasm rather than bound to the membrane.

When neutrophils were ingesting ^{14}C-*Staph. aureus* under normal laboratory testing conditions at 37 °C, however, the intracellular ^{3}H-DXM concentrations increased beyond this basal *resting* level; either because: 1) the neutrophil membrane became 'leaky' allowing greater than normal amounts of ^{3}H-DXM to enter the cell; 2) some ^{3}H-DXM could have been passively incorporated into the 'phagocytic vacuole' from the surrounding medium; or 3) the ^{3}H-DXM became attached in some manner to the *Staph. aureus*-opsonin complex, thus entering the cytoplasm via the process of 'phagocytic vacuole' formation. The latter situation appears more plausible because in most circumstances the cell-associated ^{3}H-DXM levels rose in a parallel manner to that of the ingested ^{14}C-*Staph. aureus*. This was especially evident in the reaction mixtures containing HES and Na_3-citrate. In both Figs 1 and 2 the cell-associated radioactivity of ^{3}H and ^{14}C was maintained similarly following the 35-minute ingestion period, when neutrophils were incubated with both agents. These data may indicate that intracellular ^{3}H-DXM levels increase in direct relation to the quantity of ingested ^{14}C-*Staph. aureus*.

The present experimental protocol was designed to investigate the uptake of ^{3}H-DXM under normal routine laboratory conditions in which various tests of neutrophil function (NBT, adhesion, chemotaxis and phagocytosis) are conducted at 25-37 °C for periods of 30–60 minutes. It has been previously shown in my laboratory that the technique of measuring phagocytosis described by Root et al [7] is sensitive to both temperature [6] and the presence of complement [5]. Phagocytosis is completely inhibited when incubation of the reaction mixtures occurs at 0 °C, or in the presence of complement-depleted serum. I therefore believe that the changes occurring in the cell-associated concentration of ^{3}H-DXM under the test conditions utilized in this study reflect *true* alterations in the uptake of this steroid as influenced by the presence of *Staph. aureus* in the medium. It therefore seemed logical to investigate the kinetics of cell-associated ^{3}H-DXM at 37 °C, in order to better assess the differences between *resting* and *active* states under routine testing conditions. Even though the increase (15–25%) of ^{3}H-DXM due to phagocytosis was not great, it does point out that an additional factor is present when comparing *resting* and *activated* functional aspects of neutrophils incubated with glucocorticoids. The observation that the cell-associated activity of ^{3}H-DXM rises in a parallel fashion to the quantity of ingested *Staph. aureus* must be recognized and taken into consideration as a *variable* when assessing the results between *resting* and *activated* phases of neutrophil function in the presence of glucocorticoids.

*

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Sea-Blue Histiocyte Syndrome with Bone Anomalies

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Two sisters, now 29 and 24 years old, are described. They presented a congenital storage of, most probably, phospholipids in the histiocytes of the sea-blue type or blue pigmentophages. The granules of these cells showed a PAS positivity and strong positivity for acid phosphatase, but they were negative also for non-specific esterase, naphthol-AS-D-chloracetate esterase and iron acid also for urine mucopolysaccharide. The two cases differed from all the described cases in the absence of hepatosplenomegaly and in the presence of bone changes resembling late spondyloepiphyseal dysplasia or atypical dysostosis multiplex.

During the last ten years, a rapid improvement has been achieved in the understanding of the biochemical bases of lipid thesaurismoses [6]. This has provided an explanation not only of a group of diseases known many years ago as Gaucher disease with glucocerebroside thesauration, Niemann–Pick disease with sphingomyelin and Tay–Sachs disease with GM_2-ganglioside, but also allowed a new classification into specialized subgroups. Other previously known diseases are now understood to be thesaurismoses and new ones have been discovered, so that today the group of lipidoses includes not only the diseases enumerated, but also GM_1-gangliosidosis, Fabry, Krabbe, Farber, Sjögren, Batten, Refume, Wolman disease or ceramide lactoside lipidosis and fucosidosis [1, 4]. Understanding of these diseases needs the recognition of the clinical syndrome, of the specific functional abnormality and of the primary enzyme deficiency [6].

In all the above syndromes the principal feature is a deficiency of lysosomal enzymes of the hydrolase type, where abnormal synthesis is not responsible, but where the normal degradation of lipid in blood cells and perhaps also in other cell elements is inhibited before it is complete [2]. The splitting of lipids is blocked and the intermediary products accumulate in all organ systems. Initially this occurs in the histiocytic system, but finally in almost all organs including the brain. It is valuable for biochemical analysis and, eventually for prenatal diagnosis, that the deficiency is demonstrable in cell culture, even in cell cultures from amniotic fluid [4].

The sources of the stored material are initially the lipids of blood cells, especially granulocytes and thrombocytes, but to a lesser degree also lymphocytes

and red cells. This material may take part in the formation of new material for closing the cell membrane after phagocytosis [2].

The third step, the understanding of the primary enzyme deficiency, has not yet been achieved in the thesaurismosis described by Wewalka [8], later by Moeschlin [5] and Sawitsky et al. [6] and formed the sea-blue histiocyte syndrome by Silverstein et al. [7]. The variable clinical picture characterized by splenomegaly in all but one of the cases described, by hepatomegaly in 2/3 of the cases, ending frequently in cirrhosis of the liver, and in some cases displaying macular changes,

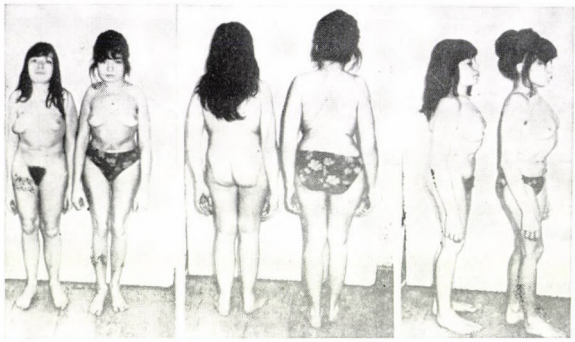

Fig. 1. The patients

lung infiltrations, neurologic symptomatology and secondary splenogenic hypoplastic bone marrow, has a common feature in the presence of many typical thesaurocytes in the bone marrow, spleen and other organs. Cytochemical reactions and biochemical examination of organs, especially of the spleen, bone marrow and liver, show a storage of phospholipids and perhaps also of glycolipids.

We have recently had the opportunity of examining this type of disease in two sisters without splenomegaly, but with marked bone changes previously not described in such cases.

The parents of the patients are healthy and a bone marrow picture of the mother showed no thesaurocytes. We had no opportunity to examine the bone marrow of the father. The mother had had two spontaneous abortions and her son had died during the first 24 hours of life. The first sister, now 29 years old, was a mature newborn and, except for frequent tonsillitis, healthy up to the age of 7 years. At this age she began to limp. Perthes disease was diagnosed in a rehabilitation department, where she stayed for eight years. Progress of the disease with bone changes and with changing physiognomy led to kyphoscoliosis and restricted movements in the hip and knee joints, deformation of the skull and of the long bones of the extremities. At the age of 16 she was obliged to walk on French crutches. The diagnosis was changed to dysostosis multiplex Pfaundler–Hurler with localized chondrodystrophy and in another orthopaedic department it was

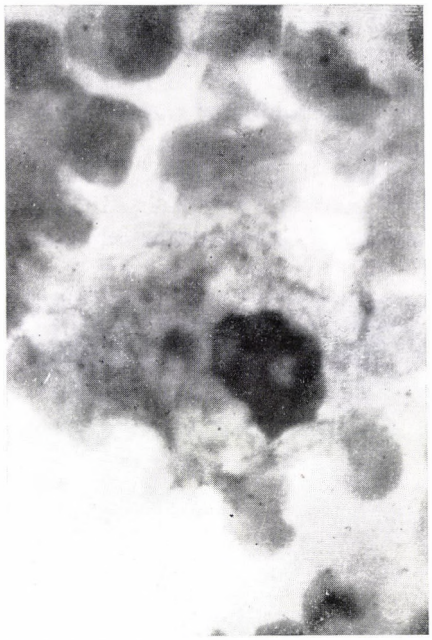

Fig. 2. Sea-blue histiocyte of bone marrow

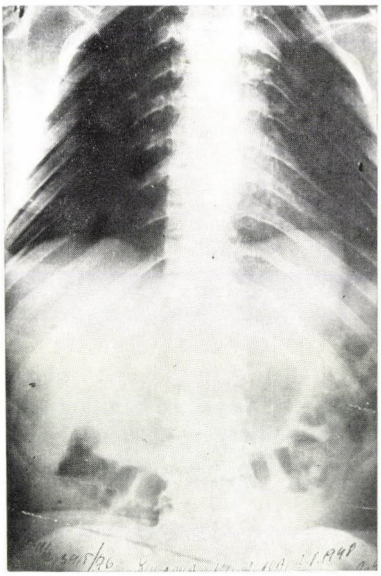

Fig. 3. Roentgenogram of thoracal spine

concluded that she had Morquio – Ullrich disease. Some years later, she married and at the end of 1975, she visited a genetic clinic asking if it was possible for her to have healthy children. With a diagnosis of mucopolysaccharidosis type VI Maroteaux – Lamy she was referred to our Haematologic Department for examination.

Her sister, now 24 years old, has an identical history, but her limp developed a little earlier and during childhood she had had "growing pains" in her knee joints. She had been hospitalized in the same rehabilitation department as her sister until the age of 16 years. She too walks on French crutches.

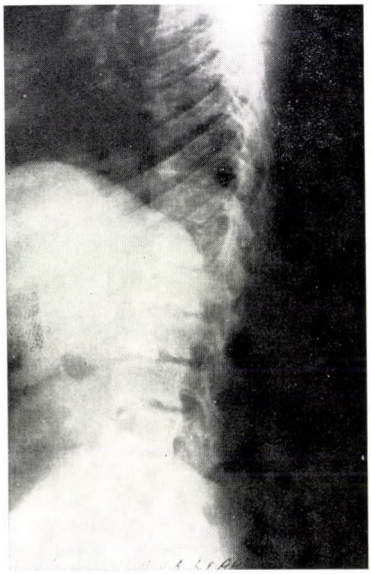

Fig. 4. Roentgenogram of lumbar spine

Both sisters are mentally normal with IQ of 100 and 110. Their appearance, shown in Fig. 1, is abnormal, but not identical with that of gargoylism. They are of short stature, 140 and 137 cm high, with long upper and lower extremities, with a short and deformed trunk, marked lumbar lordosis and restricted movements of the hip and knee joints. A slight hypertelorism and antimongoloid slant of the eyes are visible. The liver and spleen are not palpable. Haematology revealed a slightly coarser granulation in the neutrophils and occasionally coarser granules in the lymphocytes, but they were not comparable with the coarseness of the granules in the Alder – Reilly granulation anomaly or the Gasser – Mittwoch granules. All other haematological findings in peripheral blood were normal. In spite of this, a bone marrow puncture yielded a surprising number of thesaurocytes of the sea-blue histiocyte type, identical with the cells described previously in another case (Fig. 2) [3]. These cells have a small, mature, eccentric, round nucleus and extensive cytoplasm 30 to 60 μm in diameter, containing stored ma-

terial ranging from small, optically empty vacuoles to larger inclusions up to 5 μm round or irregular in shape and blue or greyish-blue in colour. Exceptionally, the inclusions have a dark blue colour and in the cytoplasm of a few such cells the rests of phagocyted blood cells are visible.

Cytochemical examination of the granules showed a slight to strong PAS positivity, especially in the Rossmann modification, and a strong acid phosphatase positivity. Non-specific esterase, naphthol-AS-D-chloracetate esterase and iron reactions were weakly positive and the other reactions were negative. Urine mucopolysaccharides were absent in both patients, the eye fundi were normal, but with

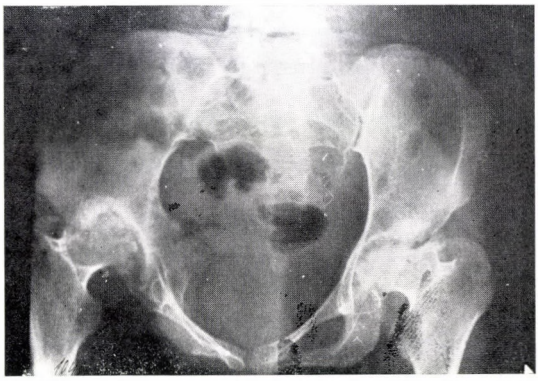

Fig. 5. Roentgenogram of acetabula and femoral epiphysis

a split lamp a "dusky" opacity of the corneal stroma was seen, with no changes at the periphery or in the depth of the cornea, resembling the changes in cornea farinata. Amino acids in blood and urine were normal and so were the palmar creases.

Very striking were the bone changes resembling dysplasia spondyloepiphysaria tarda or atypical dysostosis multiplex. In the facial region a slight hypoplasia of the mandible and hypertelorism was visible; the skull and the sella were normal. The ribs were in the roof position and had oar shape. The spine was somewhat shortened (Fig. 3), with an S-shaped scoliosis and the lateral curve had become straight. The vertebrae were dysplasic, flattened, with anterior body convexity in the lumbar area (Fig. 4). The end plates of the bodies are undulated, irregular and give the appearance of Schmorl's knots. The intervertebral distance was irregular and some intervertebral disks between Th_{12} and L_1 and between L_2 and L_3 were calcified. The acetabula and femoral epiphyses were dysplasic (Fig. 5). The long bones of the extremities were slightly shortened and their epiphyses hypoplasic (Fig. 6). The distal radio-ulnar epiphyses were oblique.

In the two sisters an atypical case of thesaurismosis was observed with thesaurosing most probably phospholipids in the histiocytes of the sea-blue histiocyte type or blue pigmentophages. Stored material in organ biopsy samples and thin fibroblast cultures could be characterized only cytochemically.

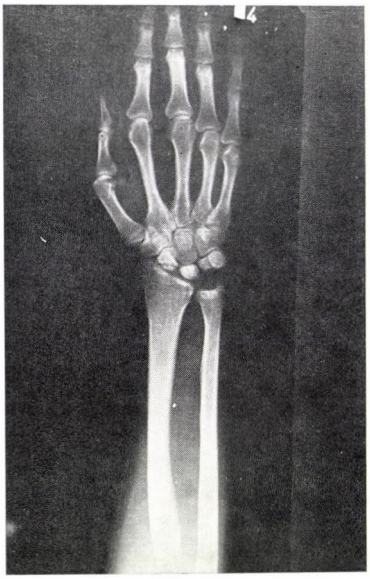

Fig. 6. Roentgenogram of hand

So far no biochemical investigations into the phospholipid storage and/or enzyme activities in the sea-blue histiocyte syndrome have been reported. These cases differ from the normal syndrome with the absence of hepatosplenomegaly, and principally with the presence of specific bone changes resembling late spondyloepiphyseal dysplasia or atypical dysostosis multiplex. This symptom has never been described in the sea-blue histiocyte syndrome.

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IgM Plasmacytoma
Report of a Case and Review of the Literature

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IgM paraproteinaemia is described in a patient with diffuse plasmacytoma involving the pleura, lymph nodes, and kidneys. Sixteen cases of pure plasmacellular tumour (diffuse or solitary plasmacytoma and plasmacellular leukaemia) associated with IgM paraproteinaemia have only been reported. These cases and experimental data indicate that neoplastic plasma cells can synthetize IgM as well as other immunoglobulins and that this particular M component is not associated solely with Waldenström's macroglobulinaemia.

Neoplastic proliferation of plasma cells is most frequently associated with production of monoclonal IgG or light chain immunoglobulin fragments, less frequently of IgA, IgD and IgE. IgM paraproteinaemia has rarely been reported in association with diffuse plasmacytoma but with Waldenström's macroglobulinaemia and other non-malignant diseases. We shall describe a patient with diffuse IgM plasmacytoma and review the previously reported cases.

Case report

C. V., a 62-years- old-man, had presented in May, 1975, with a five-year history of epigastric pain relieved by food, and a two to three months history of anorexia, pallor and fatigue. Physical examination was negative with the exception of epigastric pain on palpation. A barium meal revealed a gastric ulcer. Laboratory studies, which are summarized in Table 1, revealed the presence of an M component and an IgM level of 2800 mg/dl in the serum of a patient in whom rouleaux were present in the otherwise normal peripheral blood sample. The bone marrow aspirate was non-diagnostic with a slight elevation (18%) of normal appearing plasma cells. Other parameters often found to be abnormal in "plasma cell dyscrasias" (total protein, albumin, BUN, calcium, Bence—Jones urinary protein excretion) were all normal. The patient was discharged on melphalan (2 mg daily dose) and antacids with the diagnosis of "plasma cell dyscrasia" and gastric ulcer.

In March, 1976, he was hospitalized for evaluation of a right lower quadrant pain and cough. The laboratory data, summarized in Table 1, showed persistence

Table 1

Summary of laboratory studies

	May, 1975	March, 1976	May, 1976
Total protein (g/dl)	serum 7.4	serum 13.5	pleural fluid 6.8
Alfa-1- globulin (%)	serum 32	serum 26	
Alfa-2 globulin (%)	serum 5.5	serum 4.7	
Beta globulin (%)	serum 4.2	serum 4.4	
Gamma globulin (%)	serum 56	serum 61.9	
M component	serum +	serum +	
Immunoglobulins (mg/dl)			
IgG	serum 620	serum 750	pleural fluid 315
IgA	serum 57	serum 60	pleural fluid 35
IgM	serum 2,800	serum 5,800	pleural fluid 2,200
Sedimentation rate (1 hour)	serum 84	serum 55	
Urea nitrogen (mg/dl)	serum 15	serum 19	
Calcium (mg/dl)	serum 10.2	serum 10	
Creatinine clearance (ml/min)	serum 110	serum 113	
Bence–Jones proteinuria	—	—	
Haemoglobin (g/dl)	10.7	10.2	
White blood cells (μl)	5,700	7,000	
neutrophils (%)	30	51	
lymphocytes (%)	40	40	
monocytes (%)	22	5	
eosinophils (%)	8	4	
basophils (%)	—	—	
Platelets (μl)	300,000	250,000	
Bone marrow plasma cells (%)	18—23	18	
PTT (control 40 sec)	42	37	
Fibrinogen (mg/dl)	341	350	
Lytic bone lesions	—	—	

of the M component with a remarkable increase of serum IgM level on immunodiffusion and immunoelectrophoresis. On the second day of hospitalization the patient displayed an abdominal haematoma which at laparotomy was shown to be in the abdominal wall along the rectal muscle. On the twentieth day in hospital the patient was subjected to thoracenthesis for bilateral pleural effusion. A total protein of 6.8 g/dl was found in the pleural fluid and the immunoelectrophoresis

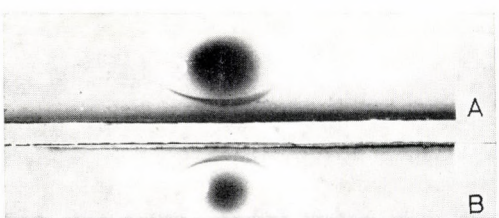

Fig. 1. Immunoelectrophoresis with IgM-specific antiserum: A) serum; B) pleural fluid

and quantitative immunoglobulin levels of the pleural fluid were similar as that found in the serum (Fig. 1). Plasma cells were found in the pleural fluid (Fig. 2). The patient was supported with whole blood transfusions, plasmapheresis and treated with prednisone (30 mg per day) and melphalan (three 80 mg courses)

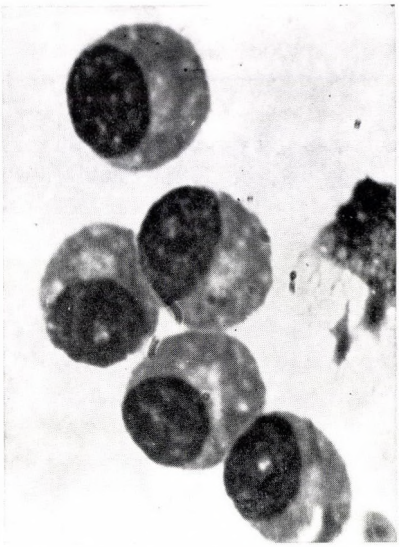

Fig. 2. Plasma cells detected in pleural fluid

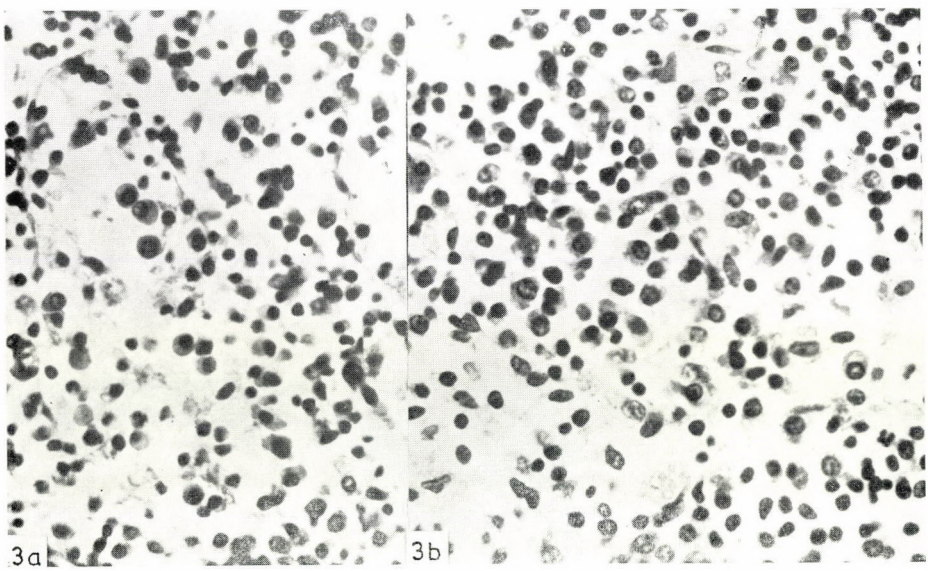

Fig. 3. Pleural mass (a) and abdominal lymph nodes (b): plasmacellular infiltrate

without significant improvement. The surgical wound failed to heal and a sudden worsening of the anaemia with fever and peritonitis appeared. The patient died in June, 1976, after four months hospitalization.

Necropsy disclosed serofibrinous pleurisy with bilateral effusions; a mass 7 cm in diameter, 2 cm thick and whitish was present on the posterior right parietal pleura at the level of the 6th – 8th ribs. The mass which was not connected to the unaffected ribs, was mostly composed of plasma cells (Fig. 3a). In the right anterior abdominal wall a huge haematoma was detected near the rectal muscle. A healed gastric ulcer was also found. The periaortic, perigastric, iliac and inguinal lymph nodes appeared enlarged (maximum 2 cm); their structure was altered and a plasma cell infiltrate was visible (Fig. 3b). The liver was fatty and the kidneys were normal on cross examination, while histology revealed a tubular eosinophilic mass and a plasmacellular infiltration in the cortex.

Methods and results of immunochemical studies

Protein electrophoresis was done on cellulose acetate. Immunoglobulins were quantitated by immunodiffusion on Behringswerke plates. Immunoelectrophoresis [19] was carried out with the following Hyland and Behringswerke antisera: total, IgG(H + L), IgG(H), IgA(H), IgM(H), IgD(H), IgE(H), Fc(IgG), kappa and lambda free and Ig-bound. IgM was separated from other serum components by gel filtration through a Sephadex G 200 column (1.5 per 90 cm; Pharmacia) [11]. Ultracentrifugation of serum diluted 1 : 30 was carried out in a Spinco (Model E) analytical centrifuge with rotor AND, at 60,000 r.p.m., at 4°C. Urine was concentrated 100 times against a 20% polyethylene glycol solution.

Analysis of the serum in March, 1976, revealed a serum total protein of 13.5 g/dl. On electrophoresis it showed a sharp peak migrating in the gamma region. Quantitative determination of immunoglobulins revealed 750 mg/dl IgG,

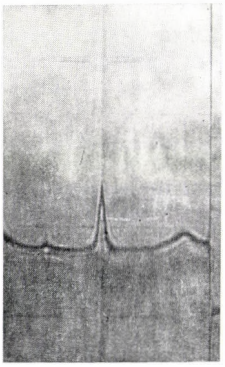

Fig. 4. Ultracentrifugal pattern of patient serum

60 mg/dl IgA, and 5800 mg/dl IgM. Serum protein immunoelectrophoresis with a rabbit anti-human IgM showed a strong precipitin line (Fig. 1A). Ultracentrifugation of the serum protein showed three different peaks, a first one (11.4%) of 26 S, a second (29%) of 18 S and a third peak (29%) comprising fractions of 4.5 S and 7 S (Fig. 4). IgM was separated from other immunoglobulins by passage through a Sephadex G 200 column (Fig. 5); its light chains proved to be lambda

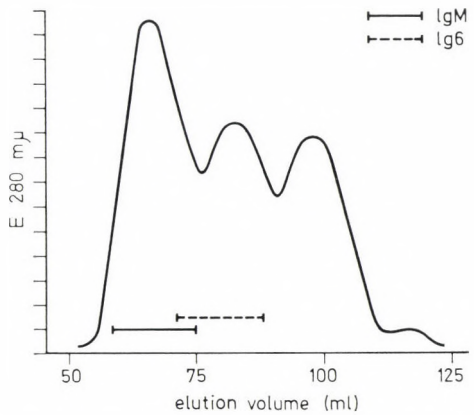

Fig. 5. Gel filtration pattern on Sephadex G 200 column with patient serum. Elution patterns of IgG and IgM are indicated

type with specific antisera. Immunoelectrophoresis and immunodiffusion of previously concentrated urinary proteins showed the presence of albumin, transferrin, and IgG, but free light chains were absent. Chemical analysis of the pleural fluid in May, 1976, revealed a total protein level of 6.8 g/dl. Quantitative determination of immunoglobulins showed 315 mg/dl IgG, 35 mg/dl IgA, and 2200 mg/dl of IgM. Immunoelectrophoresis of the pleural fluid showed a pattern similar to that of the serum (Fig. 1B).

Discussion

In a patient an IgM paraprotein containing light chains of the lambda type was found in the serum and pleural fluid, together with plasmacellular infiltrates in pleura, thoracic wall, abdominal lymph nodes in the absence of significant bone marrow plasmacytosis, lytic bone lesions, Bence – Jones proteinuria and renal insufficiency.

Although IgM paraproteinaemia is usually associated with Waldenström's macroglobulinaemia, 16 cases of IgM paraproteinaemia associated with plasma cell tumours have been reported. The main data are listed in Table 2. Though not all the cases had extensively been described, sufficient data to support the diagnosis

Table 2

Data of 16 cases of IgM plasmacytoma reported in the literature

Cases	Reference	M components	Clinical manifestations	Bone marrow
1	(18)	Macroglobulinaemia 21 S (46% of serum protein)	Diffuse plasmacytoma. Osteolytic lesions. Haemorrhagic syndrome	37% plasma cells
2	(1)	Macroglobulinaemia 19 S (47% of serum protein	Diffuse plasmacytoma. Osteolytic lesions	Plasmacytosis
3	(16)	IgM 19 S	Diffuse plasmacytoma. Osteolytic lesions	Plasmacytosis
4	(13)	IgM 19 S (15% of serum protein)	Plasma cell leukaemia	38.2% plasma cells
5	(8)	IgG + IgM 18 S	Diffuse plasmacytoma. Osteolytic lesions	Plasmacytosis
6	(23)	IgM(k). Serum IgM 10,400 mg/dl	Diffuse plasmacytoma. Osteolytic lesions. Haemorrhagic syndrome. Retinal thrombosis	75% plasma cells
7	(5)	IgM	Diffuse plasmacytoma. Osteolytic lesions	5% pleomorphic plasma cells
8	(20)	IgG(k) + IgM(k)	Plasmacytoma of the tongue	5% plasma cells
9	(10)	IgM(k) + Fc(IgG). BJ κ + Fc(IgG) in urine	Plasma cell leukaemia	Plasmacytosis
10	(15)	IgG(λ) + IgM(λ)	Retroperitoneal plasmacytoma	Not reported
11	(7)	IgM(λ). Serum IgM 1780 mg/dl	Diffuse plasmacytoma	Plasmacytosis
12	(17)	IgG(λ) + IgM(λ). Serum IgM 6000 mg/dl. BJ λ in urine	Plasmacytoma of liver	Few immature plasma cells
13	(4)	IgM(k). Serum IgM 1000 mg/dl	Diffuse plasmacytoma. Osteolytic lesions	80% plasma cells
14	(9)	IgM(k), 16.7 S, 40.5% of serum protein. IgM 4200 mg/dl	Diffuse plasmacytoma. Osteolytic lesions Haemorrhagic syndrome. Retinal thrombosis	70% plasma cells
15	(19)	IgM(k) BJK in urine	Diffuse plasmacytoma. Osteolytic lesions	19% pleomorphic plasma cells
16	(12)	IgM(λ) BJλ in urine	Diffuse plasmacytoma. Osteolytic lesions. Haemorrhagic syndrome	90% plasma cells

were given. Not included in this review are cases which, though defined as plasmacytoma, showed either a mixed lympho-plasmacellular picture suggesting Waldenström's macroglobulinaemia or had no clearly defined IgM serum M component. With the exception of Nos 1 and 2, all the cases were examined by immunoelectrophoresis. Of the cases, 11 were diffuse plasmacytomas, 3 solitary extraosseous plasmacytomas, and 2 plasmacellular leukaemias. Four of them presented with IgG + IgM a double paraproteinamia with identical kappa or lambda light chains. In one of these patients different clones of plasma cells were synthetizing the 2 immunoglobulins [8]. In one case the IgM was associated with the Fc(IgG) fragment as in gamma heavy chain disease. Light chains were determined in 10 cases; 7 were kappa and 3 lambda type.

Our patient had a typical diffuse plasmacytoma localized extramedullarly, involving the lymph nodes and kidneys. Detection of a large number of plasma cells in the pleural fluid proved *in vivo* the presence of a plasmacellular tumour, while clearcut osteolytic lesions were absent and the number of plasma cells (18–23%) in the bone marrow was increased only moderately.

Pleural localization is a rare event in plasmacytoma: only 7 cases have been reported, some of them occurring a long time ago and therefore without sufficient cytological and immunochemical data [3]. In some of these patients pleural involvement by the plasmacellular tumour followed pulmonary or bone localization. In our patient the tumour mass involving the right pleural cavity probably originated from the parietal pleura, since the lungs and ribs were not affected.

Protein study in our patient revealed a large amount of IgM(lambda) in serum and in pleural fluid. This type of M component is rarely associated with a plasma cell tumour and is usually found in Waldenström's macroglobulinaemia, which shows a mixed lympho-plasmacellular picture in connection with mast cells and histiocytes. Clinically, plasmacytomas and Waldenström's macroglobulinaemia may present similar pictures with osteolytic lesions, IgM paraproteinaemia and hyperviscosity. This makes the differential diagnosis difficult. Ultimately, the difference between the two conditions is a morphological one. Many authors seem to define as Waldenström's macroglobulinaemia cases which are typical plasmacytomas associated with IgM paraproteinaemia and as a consequence we find cases defined as Waldenström's macroglobulinaemia with lytic osseous lesions and plasma cell morphology [4], or "macroglobulinaemia-myeloma with double gammopathy" [16], or "macroglobulinaemia with bone destruction" [23]. From the literature it seems that IgM paraproteinaemia is associated only with Waldenström's macroglobulinaemia, no IgM paraproteinaemias being connected with plasmacellular tumours. Because of this it is possible that some cases of IgM plasmacytoma have not been reported in the literature since they were diagnosed as Waldenström's macroglobulinaemia.

The presence of Waldenström's macroglobulinaemia with a plasmacellular infiltrate and/or osteolytic lesions [22] have caused some authors to regard this illness and plasmacytoma as two end points of the same condition, i.e. a neoplastic proliferation of IgM-producing cells, which can express itself with different cyto-

logical features. However, this case and some others reported previously, together with what is commonly observed in experimental plasmacytoma in Balb mice [6, 14], show that pure neoplastic proliferation of plasma cells can synthetize IgM as well as other Ig classes. This observation is in accordance with what one would expect theoretically, since IgM is synthetized by plasma cells in physiologic conditions. It is not clear why plasma cell tumours associated with IgM paraproteinaemia should be so rare; IgM-producing plasma cells are constantly present and active in certain phases of the immune response. Nevertheless, both for practical and theoretical reasons the detection of a paraproteinaemia involving a particular class of immunoglobulins cannot be considered a valid criterion for differentiating various illnesses. Monoclonal complete immunoglobulins of different classes or their fragments, heavy or light chains, can be detected not only in plasmacytoma and in Waldenström's macroglobulinaemia, but can also be found in chronic lymphatic leukaemia, in lymphomas, in solid tumours and in non-neoplastic conditions [2]. It is likely that an M component could either be the expression of a neoplastic proliferation of a certain cell clone, or a reactive immunological phenomenon. It seems therefore appropriate to define an illness associated with an M component on the basis of cytological criteria and of the neoplastic or reactive nature of the cell responsible for the immunoglobulin production in that illness.

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Prognose der chronischen lymphatischen Leukämie

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(Eingegangen am 5. Januar 1978)

In einer retrospektiven Studie wurden 117 Patienten mit chronischer lymphatischer Leukämie (CLL) nach Rai klassifiziert und hinsichtlich ihrer Überlebenszeit beurteilt. Mit zunehmender Schwere des Stadiums verschlechterte sich die Prognose. Ein Wechsel des Stadiums erfolgte jeweils im Sinne einer Progredienz der CLL.

Unbehandelte Patienten wiesen die höchsten Überlebenszeiten auf. Unter den verschiedenen Behandlungsformen ergab sich bei einer Monotherapie mit Corticosteroiden die schlechteste, bei einer lokalen Strahlentherapie die beste Prognose.

Es ist seit vielen Jahren bekannt, daß die chronische lymphatische Leukämie (CLL) verschiedene Verlaufsformen aufweisen kann [2, 14, 15, 31]. Erst seit dem Vorschlag von Rai und Mitarb. [49] besteht eine nach klinischen Gesichtspunkten orientierte Stadieneinteilung der CLL, die mit der Prognose des Krankheitsbildes korreliert und eine gewisse Richtlinie gibt, wann eine Therapie eingeleitet werden sollte.

Die bisherigen Mitteilungen über die mittlere Überlebenszeit bei CLL-Patienten vom Zeitpunkt der Diagnose schwanken zwischen 1,7 bis 9 Jahre [5, 24, 29, 33, 42, 44, 59]. Einige Autoren haben die Überlebenszeit mit der klinischen Symptomatik [2, 14, 57], der Leukozytose [38], dem Verhalten der Leukozytenzahlen unter der Therapie [61], der Anämie [38, 51], der Thrombozytopenie [57] sowie mit dem Grad der lymphozytären Infiltration im Knochenmark in Beziehung gesetzt [4, 9, 29].

Die von Rai und Mitarb. [49] vorgeschlagene Stadieneinteilung wurde auf die CLL-Patienten der letzten 20 Jahre angewandt. Unter Berücksichtigung der verschiedenen klinischen und laborchemischen Parameter wurden Untersuchungen zur Prognose der Erkrankung durchgeführt.

Patientengut

Zur Auswertung gelangten die Krankengeschichten von 117 Patienten mit CLL, die von 1955 bis 1975 stationär behandelt worden sind.* Es handelt sich dabei um 80 Männer und 37 Frauen. Patienten mit unklarer Lymphozytose,

* Wir danken Herrn Prof. Dr. W. H. Hauss, em. Direktor der Medizinischen Klinik und Poliklinik der Universität Münster, für die Erlaubnis zur Auswertung der Krankenblätter.

Morbus Waldenström und Lymphosarkom wurden nicht in die Studie aufgenommen.

Die Überlebenszeiten wurden vom Zeitpunkt der Diagnosestellung berechnet [13].

Die Gruppenzuordnung erfolgte nach der Stadieneinteilung von Rai und Mitarb. [49]. Das Lebensalter lag zwischen 36 und 85 Jahren. Das mittlere Erkrankungsalter betrug zum Zeitpunkt der Diagnosestellung 58,6 Jahre.

Ergebnisse

Die Verteilung der Patienten auf die Stadien von Rai und Mitarb. geht aus Tabelle 1 hervor. Es zeigt sich, daß am häufigsten des Stadium II vertreten ist.

Bei einer Aufteilung der Patienten in die einzelnen Stadien unter Berücksichtigung der Altersdekaden ergibt sich die aus Tabelle 2 hervorgehende Verteilung.

Tabelle 1

Verteilung von 117 Patienten mit chronischer lymphatischer Leukämie (CLL) auf die Stadien von Rai [49]

Stadium	Zahl der Patienten	Anzahl in Prozent
0	6	5,1
I	25	21,4
II	40	34,2
III	19	16,2
IV	27	23,1
	117	100,0

Tabelle 2

Verteilung von 117 Patienten mit CLL auf die Stadien von Rai [49] unter Berücksichtigung des Lebensalters

Alter (Jahre)	Stadium					Insgesamt
	0	I	II	III	IV	
< 50	1	3	12	3	6	25
> 50 < 60	2	13	10	8	9	42
> 60 < 70	3	7	12	4	9	35
> 70	—	2	7	4	4	15

Es ist ersichtlich, daß keine Beziehungen zwischen der Erkrankungshäufigkeit und einer bestimmten Altersklasse bestehen.

Die prozentuale Verteilung nach dem Geschlecht der Patienten unter Berücksichtigung der Stadien von Rai geht aus Tabelle 3 hervor.

Tabelle 3

Prozentuale Geschlechtsverteilung von 117 Patienten mit CLL in die Stadien von Rai [49]

	Stadium/Prozent der Patienten				
	0	I	II	III	IV
♂	3.75	16,3	37,5	13,7	28,7
♀	8.1	32,4	29,7	18,9	10,8

Auffallend ist die wesentlich geringere Beteiligung der Frauen am Stadium IV, die anteilmäßig am Stadium I vorherrschen.

Die Zeit zwischen Diagnosestellung und Beginn der Therapie geht aus Abb. 1 hervor. Die Kurven zeigen einen exponentiellen Verlauf. Bei 50% der Patienten wurde innerhalb von 16 Monaten mit der Therapie begonnen. Dabei wurden Patienten im Stadium IV früher behandelt als in den übrigen Stadien.

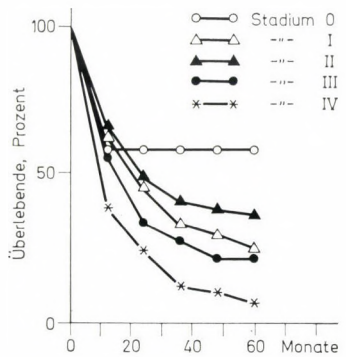

Abb. 1. Zeit zwischen Diagnosestellung und Therapiebeginn bei 117 Patienten mit chronischer lymphatischer Leukämie unter Berücksichtigung der Stadieneinteilung von Rai [49]

Bei 50% der Patienten im Stadium IV wurde innerhalb von 7 Monaten mit der Therapie begonnen, im Stadium III innerhalb von 15 Monaten, im Stadium II innerhalb von 22 Monaten und im Stadium I innerhalb von 19 Monaten. Nur 2 Patienten des Stadiums 0 wurden innerhalb eines Jahres behandelt, die übrigen blieben ohne Medikation.

Der Zeitpunkt für den Beginn der Therapie wurde recht unterschiedlich gewählt. In der Regel wurde erst im Spätstadium der CLL mit einer Therapie be-

gonnen, wenn Beschwerden infolge vergrößerter Lymphknoten und Milz, leukämischer Hautinfiltrate, Anämie, Thrombopenie oder gehäufter Infekte vorlagen.

Für die spezifisch antileukämische Behandlung fanden vor allem Zytostatika Verwendung. Bis 1966 war Cyclophosphamid das am häufigsten verwendete Zytostatikum. In den folgenden Jahren wurde es durch Chlorambucil ersetzt. Prednison als Monotherapie oder kombiniert mit Zytostatika und Röntgenbestrahlung fand ein breites Anwendungsgebiet. Daneben standen allgemein roborierende Maßnahmen und symptomatische Behandlungen der Begleiterscheinungen im Vordergrund.

Verlauf

Die retrospektive Verlaufserhebung ergab, daß 40% der Patienten vor 1965 verstarb. Bei 14 Patienten verlief die CLL über längere Zeit gutartig, bei einem Patienten über 15 Jahre. Interessanterweise ist die Geschlechtsverteilung für diese gutartigen Verläufe 1 : 1, während sie für die Gesamtheit der Patienten bei 2,2 : 1 zugunsten der Männer liegt. Bei der Aufteilung in zwei Dekaden (1955–1965 und 1966–1975) fällt bei den gutartigen Verläufen auf, daß in dem ersten Zeitraum die Geschlechtsverteilung im Verhältnis 3 : 1 zugunsten der Männer, zwischen 1966 und 1975 bei 1 : 6 zugunsten der Frauen liegt. Je 2 Patienten befanden sich im Stadium 0, I und IV, 8 Patienten im Stadium II. Die Hälfte dieser Patienten erhielt keine Therapie.

Verlaufsbetrachtung anhand von Laboratoriumswerten

46 Patienten wiesen einen konstanten Hämoglobinwert auf, davon waren 14 Patienten anämisch. Bei 37 Patienten verschlechterte sich das rote Blutbild im Verlauf der Erkrankung, bei 20 dieser Patienten entwickelte sich eine Anämie mit einem Hämoglobinwert unter 11 g/dl.

Tabelle 4

Prozentuale Überlebenszeiten von 117 Patienten mit chronischer lymphatischer Leukämie (CLL) unter Berücksichtigung der Stadien von Rai [49]

Stadium	Nach		
	2 Jahren %	5 Jahren %	10 Jahren %
0	83	58	–
I	63	30	3
II	60	37	10
III	45	15	–
IV	51	21	5

Bei 32 Patienten blieben die peripheren Thrombozytenzahlen konstant. In 11 Fällen sanken die Thrombozytenwerte zwar ab, jedoch nicht unter 100,000/mm^3. Einundzwanzig Patienten entwickelten eine ausgeprägte Thrombopenie.

Bei 31 Patienten (26,5%) entstand im Verlauf der Erkrankung eine Generalisierung der CLL, im Mittel 22,7 Monate nach Diagnosestellung, jedoch variierte der Zeitpunkt der Generalisation erheblich. Bei einem Patienten lag er bei 112 Monaten.

Die Wechsel der Stadien im Verlauf der CLL gehen aus Tabelle 4 hervor.

28 Patienten wechselten das Stadium während der Erkrankung und zwar durchweg im Sinne eines Fortschreitens der CLL; 6 bzw. 7 Patienten gelangten in das Stadium II bzw. III, während 15 in das Stadium IV übertraten.

Mortalitätsstatistik

Die Abhängigkeit der Überlebensrate vom Alter der Patienten geht aus Abb. 2 hervor. Die relativen 5-Jahresüberlebenszeiten für Patienten, die bei Beginn der Erkrankung unter 50 Jahre alt waren, liegt bei 40%, die 50–60-jährigen überlebten 5 Jahre zu 47%, die 60–70jährigen zu 33%, die über 70-jährigen nur zu 6%.

Die 10-Jahresüberlebensrate betrug für die Patienten unter 50 Jahren 10%, für die 50–60jährigen 15%. Kein Patient, der beim Beginn der Erkrankung über 60 Jahre alt war, lebte zu diesem Zeitpunkt noch.

Die Abhängigkeit der Überlebensrate vom Geschlecht geht aus Abb. 3 hervor. Nach 5 Jahren leben noch 35% der Männer und 42,5% der Frauen, nach 10 Jahren noch 4,9% der Männer und 14,8% der Frauen.

Die Abhängigket der Überlebensrate von der Lymphknotenbeteiligung zeigt Abb. 4. Die 5-Jahresüberlebensrate beträgt bei Patienten ohne Adenomegalie 47%, für diejenigen mit geringer lokaler und stärkerer Lymphombildung

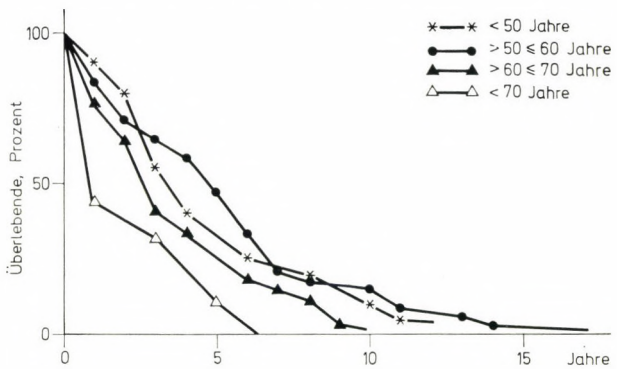

Abb. 2. Überlebenszeit von 117 Patienten mit chronischer lymphatischer Leukämie in Abhängigkeit vom Lebensalter

gleicht annähernd 42 bzw. 43%. Bei generalisierten Lymphomen liegt die Überlebensrate bei 17%.

Eine 10jährige Überlebenszeit erreichten nur 12% der Patienten ohne Lymphome und 10% der Patienten mit nur geringen lokalen Lymphknotenvergrößerungen. Von den beiden anderen Gruppen waren bereits alle Patienten verstorben. Ein Patient ohne Lymphome erreichte 17 Überlebensjahre nach Diagnosestellung.

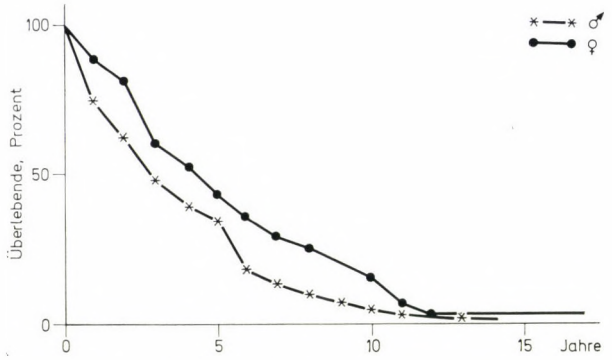

Abb. 3. Überlebenszeit von 117 Patienten mit chronischer lymphatischer Leukämie in Abhängigkeit vom Geschlecht

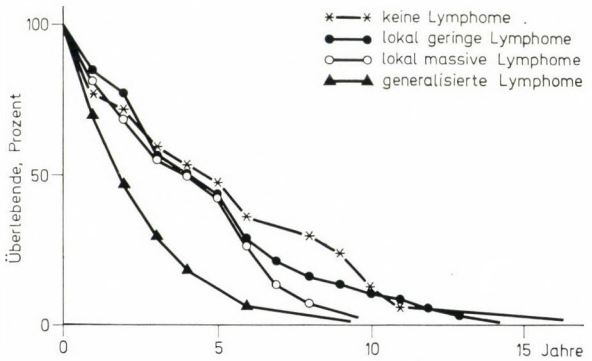

Abb. 4. Überlebenszeit von 117 Patienten mit chronischer lymphatischer Leukämie in Abhängigkeit von der Ausbildung von peripheren Lymphomen

Die Überlebensraten in Abhängigkeit von Organvergrößerungen zeigt Abb. 5. Die 5-Jahresüberlebenszeit betrug für Patienten ohne Organomegalie und für Patienten mit Splenomegalie 41 bzw. 40%. Die Überlebensrate lag aber in den ersten 4 Jahren für Patienten ohne Organvergrößerungen deutlich höher (nach 1 Jahr 89% : 75%; nach 2 Jahren 85% : 69%; nach 3 Jahren 67% : 48%; nach 4 Jahren 55% : 45%). Bei der 5-Jahresgrenze überschnitten sich die beiden Kurven,

die Überlebensrate lag für splenomegale Fälle gegenüber 3% der Patienten ohne Organvergrößerungen.

Die eindeutig schlechteste Überlebenschance hatten die Patienten mit Hepatomegalie: die 5-Jahresüberlebensrate betrug 16,5%. Keiner dieser Patienten erreichte eine 10jährige Überlebenszeit.

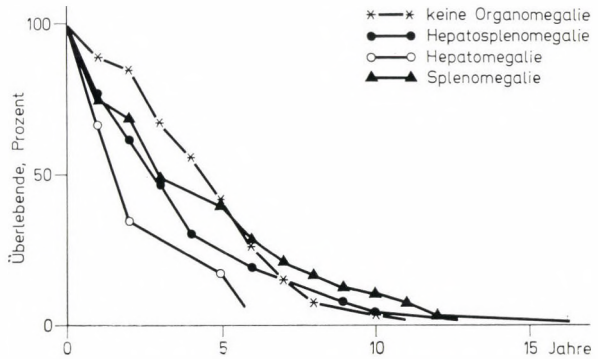

Abb. 5. Überlebenszeit von 117 Patienten mit chronischer lymphatischer Leukämie in Abhängigkeit von Hepatosplenomegalie

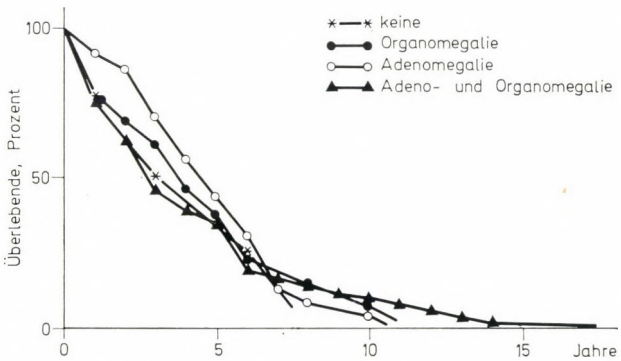

Abb. 6. Überlebenszeit von 117 Patienten mit chronischer lymphatischer Leukämie in Abhängigkeit von Organo- und Adenomegalie

Die Überlebenskurve der Patienten mit Hepatosplenomegalie lag zwischen den Kurven mit isolierter Spleno- bzw. Hepatomegalie; nach 5 Jahren lebten noch 25%, nach 10 Jahren 4%. Patienten mit einer Leberbeteiligung hatten die schlechteste Prognose.

Die Überlebensraten in Abhängigkeit von Adeno- und Organomegalie zeigt Abb. 6.

Die höchsten Überlebensraten in den ersten 5 Jahren hatten Patienten mit reiner Adenomegalie. 43% überlebten 5 Jahre, gefolgt von den Patienten mit

reiner Organomegalie mit einer 5-Jahresüberlebensrate von 38%, während Patienten mit kombinierter Adenoorganomegalie nur zu 35% überlebten.

Bei der 10-Jahresgrenze ergibt sich ein spiegelbildliches Verhalten der Kurven: es überlebten 10% der Patienten mit kombinierter Adenoorganomegalie gegenüber 8% mit Organomegalie und 5% mit Adenomegalie.

Patienten ohne periphere lymphatische Infiltrationen scheinen in den ersten 5 Jahren eine ähnliche Prognose zu besitzen wie Patienten mit Adenoorganomegalie, doch kann bei der geringen Anzahl von 4 Patienten keine allgemein gültige Aussage getroffen werden. Keiner dieser Patienten lebt bis jetzt länger als 7 Jahre nach Diagnosestellung.

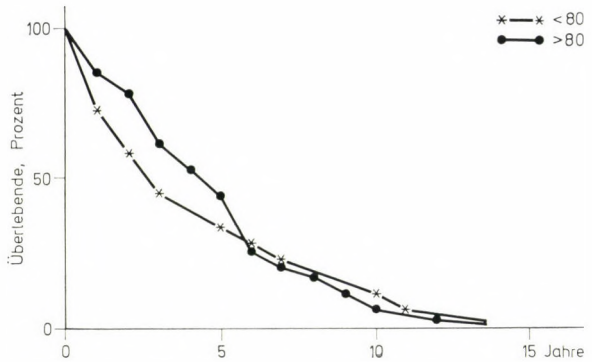

Abb. 7. Überlebenszeit von 117 Patienten mit chronischer lymphatischer Leukämie in Abhängigkeit von der Lymphozyteninfiltration im Knochenmark

Tabelle 5

Wechsel der Stadien by 117 Patienten mit chronischer lymphatischer Leukämie

Stadium 0	Zahl	Stadium I	Zahl	Stadium II	Zahl	Stadium III	Zahl	Stadium IV	Zahl
D	6	W	0	W	0	W	0	W	0
		D	25	W	6	W	2	W	2
				S	6	W	5	W	7
				D	40				
						S	7	W	6
						D	19		
								S	15
								D	27

D = Zeitpunkt der Diagnosestellung
W = Wechsel von Stadium zu Stadium
S = Summe aller Wechsel

Die Überlebensraten in Abhängigkeit von der Knochenmarkinfiltration gehen aus Abb. 7 hervor.

In den ersten 5 Jahren scheinen Patienten mit einer lymphatischen Knochenmarkinfiltration von mehr als 80% eine bessere Prognose zu haben: 45% der Patienten überlebten 5 Jahre gegenüber 34% der Patienten mit weniger als 80% Lymphozyten im Knochenmarkausstrich. Dann allerdings überschneiden sich die Kurven und die Überlebensrate von Patienten mit starker Knochenmarkinfiltration liegt im Mittel 3% unter der Überlebenskurve der Patienten mit geringerer Knochenmarkinfiltration.

Es ist jedoch zu berücksichtigen, daß beide Gruppen unterschiedlich stark vertreten sind und sich somit leicht statistische Verzerrungen ergeben können.

Die Überlebensraten in Abhängigkeit vom Stadium zeigt Tabelle 5. Die schlechteste Prognose weisen Patienten im Stadium III und IV auf.

Tabelle 6

Überlebenszeit in den einzelnen Stadien

0	Zahl	Überlebenszeit	I	Zahl	Überlebenszeit	II	Zahl	Überlebenszeit	III	Zahl	Überlebenszeit	IV	Zahl	Überlebenszeit
D	6	67	W	0	—	W	0	—	W	0	—	W	0	—
		M = 67	D	25	43,6	W	6	23,6	W	2	2	W	2	12
					M = 43,6	S	6	23,6	W	5	6	W	7	16
						D	40	45,8						
								M = 42,3	S	7	4,8	W	6	26,8
									D	19	46,0			
											M = 32,3	S	15	19,8
												D	27	44,8
														M = 34,4

D = Zeitpunkt der Diagnosestellung
W = Wechsel der Stadien
S = Summe aller Wechsel
M = mittlere Überlebenszeit

Die Beantwortung der Frage, wie lange die Patienten beim Wechsel der Stadien jeweils in einem Stadium verharrten, ergibt sich aus der Tabelle 6. Es zeigt sich, daß die mittleren Überlebenszeiten im Stadium III und IV deutlich niedriger sind im Vergleich zu den Stadien 0–II. Die Verweildauer in einem gewechselten Stadium ist häufig sehr kurz, daher beträgt die Überlebenszeit z. B. im Stadium IV bei Patienten, die dieses Stadium erst im Verlauf der CLL erreichten, 19,8 Monate

gegenüber 44,8 Monaten bei Patienten, die sich schon bei der Diagnosestellung im Stadium IV befanden.

Überlebensrate in Abhängigkeit von der Therapie

Die Überlebenszeit der unbehandelten Patienten war in den ersten 8 Jahren um durchschnittlich 7,4% höher als die der behandelten. Die 5-Jahresüberlebensrate lag für die unbehandelten Patienten bei 44%, für die behandelten bei 33%, bei der 10-Jahresgrenze bei 4 bzw. 7%.

Die unbehandelten Patienten wiesen meist sehr gutartige Verläufe auf. Beide Gruppen sind in ihrer Zusammensetzung also nicht direkt vergleichbar.

Da die Therapieform je nach der klinischen Ausprägung der CLL gewählt wurde, sind auch folgende Gruppen nicht direkt miteinander vergleichbar. Wir möchten jedoch nicht auf eine Gegenüberstellung der einzelnen Therapieformen verzichten (Tabelle 7).

Tabelle 7

Mittlere prozentuale Überlebenszeiten von 117 Patienten mit chronischer lymphatischer Leukämie in Abhängigkeit von der Therapie

Therapie	Nach		
	2 Jahren %	5 Jahren %	10 Jahren %
Keine Therapie	81	44	4
Prednison	61	30	4
Zytostatika	77	57	10
Zytostatika/Prednison	59	31	2
Bestrahlung	80	45	10

Lokale Bestrahlungen und Zytostatika als jeweilige Monotherapie ermöglichten also die höchsten Überlebensraten. Sie gleichten der der unbehandelt gebliebenen Patienten, während Prednison als Monotherapie die schlechtesten Ergebnisse aufwies.

Diskussion

Über den Zeitpunkt der klinischen Manifestation der chronischen lymphatischen Leukämie (CLL) finden sich in der Literatur unterschiedliche Angaben. Das Erkrankungsalter in der eigenen Studie liegt zwischen 36 und 85 Jahren bei einem mittleren Lebensalter von 58,6 Jahren. Dieses Ergebnis stimmt mit den Mitteilungen vieler Autoren überein [2, 3, 14, 18, 19, 49, 57, 58]. Die CLL gilt als eine Erkrankung des höheren Lebensalters [2, 15, 20, 21, 24, 25, 26, 31, 58].

Bezüglich der Geschlechtsverteilung haben wir in einer früheren Mitteilung [30] darauf hingewiesen, daß in den letzten Jahren in zunehmendem Ausmaße Frauen an der CLL erkranken.

Die numerische Verteilung der Patienten auf die Stadien von Rai und Mitarb. [49] ließ als häufigste Manifestation die Lymphombildung erkennen. Diese Beobachtung stimmt mit der Mitteilung von mehreren Autoren [6, 47, 49] überein. In einer Studie von Hansen [28] fanden sich die Patienten vorwiegend im Stadium III.

Bei einer Altersverteilung der Patienten auf die Stadien von Rai und Mitarb. ergaben sich in unserer Studie keine Beziehungen zwischen dem Lebensalter und dem Stadium der Erkrankung.

Die von vielen Autoren [6, 29, 44, 45, 49] beobachtete bessere Prognose der CLL bei Frauen ist möglicherweise darauf zurückzuführen, daß Frauen anteilmäßig an den Stadien 0 und I vorherrschen und nur wenige im Stadium IV zu finden sind. Demgegenüber konnten einige Autoren [6, 9, 47] keine geschlechtsspezifischen Unterschiede bezüglich der Prognose nachweisen. Bei einer Analyse der wichtigsten Laborparameter und klinischen Befunde, die in die Stadieneinteilung von Rai und Mitarb. [49] eingehen, ergab sich, daß in einem hohen Prozentsatz eine Progredienz der Erkrankung festzustellen war. Bei einer Analyse der Beziehungen zwischen Lebensalter und Prognose der Erkrankung zeigte sich unter Berücksichtigung der 5-Jahresüberlebenszeit, daß die 50- bis 60jährigen zu 47% lebten, während der Anteil der über 70jährigen nur 6% betrug. Die 10-Jahresgrenze erlebte allerdings keiner der Patienten jenseits des 60. Lebensjahres. Diese Ergebnisse stimmten mit den Mitteilungen von anderen Autoren [4, 6, 29, 44, 45] gut überein. Im Gegensatz dazu fanden Dameshek und Gunz [15] sowie Holmes und Westphal [31], daß sich die Prognose mit zunehmendem Alter bessert.

Es besteht eine Abhängigkeit der Überlebensrate vom Ausmaß der Lymphombildung in dem Sinne, als bei generalisierter Lymphknotenschwellung die Prognose schlechter ist als bei nur mäßiger oder fehlender Ausprägung. Vergleichbare Untersuchungen finden sich bei Phillips und Mitarb. [47] sowie Hansen [28]. Die Autoren konstatieren mit zunehmender Lymphknotenschwellung eine Verschlechterung der Prognose. Von Bogges und Mitarb. [6] sowie Dameshek [14] stammt der Hinweis, daß sich bei Befall der Tonsillen eine sehr schlechte Prognose ergibt. Eine Manifestation an den Tonsillen konnten wir bei unseren Patienten nicht nachweisen.

Bei der Frage nach Beziehungen zwischen Befall von Leber und Milz und Prognose der Erkrankung fanden wir bei Patienten mit und ohne Organbefall die gleiche 5-Jahresüberlebenszeit. In den ersten vier Jahren ergaben sich jedoch deutliche Unterschiede in der Weise als eine Organmanifestation mit einer schlechteren Prognose vergesellschaftet war.

Bei isoliertem Leberbefall fanden wir die schlechteste Prognose. Phillips und Mitarb. [47] berichteten über ähnliche Ergebnisse. Die prognostische Bedeutung der Splenomegalie wird von anderen Autoren [4, 15, 29, 32] unterschiedlich

beurteilt. Darüber hinaus wurde die Lebervergrößerung nicht regelmäßig berücksichtigt. Nach Untersuchungen von Coeur und Mitarb. [9, 10] ergab sich kein Unterschied hinsichtlich der Überlebenszeit bei Patienten mit und ohne Hepatomegalie. Zwischen der lymphozytären Infiltration im Knochenmark und der Prognose der Erkrankung bestanden nach unseren Untersuchungen in Übereinstimmung mit Phillips und Mitarb. [47] keine Beziehungen. Demgegenüber stellten verschiedene Autoren [8, 10, 22, 23, 46, 47] heraus, daß eine vermehrte Lymphozyteninfiltration im Knochenmark mit einer schlechteren Prognose vergesellschaftet ist.

Bei der Analyse der Überlebensraten in Abhängigkeit vom Stadium der Erkrankung bestätigen wir die Beobachtungen von Rai und Mitarb. [49] sowie Phillips und Mitarb. [47], daß mit zunehmender Schwere des Stadiums die Überlebenszeit sinkt. Auch wir fanden keinen Unterschied zwischen dem Stadium III und IV. Im Stadium IV ist eine gleichzeitig bestehende Anämie mit einer schlechteren Prognose belastet als eine alleinige Thrombopenie. Die Frage, ob diese Beobachtung dazu berechtigt, das Stadium IV neu zu definieren im Sinne einer Thrombopenie mit Anämie erscheint berechtigt, wenn man die 5- und 10-Jahresüberlebenszeiten berücksichtigt, die bei Patienten mit Anämie deutlich niedriger liegen als bei Patienten mit Thrombopenie.

Es erschien uns von Bedeutung, der Frage nachzugehen, wie sich ein Wechsel des Stadiums auf die Prognose der Erkrankung auswirkt. Aus unserer Studie ergab sich zunächst, daß sich eine Änderung des Stadiums immer im Sinne einer Progredienz der Erkrankung manifestiert. Darüber hinaus verschlechterte sich die Prognose zunehmend, wenn infolge Fortschreitens der Erkrankung ein höheres Stadium erreicht wurde.

Die Frage, welchen Einfluß die Therapie auf die CLL ausübt, ist unter Berücksichtigung der verschiedenen Medikationen und des unterschiedlichen Therapiebeginns nicht sicher zu beantworten. Von Wichtigkeit erscheint uns jedoch die Feststellung, daß bei alleiniger Monotherapie mit Corticosteroiden die schlechtesten Überlebenszeiten nachzuweisen waren, während die Patienten, die mittels lokaler Strahlentherapie behandelt wurden, die beste Prognose aufwiesen. Untersuchungen von Phillips und Mitarb. [47] bestätigten unsere Beobachtung, daß unbehandelte CLL-Patienten länger leben als behandelte Fälle. Hier ist jedoch das leichtere Stadium der Erkrankung zu berücksichtigen. Die Autoren fanden jedoch keinen Unterschied zwischen den einzelnen Therapieformen und der Prognose der Erkrankung. Untersuchungen von Osgood und Mitarb. [43, 44] haben demgegenüber ergeben, daß Patienten, die strahlentherapeutisch behandelt wurden, die beste Prognose aufwiesen.

Die Frage, ob weitere Faktoren, wie eine Verminderung der Gammaglobuline, die Prognose wesentlich beeinflussen, wird in der Literatur unterschiedlich beurteilt. So fand Hansen [28] keinen signifikanten Unterschied zwischen Patienten mit erniedrigten und normalen Gammaglobulinen. Demgegenüber stellten Bernadou und Mitarb. [4] fest, daß sich mehr Patienten mit erniedrigten Gammaglobulinspiegeln unter den Kurzlebenden fanden als unter den Langlebenden.

Die Frage nach der Bedeutung des ^{3}H-Thymidinindexes im peripheren Blut [40, 53, 54, 55, 56, 61] und den Lymphknoten [11, 12, 13] für die Prognose der Erkrankung ist noch nicht geklärt. Auch immunologische Untersuchungen zur Identifizierung der Lymphozytensubpopulation haben bislang noch keinen entscheidenden Beitrag zum Wohle der Patienten erbracht [7, 16, 36, 43, 48, 50, 52, 60].

Die Forderung nach neuen Behandlungsformen der CLL ergab sich für viele Arbeitsgruppen. Die Frage, ob eine aggressive Chemotherapie [1, 3, 17, 27, 28, 37, 39, 53, 54, 59] oder eine Strahlentherapie [34, 35, 37, 41, 42] eine entscheidende Besserung der Prognose bringen wird, muß anhand von kontrollierten Studien analysiert werden.

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Prognosis of Chronic Lymphatic Leukaemia

In a retrospective study 117 patients with chronic lymphatic leukaemia (CLL) were classified according to Rai and analyzed with regard to their survival time. With increasing severity of the stage of the disease the prognosis becomes worse. A change of the stage was always in the sense of progredience of CLL. Untreated patients had the longest survival time. Among the various forms of treatment, monotherapy with corticosteroids had the worst, local irradiation therapy the best prognosis.

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Chronische lymphatische Leukämie und maligne Tumoren

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Von 117 Patienten mit chronischer lymphatischer Leukämie (CLL) wiesen 10 maligne Tumoren auf. In 2 Fällen wurde ein Doppelkarzinom nachgewiesen. Die Karzinomhäufigkeit stieg mit zunehmendem Schweregrad der CLL klassifiziert nach Rai und Mitarb. Die eigenen Beobachtungen wurden mit den Mitteilungen in der Literatur verglichen.

Das gemeinsame Vorkommen von Leukämie und malignen Tumoren ist so lange bekannt wie die Leukämie selbst als eine Krankheitseinheit angesehen wird. Whipham [25] stellte im Jahre 1878 als erster die Frage, ob Beziehungen zwischen dem Vorkommen einer Leukämie und der Manifestation eines Karzinomleidens bestehen.

In einer eigenen Studie untersuchten wir die Beziehungen zwischen der Schwere der CLL und dem Auftreten von Malignomen.

Patientengut und Methodik

Zur Auswertung gelangten 117 Patienten mit gesicherter CLL, die in den Jahren 1955 bis 1975 in der Medizinischen Klinik und Poliklinik der Universität Münster behandelt worden sind.* Es handelt sich um 37 Frauen und 80 Männer. Das Durchschnittsalter der Patienten zum Zeitpunkt der Diagnose CLL beträgt 58 Jahre. Es wurden die weiteren Krankheitsverläufe ausgewertet und die Patienten mit histologisch gesicherten malignen Tumoren herausgesucht. Die CLL wurde entsprechend dem Vorschlag von Rai und Mitarb. [20] klassifiziert.

Ergebnisse

Wir fanden bei 10 Patienten mit CLL Malignome verschiedener Organe. Es handelt sich dabei um 3 Frauen und 7 Männer, deren Lebensalter zum Zeitpunkt der Diagnose der CLL und des malignen Tumors sowie dessen Art und Lokalisation aus Tabelle 1 hervorgehen.

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Das mittlere Lebensalter zum Zeitpunkt der Diagnose CLL beträgt bei den Patienten 64 Jahre und liegt somit um 6 Jahre höher als beim Gesamtkollektiv der CLL-Patienten. Zum Zeitpunkt der Diagnose des Malignoms betrug das durchschnittliche Lebensalter 66,4 Jahre. In 4 Fällen wurde die Diagnose CLL und maligner Tumor anläßlich des ersten stationären Aufenthaltes gestellt. Bei den übrigen 6 Patienten betrug das zeitliche Intervall bis zur Diagnose des Tumors 1 bis 11 Jahre.

Am häufigsten wurde ein Bronchialkarzinom nachgewiesen. Die übrigen aufgeführten Karzinome kamen in Einzelfällen vor. Bei 2 Patienten wurden Doppelkarzinome beobachtet. In einem Falle ergab sich die Diagnose des Bronchialkarzinoms erst bei der Sektion. Röntgenologisch wurden die bestehenden

Tabelle 1

Vorkommen von malignen Tumoren bei 10 Patienten mit chronischer lymphatischer Leukämie (CLL)

Lfd. Nr.	Patient	Geschlecht	Lebensalter (Jahre) zum Zeitpunkt der Diagnose		Art und Lokalisation des Tumors
			der CLL	des Tumors	
1.	K. K.	♂	56	67	Bronchial-Ca Sigma-Ca
2.	L. A.	♂	58	58	Bronchial-Ca
3.	K. J.	♂	59	59	Lesh-Zellen-Ca
4.	Z. G	♀	59	61	Schilddrüsen-Ca Colon-Ca
5.	D. A.	♀	60	60	Rektum-Ca
6.	B. A.	♂	60	64	Bronchial-Ca
7.	F. A.	♀	64	67	Melanom
8.	H. F.	♂	69	70	Bronchial-Ca
9.	F. A.	♂	71	74	Prostata-Ca
10.	A. F.	♂	84	84	Osteosarkom

Hilusverdichtungen als Lymphome im Rahmen der CLL gedeutet, die sich autoptisch als Metastasen des Bronchialkarzinoms erwiesen. Im übrigen bestanden histologisch die Zeichen der CLL.

Berücksichtigt man die Stadieneinteilung der CLL entsprechend dem Vorschlag von Rai und Mitarb. [20], so findet sich je ein Fall in den Stadien 0 bis II, 3 Fälle im Stadium III und 4 Fälle im Stadium IV. Die Patienten mit Doppelkarzinomen fanden sich in den Stadien 0 sowie IV. Von den insgesamt 10 Fällen wurden 5 behandelt, 5 blieben bis zur Ca-Diagnose ohne spezifische Therapie. Bei einem Patienten mit Prostatakarzinom wurden nach der Operation extrakorporale Blutbestrahlungen durchgeführt. Im übrigen wurde bei 2 Patienten allein Prednison verabreicht sowie in einem Falle Cyclophosphamid in Kombination mit Prednison.

In 2 Fällen wurde eine lokale Strahlentherapie durchgeführt bei zusätzlichen Gaben von Prednison. Bei einem Patienten trat die CLL in den Hintergrund, während sich ein Bronchialkarzinom entwickelte.

Bei dem 60jährigen Patienten (Nr. 6, B. A.) wurde im Jahre 1969 die Diagnose CLL gestellt. Es bestanden damals Lymphknotenschwellungen sowie eine Hepatosplenomegalie. Im peripheren Blut fanden sich 16,000 Leukozyten/μl bei 74% kleinen Lymphozyten im Differentialausstrich. Anhand des Sternalpunktates wurde die Diagnose CLL gesichert. Wegen des guten Allgemeinbefindens wurde von einer Therapie Abstand genommen. Im Jahre 1972 mußte der Patient wegen eines totalen av-Blocks zur Schrittmacherimplantation stationär aufgenommen werden. Bei der klinischen Untersuchung waren Leber und Milz nicht palpabel. Die Leukozytenzahl betrug 4500/μl bei normaler Verteilung im Differentialausstrich.

Ein Jahr später kam der Patient erneut zur stationären Aufnahme. Jetzt wurde die Diagnose eines Bronchialkarzinoms mit Hilfe einer bronchoskopisch gewonnenen Probeexzision histologisch gesichert. Der Patient wurde mit ^{60}Co-Bestrahlungen behandelt. Darunter war es zu einem weiteren Abfall der Leukozytenzahl auf 3000/μl gekommen. Der Patient verstarb an den Folgen einer kardiorespiratorischen Insuffizienz.

Bei einem 56jährigen Patienten wurde im Jahre 1960 die Diagnose CLL gestellt. Es bestand eine Splenomegalie ohne Lymphknotenschwellungen. Im Blutbild fanden sich 42,000 Leukozyten per μl bei 90% Lymphozyten im Differentialausstrich. Im Sternalpunktat wurde die Diagnose der CLL bestätigt. Wegen des guten Allgemeinbefindens wurde von einer spezifischen Therapie der CLL Abstand genommen. Der Patient fühlte sich beschwerdefrei und wurde 11 Jahre später in einem schlechten Allgemeinbefinden erneut stationär aufgenommen. Es bestanden nunmehr eine Anämie mit einem Hb-Wert von 9 g/dl, eine Leukozytose von 62,000/μl bei 90% Lymphozyten im Differentialausstrich.

Röntgenologisch fand sich eine knollige Verdichtung des oberen Mediastinums. Trotz intensiver Maßnahmen einschließlich lokaler Strahlentherapie verstarb der Patient an den Folgen einer kardiorespiratorischen Insuffizienz. Bei der Sektion fand sich ein undifferenziertes Bronchialkarzinom mit Stenosierung der Hauptbronchien und Infiltration des Herzbeutels sowie der großen Gefäße. Als weiterer Befund wurde ein Adenokarzinom des Sigmas nachgewiesen.

Diskussion

Über die Häufigkeit von malignen Tumoren bei Patienten mit CLL finden sich in der Literatur unterschiedliche Angaben. Hansen [10] stellte eine Übersicht über die wichtigsten Publikationen zusammen, die über die Häufigkeit von malignen Tumoren bei CLL berichten (Tabelle 2).

Die prozentualen Angaben schwanken zwischen 2,5 [8] und 34,4% [12]. Der von uns gefundene Anteil von 8,5% an malignen Tumoren stellt einen re-

Tabelle 2

Gleichzeitiges Vorkommen von chronischer lymphatischer Leukämie und malignen Tumoren in verschiedenen Publikationen

Autoren			Zahl d. Häufigkeit malig. Pat. m./solider Tumoren		
			CLL	Anzahl d. Pat.	%
Schreiner u. Wehr	[21]	(1934)	38	4	10,5
Osgood u. Seaman	[18]	(1952)	102	10	9,8
Beresford	[2]	(1952)	106	20	18,9
Klima u. Herzog	[14]	(1956)	201	16	8,0
Scott	[22]	(1957)	212	10	4,7
Pisciotta u. Hirschboeck	[19]	(1957)	86	11	12,8
Moertel u. Hagedorn	[17]	(1957)	739	31	4,2
Gross u. Mitarb.	[8]	(1958)	255	10	2,5
Lawrence u. Donald	[16]	(1959)	162	14	8,6
Förster u. Reimer	[7]	(1959)	160	9	5,6
Faber u. Borum	[6]	(1962)	760	31	4,1
Helbig u. Voigt	[11]	(1963)	120	7	5,8
Bousser u. Mitarb.	[5]	(1964)	253	17	6,7
Gunz u. Angus	[9]	(1965)	86	19	22,1
Stacher u. Böhnel	[23]	(1966)	285	11	3,9
Berg	[3]	(1967)	381	19	5,0
Hyman	[12]	(1969)	209	72	34,4
Stavraky u. Mitarb.	[24]	(1970)	258	13	5,0
Hansen	[10]	(1973)	189	31	16,4
Heilmann u. Schreck		(1977)	117	10	8,5

präsentativen Mittelwert dar. Unter den bei CLL beobachteten Tumoren wird den Hauttumoren im nordischen Schrifttum eine besondere Rolle beigemessen [1, 10]. Bei unseren Patienten wurden keine Hauttumoren beobachtet.

Auffällig ist in unserem Patientengut die Häufigkeit der Bronchialkarzinome.

Hyman [12] weist auf das häufige Vorkommen von Kolonkarzinomen bei CLL-Patienten hin. Als Ursache für die gehäufte Karzinomentstehung bei Patienten mit CLL wird der Immundefekt bei dieser Erkrankung angeschuldigt.

Stacher und Böhnel [23] diskutieren eine leukämoide Reaktion bei Patienten mit Karzinomen, so daß eine CLL vorgetäuscht werden kann. Bichel [4] teilte 2 Fälle mit, bei denen im Verlaufe eines sich entwickelnden Magenkarzinoms die CLL völlig verschwand. Beide Patienten bekamen jedoch Strahlentherapie. Der von uns beschriebene Fall einer Rückbildung der CLL unter der Manifestation eines Bronchialkarzinoms wies bereits vor der Strahlentherapie ein normales Blutbild auf. Die Diskussion über die Ursachen der nachgewiesenen Häufigkeit von Malignomen bei CLL-Patienten ist noch nicht abgeschlossen. Vielleicht führen die nunmehr möglichen immunologischen Untersuchungen zu einer besseren Erkenntnis.

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Chronic Lymphatic Leukaemia and Malignant Tumours

Ten of 117 patients with chronic lymphatic leukaemia (CLL) showed malignant tumours. In 2 cases a double carcinoma was diagnosed. The frequency of carcinoma increased with the progredience of the stage of disease classified according to Rai et al. The observations of the authors are compared with data published in the literature.

In Memoriam

JACQUES ROSKAM
1890 – 1977

The scientific community of coagulationists has lost with Jacques Roskam one of its best-known pioneers. Roskam became interested in biology through Edouard Van Beneden and worked in the Physiological Laboratory of Léon Frédéricq from the very beginning of his medical studies. He was promoted to M.D. in 1914 and was soon afterwards drafted for military service. At this time he became prisoner of war near Lüttich, but he escaped and joined again the Belgian forces. During the First World War he had the opportunity to work as a pathologist with Prof. Nolf. Here he had the first contact with research on haemostasis and blood platelets.

At this point in time Roskam was already an excellent organizer. He built a hospital for infectious diseases in Doorntje Houthem and was decorated by the American Red Cross for his merits.

In 1923 he submitted his thesis at the University of Liège, where in 1926 he became Chargé de cours, in 1929 extraordinary and in 1933 full professor of pathology and special therapy of internal diseases. In 1960 he became emeritus, but he continued to work in his speciality. His last paper on haemostasis was published in 1963.

At the age of 40 he was elected corresponding member of the Academie Royale de Médecine de Belgique and became full member in 1945; in 1958 he gained presidency of the Academy.

His scientific interest was centred in the study of haemostasis and of the factors involved in the formation of the haemostatic plug, especially in the platelets. He described the morphological details leading to the formation of the haemostatic plug: adhesion, aggregation and viscous metamorphosis of the platelets. He was the first to postulate the adsorption of plasmatic clotting factors to the surface of the platelets, calling this the *atmosphère plasmatique* of the platelet, a conception which has been more and more acknowledged recently. He realized the importance of the vascular wall for the formation of the haemostatic plug, and he and his coworker Bounameaux were the first to study the adhesion of platelets to collagen and the induction of aggregation of platelets by collagen. This observation makes him the father of some important research on platelet function during the last decade. He realized also the similarities between haemo-

static plug and arterial thrombosis. Much of his work was devoted to the understanding and standardization of the bleeding time method. From a practical point of view he studied the influence of drugs on haemostasis.

He devoted important research also to the effect of salicylate on the hypothalamus, the secretion of ACTH and its antiinflammatory action.

Roskam was always intimately connected with his University as an academic teacher and in the desire to improve both teaching methods and the medical curriculum.

Roskam was a man with a special, clear, scientific spirit. He worked with precision and was full of the desire to solve scientific problems. Outside of his scientific work he was very much interested in the fine arts such as painting, music and literature. He especially liked the Spanish painters.

We lost in Roskam one of the most important contributors to our knowledge of haemostasis. His work will continue to stimulate and grow along with our future research in this field.

E. Deutsch
Vienna

Book Reviews

Immunology of Receptors. Ed. by B. Cinader. Immunology Series Vol. 6. Marcel Dekker Inc., New York and Basel 1977. 524 pages, 34 tables, 32 figures. Price: SFr 175.—

The book contains 28 special contributions on the immunology of receptors, one of the central themes of modern biology, and a brilliant introductory chapter written by the Editor.

The first chapters deal with the physicochemical aspects of receptor-ligand interactions (DeLisi and Metzger) and with the changes in membrane behaviour as a consequence of ligand-binding (Bornens, Karsenti and Avrameas; Freedman and Gelfand). The changes in triggering ligand following the interactions with the receptor site may result in binding of the altered macromolecule to another receptor site. The biological effect of cholera toxin is a good model for this type of receptor-ligand interaction (Craig and Cuatrecasas; Holmgren and Lindholm). The chemical characterization of cell receptors is an important step to the understanding of their function. Several excellent chapters are devoted to the structure and function of Fc receptors of different cells, to their relationship to other membrane structures, to their binding to Ig determinants, etc., calling attention to their importance in the regulation of B cell function and to their influence on T helper function (e.g. chapters by Dorrington; Halloran and Schirrmacher; Ryan and Henkart; Paraskevas, Lee and Orr). The term "acceptor" refers to a receptor site with which T cell factors specifically interact. The factor-acceptor model is one of the central items for understanding the different forms of cellular interactions in immune phenomena. Tada, Taniguchi and Takemori review the nature and role of acceptor sites on both B and T cells for different T cell factors with respect to the induction and regulation of the immune response. The problem whether IgD is a lymphocyte receptor and the possible relationships between the different steps of Ig expression as well as the developmental stages of B lymphocytes are discussed by Pernis and Forni. Different aspects of the regulation of immune response are also analyzed in several chapters (e.g. Lake and Mitchison; Adler). Since receptors appear to relay similar signals whether they are occupied by their natural ligands or by antibodies to receptors, auto-antibodies directed against receptors interfere with access of, or responsiveness to, the triggering molecules (e.g. hormones). Cells triggered by such antibodies may be turned off by continuous stimulation. The role of antibodies against acetylcholine, insulin, and thyrotropin receptors and their possible role in autoimmune diseases are discussed in the second part of the work (Lennon; Smith; Flier, Kahn, Jarrett and Roth).

This excellent monograph offers insight into the current thoughts in a rapidly developing area of research. It is recommended to both clinicians and experimentalists who are interested in immunology, biochemistry, molecular biology, genetics, and virology.

J. Gergely

Dyserythropoiesis. Ed. by S. M. Lewis, R. L. Verwilghen. Academic Press, London—New York—San Francisco 1977. 350 pages, 167 illustrations. Price: £ 11.80.

Dyserythropoiesis is a collective term designating diseases of various aetiology in

which anaemia occurs. This was one of the subjects discussed at a symposium held in Belgium in 1976 on which the volume is based.

The first chapter discusses definitions, and diagnostics based on clinical, biochemical and morphological characteristics. Dyserythropoiesis is divided into primary and secondary forms; the latter involves diseases of known and unknown pathogenesis. Chapter 2 discusses normal erythropoiesis and disorders occurring in other diseases. The other chapters deal in detail with the single disorders (congenital dyserythropoietic anaemia type I, II and III, other congenital dyserythropoietic anaemias, megaloblastic anaemia, thalassaemia, myelodysplastic syndrome). The part discussing pathophysiology deals with the role of red blood cell antigens, erythroid cell membrane, red blood cell enzyme abnormalities and foetal haemoglobin production.

The last three chapters discuss ultrastructural details. Chapter 19 describes the ultrastructure of normal and disturbed erythropoiesis with 93 illustrations (some of which are not the best in quality). Chapter 20 (Experimental Dyserythropoiesis) discusses the effect of certain compounds (phenylhydrazine, lead, thiamphenicol) on the erythroid precursors. The last chapter studies the pathogenetic mechanisms involved in dyserythropoiesis from the ultrastructural aspect.

The book is well-edited, the chapters although concise are easy to follow, the topics are discussed from several aspects. Each chapter is followed by a carefully compiled list of references. The illustrations are generally good. This volume is recommended to all workers in the field who are interested in obtaining new information.

Gy. Lelkes

The Immunopathology of Lymphoreticular Neoplasms. Ed. by J. J. Twomey, R. A. Good. *Comprehensive Immunology*. Vol. 4. Plenum Press, New York—London 1978. 763 pages. Price: $ 54.—

The fourth to appear in the "Comprehensive Immunology" series, this volume is both topical and timely. The fast development in the field of immunology had a considerable impact on the understanding of pathological processes in the lymphoid malignancies. About a quarter of the book is focussed on the theoretical background of lymphoproliferative diseases. There is a concise introduction on the development of lymphoid tissues and the regulation of the immune response. Since the book contains few informations after 1976, some of the new findings that added to our understanding of the cellular interactions in the immune response are necessarily lacking from the volume.

There has been a considerable amount of excitement during the past ten years about the epidemiological findings in lymphoreticular neoplasia as well as about the association of immunodeficiency with these tumours. It is clear from the appropriate chapters in this book that these associations, although theoretically most relevant, need to be further investigated. Histological classification is based on the Lukes—Parker studies which aim at combining the morphological and immunological approach. There is an excellent summary by Siegal on the cytoidentity of lymphoid tumours.

Discussion of the various clinical entities is split into primarily B-cell or T-cell malignancies, including an enlightening chapter on the association of B-cell proliferations with EBV infection.

The last section of the book contains chapters on various paediatric aspects of lymphomas. The classically difficult distinction between leukaemia and lymphoma, however, would deserve a more lengthy discussion.

The greatest merit of the book is undoubtedly the fact that some of the best "authorities" on the particular aspects were asked to contribute. Rather than theorizing about the newest findings, the volume gives good, solid information and is recommended first of all to clinicians interested in the field.

T. Révész

Thrombosis, Animal and Clinical Models. Ed. by H. J. Day, A. B. Molony, E. E. Nishizawa, R. H. Rynbrandt. *Advances in Experimental Medicine and Biology.* Vol. 102. Plenum Press, New York—London 1978. 337 pages. Price: $ 42.00

The volume reviews the Brook Lodge Conference held in October 1977. Twenty papers discuss the mechanism of thrombosis, the possibilities of diagnosing susceptibility to thrombosis and the problems of prevention. Many factors are involved in thrombogenesis, therefore the problem is extremely complex. In recent years much knowledge has accumulated regarding early stage of thrombogenesis, the interaction of thrombocytes and blood vessels, and the mechanism of atherosclerosis. The authors review and critically analyse present-day knowledge on the subject, and give a general description of the process, followed by detailed information.

The trigger activating the components of haemostasis is the injury of the endothelium. Factors which may induce endothelial injury in healthy individuals, in various diseases and in animal experiments are discussed in detail.

Besides the rheological conditions and the role of lipids, the Conference discussed platelet reactions playing a decisive role in thrombogenesis. The increased adhesivity and increased sensitivity to aggregation inducers, the role of released biological substances in atherogenesis and in thrombogenesis, drugs inhibiting platelet reactions and problems related to prevention of thrombosis were the subjects of discussions. The papers involved with the possibilities of detecting thrombosis are of special interest for diagnosticians. The critical analyses of newly introduced laboratory methods such as increased sensitivity to aggregation inducers of platelets, the detection of circulating thrombocyte aggregates, the detection of the "light" thrombocytes, which have undergone a release reaction, detection of proteins indicating release reactions (e.g. of β-thromboglobulin and platelet factor 4), give the reader the impression that these only indicate processes which are already over and associated reactions, but are not suitable for prediction of thrombosis or for detecting predisposing conditions.

The volume is of interest to clinicians, laboratory and research workers involved in problems of haemostasis and thrombosis.

*Susan Előd*i

The HLA System. British Medical Bulletin. Vol. 34. No. 3. The British Council, 65 Davies St., London 1978. 324 pages. Price: £ 5.—

As W. F. Bodmer says in his introductory paper, the aim of this issue is to provide a general review of the HLA system in all its various aspects, but emphasizing in particular the clinical applications.

V. C. Joysey and E. Wolf deal with the definition of the HLA-A, B and C antigens and consider the problems associated with serum supply and with cross-reactions between the antigens. The specificities detected by the T cells and the consequent reactions are discussed by B. A. Bradley and H. Festenstein. Then J. G. Bodmer describes techniques for identifying Ia antigens in lymphatic leukaemia cells, and discusses the relationship of HLA-DRw antigens to the HLA-D locus and other antigens. C. J. Barnstable, E. A. Jones and M. J. Crumpton discuss the isolation of HLA-A, B, C and DRw antigens, the derivation of their structure and the genetic control of antigen expression. Three types of genetic variation have been described for complement components; these are reviewed by P. J. Lachmann and W. J. Horbst, who discuss component loci, complement evolution and the consequences of complement deficiency in some human diseases. Subsequently A. Munro and H. Waldmann assess current thinking of MHS function in relation to T-cell function, in particular to the T-cell factor, the generation of T-cell receptor diversity, and the interactions of various T-cell effector functions.

Most data on the influence of matching donor and recipient antigens of the HLA system on the outcome of tissue allografts derive from work on renal and corneal transplantation; P. J. Morris, J. R. Batchelor and H. Festenstein confine their paper mainly to those fields of research. A. Ting, K. A.

Williams and P. J. Morris deal with studies on renal transplants and conclude that more studies are necessary to determine which assays are the most important for donor-recipient selection, and for predicting rejection. H. M. Dick, in an introductory paper on the relationship between HLA and diseases, touches on alternative mechanisms of association and discusses the problems that arise in designing relevant studies. J. A. Sachs and D. A. Brewerton suggest that the association in some populations indicates a disease-susceptibility gene very closely linked to the HLA-B locus. Still, familial incidence and the manifestations of the disease cannot be explained solely by the presence of such a gene, and a multi-gene basis needs to be postulated. The significance of the association between HLA and multiple sclerosis is discussed by J. R. Batchelor, A. Compston and W. I. McDonald. Thereafter, A. G. Cudworth and H. Festenstein discuss the evidence provided by population and family studies for the hypothesis that HLA-linked immune response genes control susceptibility to diabetes, pointing out that the major susceptibility to insulin-dependent diabetes is conferred by genes in the HLA chromosomal region. This observation has provided the basis for a new classification of the disease. The last paper, by W. F. Bodmer and J. G. Bodmer, summarizes the data on HLA variation in the human population. They try to explain the polymorphism and disease associations in terms of known mechanisms and HLA system functions and consider the evolution of the HLA region and the functional significance of such a complex cluster of genes. They conclude that the HLA system provides a fine example of the interaction between basic biological research and the more practical needs of the clinician.

This volume of the British Medical Bulletin besides being an excellent survey of the HLA-system, includes also the latest knowledge on the subject. It should be of prominent interest for theoreticians and clinicians. It provides important and most outstanding information for researchers working in the field of biology, biochemistry, immunology and genetics, for general practitioners and clinicians with special regard to internists, haematologists, paediatricians and dermatologists.

G. Gy. Petrányi

Abstracts

Stoichiometric and site characteristics of the binding of iron to human transferrin. P. Aisen, A. Leibman, J. Zweier (Department of Biophysics and Medicine, Albert Einstein College of Medicine, Bronx, New York). *J. biol. Chem. 253*, 1930 (1978).

Stoichiometric or thermodynamic equilibrium constants for the binding of Fe^{3+} to transferrin were evaluated by the method of equilibrium dialysis using citrate as a competing complexing agent, near pH 6.7 and near pH 7.4. In each case K_1 was substantially greater than K_2, the effect being more marked at lower pH. These overall stability constants for the binding of iron, which take account of the participation of bicarbonate and protons in the reactions of binding, decrease with increasing pH. This may reflect the influence of the protein's negative charge, which increases with pH, on the critical role of the anion in metal binding. However, at any pH and pCO_2 apparent stability constants may be defined, and these increase with increasing pH as a result of the inverse dependence of iron binding on the fourth power of the hydrogen ion concentration. At pH 7.4 and atmospheric pCO_2, the apparent stability constants K_1' and K_2' are $4.7\times 10^{20}\ M^{-1}$ and $2.4\times 10^{19}\ M^{-1}$, respectively.

Using the urea gel electrophoresis method of Makey and Seal, relative concentrations of the two species of monoferric transferrin were measured in each preparation at equilibrium. This made it possible to estimate the four intrinsic site constants for the binding of iron to transferrin. A slight negative cooperativity was evident in the binding of iron to each site.

Although one site, designated the a-site is more strongly binding than the b-site, it is not necessarily more accessible to all complexes of iron. Thus, iron as ferric citrate, ferric oxalate, ferrous ammonium sulfate and ferric chloride preferentially occupy the b-site when presented to transferrin under conditions in which an equilibrium distribution of iron between the binding sites of the protein would not be expected. Iron as ferric nitrilotriacetate, however, is directed toward the a-site. This is also the site which retains iron at low pH.

On the basis of these observations single site transferrins were prepared, and a difference in their EPR spectra was demonstrated under near physiologic conditions.

A. Egyed

Binding sites of iron transferrin on rat reticulocytes. Inhibition by specific antibodies. C. van der Heul, M. J. Kroos, H. G. van Eijk (Department of Internal Medicine I and Chemical Pathology, Medical Faculty, Erasmus University, Rotterdam, The Netherlands). *Biochim. biophys. Acta (Amst.) 511*, 430 (1978).

In the process of iron uptake by precursors of the erythrocytes probably more than one membrane component is involved; besides the specific transferrin receptor, another membrane component with a high iron activity after incubation with ^{59}Fe can be isolated. A striking resemblance exists between rat and human reticulocyte components which are involved in the process of iron uptake. Incubation of reticulocytes

with F_{ab} fragments of an antibody against the membrane receptor for transferrin causes a concentration-dependent decrease in transferrin binding and iron uptake. The membrane receptor complex isolated is still heterogeneous; analytical ultracentrifugation studies suggest a molecular weight lower than 230 000. Intact immature red cells are necessary for specific binding of transferrin with the receptor followed by iron uptake. This is the only mechanism for iron uptake. Immunofluorescence studies showed that the receptor for transferrin is localized at the outside of the cell membrane.

A. Egyed

Chemical modification of the arginines in transferrins. T. B. Rogers, T. Børresen, R. E. Feeney (Department of Food Science and Technology, University of California, Davis, California). *Biochemistry 17*, 1105 (1978).

Phenylglyoxal, 2,3-butanedione, and 1,2-cyclohexanedione were used to modify the arginines in ovotransferrin and human serum transferrin. As much as 88% of the arginines of iron-free ovotransferrin was modified with a 7.5-fold excess of 2,3-butanedione in 200 mM borate. The iron-binding activity decreased to 50% in this reaction. The rate and extent of arginine modification and inactivation of the protein were directly related to the concentration of borate in solution. When borate was removed from the modified protein solution, the iron-binding activity increased from 50 to 80% and some of the arginine was regenerated. Cyclohexanedione also inactivated the protein in borate buffer but was not used further due to apparent side reaction. When iron-free ovotransferrin or human serum transferrin was modified with a 7.5-fold molar excess of phenylglyoxal, as compared to arginines, in bicarbonate buffer, a pseudo-first-order loss in arginine and activity was observed. There was no significant modification of arginines or inactivation of the protein when the phenylglyoxal reaction was done in borate buffer. Analysis of the reaction data by a kinetic method or a statistical analysis suggests that there is one critical arginine involved in the binding of each iron atom to the protein. Arginine may function as an essential residue in the anion-binding site of transferrin, i.e. the bicarbonate-binding site.

A. Egyed

Erythrocytic mechanism of sickle cell resistance to malaria. M. J. Friedman (The Rockefeller University, New York, N. Y., USA). *Proc. nat. Acad. Sci. (Wash.) 75*, 1994 (1978).

The physiological basis of resistance to falciparum malaria of individuals with sickle cell trait had been unclear until erythrocytic *Plasmodium falciparum* cultures allowed a direct investigation of the development of the malaria parasite in cells with sickle cell hemoglobin. In a high (18%) oxygen atmosphere, there is no apparent sickling of cells, and the growth and multiplication of *P. falciparum* is identical in normal (AA), hemoglobin S homozygous (SS) and hemoglobin S heterozygous (SA) erythrocytes. Cultures under low (1 to 5%) oxygen, however, showed clear inhibition of growth. The sickling of SS red cells killed and lysed most or all of the intracellular parasites. Parasites in SA red cells were killed primarily at the large ring stage, probably as a result of a disruption of the parasite metabolism. Incubation in cyanate prior to culture reversed the resistance of SA erythrocytes to plasmodium growth, but had no effect on SS red cell sickling or resistance. Thus, the mechanism of resistance *in vivo* may be due solely to intraerythrocytic conditions.

Ilma Szász

Human erythrocyte membrane acetylcholinesterase. Incorporation into the lipid bilayer structure of liposomes. E. R. Hall, U. Brodbeck (Medizinisch-Chemisches Institut der Universität Bern, Bern, Schweiz). *Europ. J. Biochem. 89*, 159 (1978).

Human erythrocyte acetylcholinesterase was incorporated into liposomes of different phospholipid composition by detergent depletion methods. Complete incorporation of activity into liposomes was achieved. Either by gel filtration or by dialysis, 99.96% of the sodium deoxycholate originally present was removed. The preferred method of liposome formation involved the use of dialysis followed by gel filtration, as gel filtration

alone resulted in liposomes different in size. The liposomes had a diameter of about 30 nm (determined by electron microscopy and gel filtration). Studies involving the use of Triton X-100 and proteolytic enzymes revealed that at least 70% of the incorporated activity was located on the outer side of the liposomes; this percentage was even higher in small liposomes.

G. Gárdos

Erythrocyte membrane proteins. Sequential accumulation in the membrane during reticulocyte maturation. N. D. Light, M. J. A. Tanner (Department of Biochemistry, University of Bristol, Bristol). *Biochim. biophys. Acta (Amst.) 508*, 571 (1978).

Reticulocytes of increasing maturity were separated by dextran gradient centrifugation. Accumulation in the membrane of the anion transport protein and other erythrocyte membrane proteins was studied during reticulocyte maturation by separating reticulocytes after incubation with ^{35}S-methionine. Incorporation of the reticulocyte membrane proteins was shown to be sequential, the anion transport being inserted at early stage of the cells' maturation.

B. Sarkadi

Red cell aging. II. Anomalous electrophoretic properties of neuraminidase treated human erythrocytes. F. J. Nordt, R. J. Know, G. V. F. Seaman (Department of Neurology, University of Oregon, Health Sciences Center, Portland, Oreg., USA). *J. Cell Physiol. 97*, 209 (1978).

Desialylation of human red blood cells (RBC) by *Vibrio cholerae* neuraminidase (VCN) was found to produce cells with electrophoretic properties which were inconsistent with the view of a simple loss of N-acetyl-neuraminic acid (NANA), as the sole effect of VCN treatment. Modification of human RBC with 50 to 350 U VCN/10^{10} RBC at 37 °C for one hour releases 90 to 100% of NANA and produces a progressive decrease towards zero in electrophoretic mobility when measured in 0.15 M NaCl (pH 7.2) at 25 °C. The appearance of positive groups on the desialylated cells was indicated by the VCN-treated cells displaying positive mobilities below pH ~ 5.5 and increased negative mobilities at pH ~ 9 as well as substantial increases in their mobility at neutral pH following treatment with formaldehyde. Adsorption to the RBC of about 95% of the VCN activity at 0 °C did not produce any significant change in electrophoretic mobility, thus indicating that the observed changes in the electrophoretic properties of the RBC following VCN treatment could not be attributed to adsorption of VCN. These studies indicate that the cationic charge troups which appear at the electrophoretic surface of the RBC after VCN treatment are probably endogenous in origin. It is suggested that this alteration rather than simple NANA release may operate to shorten the survival time of desialylated red cells.

Ilma Szász

Reversible shift between two states of Ca^{2+}-ATPase in human erythrocytes mediated by Ca^{2+} and a membrane-bound activator. O. Scharff, B. Foder (Department of Clinical Physiology, Finsen Institute, Copenhagen). *Biochim. biophys. Acta (Amst.) 509*, 67 (1978).

The (Ca^{2+}—Mg^{2+})-dependent ATPase from human erythrocytes occurred in two different states, A-state and B-state, depending on the membrane preparation. The A-state showed low maximum activity (V) and the Ca^{2+} activation was characterized by a Hill coefficient, n_H, of about 1 and a Michaelis constant, K_{Ca}, of about 30 μM. The B-state showed high V, an n_H above 1, which indicates positive cooperativity of Ca^{2+} activation, and a K_{Ca} of about 1 μM. With varying ATP concentrations, both the A-state and the B-state showed negative cooperativity and slightly different values for K_m. The B-state was shifted towards the A-state when the membranes were exposed to low Ca^{2+} concentrations. The shift reached 50% at approx. 0.5 μM Ca^{2+}. At low Ca^{2+} concentrations an activator was released from the membranes. The A-state was shifted in direction of the B-state when the membranes were exposed to Ca^{2+} in the presence of the activator. The shift reached 50% at about 30 μM Ca^{2+}. Recovery of the high V was time-dependent and lasted several minutes. Increasing concentrations of Ca^{2+} and acti-

vator accelerated the recovery. It is suggested that the A-state and the B-state correspond to enzyme free of activator and enzyme associated with activator, respectively. Furthermore, the two states may represent a resting and an active state, respectively, of the calcium pump.

Ilma Szász

Influence of Ca^{2+} on red cell deformability and adaptation to sphering agents. H. Rogausch (Physiologisches Institut der Universität Marburg, Marburg, FRG). *Pflügers Arch. 373*, 43 (1978).

The following results indicate the close connection between Ca^{2+} and red cell deformability: ATP-depleted red cells have a lowered deformability, but chlorpromazine and papaverin restore the deformability to normal despite unchanged low ATP-concentrations of the cells. Both drugs are known to displace membrane-bound calcium.

Without significantly decreasing the red cell ATP-content, the calcium ionophore A23187 reduces the deformability in the presence of 10^{-4} to 10^{-3} mol external calcium. The ionophore is ineffective in reducing the red cell deformability when the cells are suspended in a calcium-free medium.

The sphering agent lysolecithin is less effective in reducing red cell deformability, when the external calcium concentration is kept low. Without lysolecithin, external Ca^{2+} is ineffective in reducing red cell deformability for several hours.

The results are interpreted as showing a calcium-induced reduced mobility of the protein moiety in the red cell membrane.

Ilma Szász

Selective alteration of erythrocyte deformability by SH-reagents. Evidence for an involvement of spectrin in membrane shear elasticity. T. M. Fischer, C. W. M. Haest, M. Stöhr, D. Kamp, E. Deuticke (Abteilung Physiologie, Medizinische Fakultät, Rheinisch-Westfälische Technische Hochschule, Aachen, BRD). *Biochim. biophys. Acta (Amst.) 510*, 270 (1978).

In order to elucidate the molecular basis of membrane shear elasticity, the effect of membrane protein modification by SH-reagents was studied on the deformability of human erythrocytes. Deformability was quantified by measuring the elongation of erythrocytes subjected to viscometric flow in a transparent cone-plate viscometer. Impermeable SH-reagents proved to have no mechanical effect. Many, but not all, permeable SH-reagents markedly reduced the elongation. Among these, bifunctional SH-reagents (e.g. diamide, tetrathionate and N,N-p-phenylenedimaleimide) able to cross-link membrane SH-groups were more effective than monofunctional SH-reagents (e.g. N-ethylmaleimide and ethacrynic acid). The bifunctional SH-reagents produced a 50% reduction of elongation after modification of less than 5% of the membrane SH-groups. In contrast, for a comparable effect, more than 20% of the SH-groups had to be modified by the monofunctional reagents. The effect of SH-oxidizing agents was fully reversible after treatment with disulphide-reducing agents. All bifunctional SH-reagents induced a dimerization of a small fraction of spectrin. Analysis of the distribution of the diamide-induced disulphide bonds among the various membrane protein fractions showed that this agent preferentially acts on the spectrin polypeptides.

The results provide direct experimental evidence that the native arrangement of spectrin is essential for the shear resistance of the erythrocyte membrane and that introduction of small numbers of intermolecular cross-links as well as modification within the molecule lead to a rapid loss of this function.

Ilma Szász

Isolation of phospholipase A_2 from sheep erythrocyte membranes in the presence of detergents. R. M. Kramer, C. Würthrich, Ch. Bollier, P. R. Allegrini, P. Zahler (Department of Biochemistry, University of Bern, Bern, Switzerland). *Biochim. biophys. Acta (Amst.) 507*, 381 (1978).

Isolation of phospholipase A_2 from sheep erythrocyte membranes was carried out by a combination of (1) extraction of membranes at low ionic strength, (2) solubilization of extracted membranes with sodium dodecyl

sulphate, (3) replacement of dodecyl sulphate with cholate by means of gel exclusion chromatography, and (4) affinity chromatography on dialkyl-phosphatidylcholine-Sepharose in the presence of cholate. The phospholipase was prepared with good yield and purified to near homogeneity, as judged by sodium dodecyl sulphate gel electrophoresis. The protein is a minor component of the sheep erythrocyte membrane and has an apparent molecular weight of 18 500.

B. Sarkadi

Mechanism of the modulation of the phytohemagglutinin-P agglutination of the human erythrocyte by metabolic state. J. A. Singer, M. Morrison (St. Jude Children's Research Hospital, Department of Biochemistry, Memphis, Tenn.). *Biochim. biophys. Acta (Amst.) 506*, 202 (1978).

The agglutination of human erythrocytes by phytohemagglutinin-P can be changed depending upon the metabolic state of the cell. The agglutination of intact cells depends upon the ATP content of the cell. Agglutination of resealed membranes from ATP depleted erythrocytes has been shown to be a function of the concentration of ATP in the resealing media. β,γ-methylene-ATP competitively inhibits the effect of ATP within resealed membranes.

The route of entry of adenosine into the cell was also found to influence agglutinability; in nucleoside transport-inhibited cells, there was a distinct maximum of agglutination vs. adenosine concentration not observed in uninhibited cells.

The influence of cell shape on agglutination was also considered and agglutination was found to correlate with the percentage of cells with disk morphology. Alterations of the shape of erythrocytes produced by lysophosphatidyl choline treatment, however, were not accompanied by any change in agglutinability.

An attempt was made to evaluate the relationship between agglutination and the surface charge of the cell. Partition of resealed membranes in an aqueous two-polymer phase system which is primarily determined by surface charge, decreased as a function of the ATP concentration in the lysis and resealing medium, while agglutination of these preparations increased concomitantly.

The results suggest that transitions in morphology, surface charge, and phytohemagglutinin-P agglutination of human erythrocytes have at least one ATP-mediated event in common.

B. Sarkadi

Classification and localization of hemoglobin binding sites on the red blood cell membrane. N. Shaklai, J. Yguerabide, H. M. Ranney (Departments of Medicine and Biology, University of California, San Diego, La Jolla, California). *Biochemistry 16*, 5593 (1977).

The binding of hemoglobin to the red cell membrane was characterized over a wide range of free hemoglobin concentrations by measurement of membrane-bound and supernatant hemoglobin. Scatchard analysis of the binding data revealed two classes of sites: high affinity sites with a binding constant of $1 \times 10^8\ M^{-1}$ and 1.2×10^6 sites per cell, and a second, low affinity class of sites with a binding constant of $6 \times 10^6\ M^{-1}$ and 6×10^6 sites per cell. The low affinity sites are shown to be nonspecific and appear to be a result of the ghost preparation. The high affinity sites are shown to be specific to the inner surface of the red cell membrane. The competition of hemoglobin and glyceraldehyde-3-phosphate dehydrogenase suggests band 3 proteins as a potential binding site for hemoglobin.

Ilma Szász

Interaction of hemoglobin with red blood cell membranes as shown by a fluorescent chromophore. N. Shaklai, J. Yguerabide, H. M. Ranney (Departments of Medicine and Biology, University of California, San Diego, La Jolla, California). *Biochemistry 16*, 5585 (1977).

Hemoglobin quenching of the fluorescence intensity of 12-(9-anthroyl)stearic acid (AS) embedded in the red blood cell membrane occurs through an energy transfer mechanism and can be used to measure the binding of hemoglobin to the membrane.

The binding of hemoglobin to red cell membranes was found to be reversible and electrostatic in nature. Using a theory of energy transfer based on Förster formulation, the quantitative data for the binding were derived. The number of binding sites was found to be $1.4 \pm 0.2 \times 10^6$ molecules per cell and the binding constant was 0.85×10^8 M^{-1}.

Ilma Szász

Binding of 2,3-diphosphoglycerate by spectrin and its effect on oxygen affinity of hemoglobin. N. Shaklai, L. Benitez, H. M. Ranney (Department of Medicine, University of California, San Diego, La Jolla, California). *Am. J. Physiol. 234*, C36 (1978).

The relationships between spectrin, a structural protein of the red blood cell membrane facing the cytoplasm, and hemoglobin were studied. The oxygen-binding properties of stripped hemoglobin were not altered by the presence of spectrin, but the interaction of hemoglobin with organic phosphates was reduced by the addition of spectrin. The presence of the enzyme glyceraldehyde 3-phosphate dehydrogenase, another component of the red blood cell membrane used as a control, did not change the oxygen affinity of either stripped hemoglobin or of hemoglobin solutions containing phosphates. Binding studies using the gel filtration method at pH 7.3 indicated reversible binding of 2,3-diphosphoglycerate to spectrin. A unit of 220,000 daltons was calculated to have seven binding sites and a binding constant of 1.2×10^4 M^{-1}. A mechanism is proposed in which spectrin may facilitate oxygen transport for hemoglobin molecules reaching the membrane.

B. Sarkadi

Spectrin bands 1 and 2 are antigenically distinct and are not composed of subunits. F. H. Kirkpatrick, D. J. Rose, P. La Celle (Department of Radiation Biology and Biophysics and of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.). *Arch. Biochem. Biophys. 186*, 1 (1978).

Human erythrocyte membranes and freshly isolated spectrin were separated into their constituent peptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The peptides were electrophoresed from slices of such gels into agarose gels containing anti-spectrin antibodies and Triton X-100. In fresh preparations, precipitin arcs were observed only against peptides migrating as bands 1 and 2. It was found that bands 1 and 2 did not cross-react. There are two major arcs from band 1 and one principal arc from band 2, plus minor splitting of these arcs. None of the band 1 arcs fused with band 2 arcs. In fresh erythrocyte ghosts only bands 1 and 2 reacted with anti-spectrin; bands 2.1, 3, and 5, in particular, showed no precipitin arcs. However, in aged ghosts, arcs appeared in the band 3 region; in aged isolated spectrin, arcs appeared in the band 2.1 region; and in trypsin-degraded spectrin, reactive species occurred in all molecular weight classes. It is concluded that spectrin has no subunits smaller than 220,000 molecular weight and that bands 1 and 2 are immunochemically distinct.

G. Gárdos

Spectrin as a stabilizer of the phospholipid asymmetry in the human erythrocyte membrane. C.W.M. Haest, G. Plasa, D. Kamp, B. Deuticke (Abteilung Physiologie, Medizinische Fakultät, Technische Hochschule, Aachen, F.R.G.). *Biochim. biophys. Acta (Amst.) 509*, 21 (1978).

After treatment of intact human erythrocytes with SH-oxidizing agents (tetrathionate and diamide) phospholipase A_2 cleaves approx. 30% of the phosphatidylserine and 50% of the phosphatidylethanolamine without causing hemolysis. These phospholipids are scarcely hydrolysed in fresh erythrocytes and are assumed to be located in the inner lipid layer of the membrane. The enhancement of the phospholipid cleavage is now shown to be accompanied by a 50% decrease of the membrane SH-groups and a cross-linking of spectrin, located at the inner surface of the membrane, to oligomers of $<10^6$ dalton. Blocking approx. 10% of the membrane SH-groups with N-ethylmaleimide suppresses both the polymerization of spectrin and the enhancement of the phospholipid cleav-

age. N-Ethylmaleimide, under these conditions, reacts with three SH-groups per molecule of spectrin, 0.7 SH-groups per major intrinsic 100 000 dalton protein (band 3) and 1.1 SH groups per molecule of an extrinsic protein of 72 000 daltons (band 4.2). Blocking studies with iodoacetamide demonstrate that the SH groups of the 100 000-dalton protein are not involved in the effects of the SH-oxidizing agents. It is suggested that a release of constraints imposed by spectrin enables phosphatidylserine and phosphatidylethanolamine to move from the inner to the outer lipid layer of the erythrocyte membrane and that spectrin, in the native erythrocyte, stabilizes the orientation of these phospholipids to the inner surface of the membrane.

Ilma Szász

The breakdown of spectrin produced by ultrasonication. J. R. Harris, P. Marshall, I. Naeem (Biomembrane Unit, Division of Biochemistry, Faculty of Sciences, North East London Polytechnic, London). *Biochim. biophys. Acta (Amst.) 534*, 173 (1978)

Carefully controlled ultrasonication of human erythrocyte membranes (ghosts) has been found to produce a selective breakdown of the two spectrin polypeptides. This breakdown increases with the time and the intensity of ultrasonication. The 240,000 molecular weight spectrin polypeptide is slightly more susceptible to ultrasonication than the 220,000 molecular weight polypeptide. Isolated spectrin behaves in an identical manner when ultrasonicated. When highly purified samples of spectrin are ultrasonicated, the progressive reduction in molecular weight of the products with increasing time is clearly apparent.

G. Gárdos

Enzymatic synthesis and rapid translocation of phosphatidyl-choline by two methyltransferases in erythrocyte membranes. F. Hirata, J. Axelrod (Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland). *Proc. nat. Acad. Sci. (USA) 75*, 2348 (1978).

The synthesis of phosphatidylcholine from phosphatidylethanolamine is carried out by two methyltransferases in erythrocyte membranes. The first enzyme uses phosphatidylethanolamine as a substrate, requires Mg^{2+}, and has a high affinity for the methyl donor, S-adenosyl-L-methionine. The second enzyme methylates phosphatidyl-N-monomethylethanolamine to phosphatidylcholine and has a low affinity for S-adenosyl-L-methionine. The first enzyme is localized on the cytoplasmic side of the membrane and the second enzyme faces the external surface. This asymmetric arrangement of the two enzymes across the membrane makes possible the stepwise methylation of phosphatidylethanolamine localized on the cytoplasmic side and facilitates the rapid transmembrane transfer of the final product, phosphatidylcholine, to the external surface of the membrane. A mechanism for an enzyme-mediated flip-flop of phospholipids from the cytoplasmic to the outer surface of erythrocyte membranes is described.

Ilma Szász

Evidence suggesting direct oxidation of human erythrocyte membrane sulfhydryls by copper. J. M. Salhany, J. C. Swanson, K. A. Cordes, S. B. Gaines, K. C. Gaines (Departments of Biomedicinal Chemistry and Internal Medicine and the Cardiovascular Center, University of Nebraska, Medical Center and VA Hospital, Omaha, Nebraska). *Biochem. biophys. Res. Comm. 82*, 1294 (1978).

Results are presented which suggest that cupric ion can directly oxidize the sulfhydryls of human erythrocyte membrane proteins leading to the formation of disulfide links. When packed ghosts were incubated in cupric sulfate (0.3 to 0.7 mM) at pH 8, and electrophoresed on sodium dodecyl sulfate polyacrylamide gels in the absence of dithiothreitol bands 1, 2 (spectrin), 4.2 and 5 (actin) diminished in intensity concomitant with the appearance of high molecular weight material. Band 3 moved to its dimeric position on the gel. Evidence that these crosslinks result from formation of new disulfide links due to direct copper binding includes: (a) reversal of crosslinking upon addition of dithiothreitol; (b) blockage of the effect by N-ethylmaleimide, EDTA and mercuric chloride. The effect of copper was observed under N_2, suggesting that it is not related to

air oxidation. Furthermore, the crosslinking effect does not require high copper concentrations if the ghost concentration is low. The possible implication of these results with regard to copper induced hemolytic anemias is briefly discussed.

Ilma Szász

The intramembranous segment of human erythrocyte glycophorin A. H. Furthmayr, R. E. Galardy, M. Tomita, V. T. Marchesi (Department of Pathology and Section of Cell Biology, Yale University, New Haven, Connecticut). *Arch. Biochem. Biophys. 185*, 21 (1978).

The intramembranous segment of glycophorin A has been localized to a 35-amino acid peptide. This has been isolated by a new procedure in which acid-insoluble peptides of a tryptic digest of detergent-purified glycophorin A are fractionated by countercurrent distribution. Amino acid sequence analyses, using both manual and automatic Edman degradation techniques, indicate that this peptide has a unique sequence in contrast to earlier work. Ambiguities at three positions have been resolved, and sequencing errors at two additional positions have been corrected. One segment of this peptide has an uninterrupted stretch of 22 uncharged amino acids, and it is likely that this is the part which spans the lipid bilayer of the membrane. The complete 35-residue peptide has an apparent molecular weight in the 6000—8000 range, when analyzed on sodium dodecyl sulfate gels, suggesting that it forms dimers under these conditions. This result is consistent with the earlier proposal that intact glycophorin A molecules exist as dimers in sodium dodecyl sulfate which are stabilized by noncovalent associations between hydrophobic segments of their polypeptide chains.

Ilma Szász

Asymmetry of the hexose transfer system in human erythrocytes. Experiments with non-transportable inhibitors. G. F. Baker, D. A. Basketter, W. F. Widdas (Department of Physiology, Bedford College, London, England). *J. Physiol. 278*, 377 (1978).

The asymmetrical nature of sugar affinity for the hexose transfer system in human red cells has been demonstrated using purified 4,6-O-ethylidene-α-D-glucopyranose (ethylidene glucose) to inhibit the exchange of glucose, 3-O-methyl glucose and galactose. The half-saturation concentration for ethylidene glucose inside the cell is estimated at ca. 110 mM whereas on the outside the value for exchange inhibition is ca. 11 mM. The asymmetries of affinities of two related non-transportable inhibitors 1,2-O-isopropylidene-D-glucofuranose and methyl-2,3-di-O-methyl-α-D-glucopyranoside have also been studied. From experiments at varying concentrations and on theoretical grounds the half-saturation concentration for non-transportable inhibitors on the outside surface is shown to be over-estimated by measuring inhibition of exchange. In consequence the actual asymmetry of affinities may be greater than observed. Experiments with ethylidene glucose also suggest that conformational changes redistributing components of the hexose transfer system between inward and outward facing modes may occur.

B. Sarkadi

Asymmetry of the hexose transfer system in human erythrocytes. Comparison of the effects of cytochalasin B, phloretin and maltose as competitive inhibitors. D. A. Basketter, W. F. Widdas (Department of Physiology, Bedford College, London, England). *J. Physiol. 278*, 389 (1978).

Cytochalasin B inhibits glucose transfer in human red cells. With glucose exit the inhibition is typically non-competitive, but hexose exchange is competitively inhibited. At 16°C the inhibitory constant for inhibition of 3-O-methyl glucose exchange is estimated at 1.1×10^{-7} M while that for inhibition of glucose exit is 5.0×10^{-7} M. Uptake of labelled cytochalasin B includes a saturable component which when correlated with the inhibition of exchange corresponds to a maximal binding of ca. 2.4×10^5 molecules per cell. The kinetic parameters are compared with those for maltose (a competitive inhibitor acting on the outside only) and phloretin (an inhibitor acting both inside and outside). Kinetic evidence suggests that cytochalasin B reacts with the inside of the hex-

ose transfer system and that the anomalous inhibitory characteristics are due to the chemical asymmetry of the system. Independent evidence in support of this view is discussed.

B. Sarkadi

Preparation and analysis of seven major, topographically defined fragments of band 3, the predominant transmembrane polypeptide of human erythrocyte membranes. T. L. Steck, J. J. Koziarz, M. K. Singh, G. Reddy, H. Köhler (Departments of Biochemistry, Medicine and Pathology, The University of Chicago, Chicago, Ill., USA). *Biochemistry 17*, 1216 (1978).

Band 3 is the ~90,000-dalton membrane-spanning polypeptide believed to facilitate anion transport in the human erythrocyte membrane. Previous studies have shown that digestion of this protein while still membrane-bound generates large, topographically defined, overlapping fragments which account for all or nearly all of its mass. Seven of these fragments have now been purified utilizing selective membrane solubilization, gel filtration, and preparative gel electrophoresis in sodium dodecyl sulfate. Amino acid analysis revealed that fragments derived from the outer surface and membrane-spanning regions of band 3 were distinctly hydrophobic, while cytoplasmic surface segments were comparatively polar; these compositional data parallel the solubility in aqueous solutions and the mode of membrane association of the various fragments. Digestion of intact cells with chymotrypsin generated a 38,000-dalton outer surface and a 55,000-dalton transmembrane fragment. The sums of their apparent molecular weights and of their compositions approximated the band 3 polypeptide, suggesting a single site of cleavage at the extracellular face. Chymotryptic digestion at both membrane surfaces produced a 17,000-dalton transmembrane fragment having the most hydrophobic composition. Mild trypsin digestion released a 41,000-dalton segment from the cytoplasmic pole of band 3, which could further be cleaved by trypsin to yield 22,000- and 16,000-dalton subfragments. Compositional analysis showed that the sum of these two subfragments was nearly equivalent to the 41,000-dalton tryptic fragment.

These data indicate the presence of a small lysin-rich segment near the juxtamembrane region of the 41,000-dalton fragment, which was lost upon further cleavage. The missing segment may overlap with the 17,000-dalton chymotryptic core. A 23,000-dalton fragment cleaved by S-cyanylation from the cytoplasmic terminal region of band 3 had nearly the same composition as the 22,000-dalton tryptic fragment, indicating that these two species are coextensive. Furthermore, end-group analysis suggested the presence of blocked amino termini on both the 22,000- and 23,000-dalton fragments, supporting previous reports that the amino terminus of the band 3 polypeptide is located on the cytoplasmic side of the membrane.

G. Gárdos

Membrane protein phosphorylation of intact normal and hereditary spherocytic erythrocytes. L. C. Wolfe, S. E. Lux (Division of Hematology-Oncology, Department of Medicine, Children's Hospital Medical Center and Sidney Farber Cancer Institute, and the Department of Pediatrics, Harvard Medical School, Boston, Mass., USA). *J. biol. Chem. 253*, 3336 (1978).

Membrane protein phosphorylation was measured in intact human erythrocytes with an assay which simultaneously monitored $^{32}PO_4$ incorporation into ATP and membrane protein substrates. The pattern of phosphorylation in intact cells differed from that seen with isolated ghosts and likely reflected contributions of cytoplasmic protein kinases and phosphatase(s) as well as membrane protein kinases. Band 2 of spectrin, band 3, and a band in the 4.5 region were labeled. Dibutyryl cAMP (1 mM) did not alter intact cell phosphorylation. The data indicate that spectrin contains approximately 1 to 2 mol of exchangeable phosphorus/mol of spectrin, depending on the fraction of intracellular ATP available for isotopic exchange. The degree and pattern of phosphorylation of isolated erythrocyte ghosts varied, depending on the assay conditions. In the Greenquist and Shohet

assay [Greenquist, A. C., Shohet, S. B.: *FEBS Lett. 48*, 133 (1974)] phosphorylation of HS ghosts was normal after a 5-min incubation and was variably abnormal at 60 min. In the Avruch and Fairbanks assays [Avruch, J., Fairbanks, G.: *Biochemistry 13*, 5507 and 5514 (1974)] phosphorylation of HS ghosts was normal under all incubation conditions.

It is concluded that the intact erythrocyte assay may be the optimal screening test for detecting pathologically significant dysfunctions of membrane protein phosphorylation and that studies in erythrocyte ghosts may lead to erroneous conclusions about the status of membrane protein phosphorylation in the intact erythrocyte. The problem is illustrated by hereditary spherocytosis where phosphorylation of intact erythrocytes, presumably the physiologically relevant situation, is normal.

Ilma Szász

Reconstitution of band 3, the erythrocyte anion exchange protein. A. H. Ross, H. M. McConnell (Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, Calif.). *Biochem. biophys. Res. Comm. 74*, 1318 (1977).

Band 3, the erythrocyte membrane protein thought to be responsible for anion transport, was purified to near homogeneity using a concanavalin A affinity column. Band 3 was then combined with egg lecithin, erythrocyte lipid, cholesterol, and glycophorin, the major erythrocyte sialoglycoprotein, to form vesicles capable of rapid sulfate transport. The transport activity was sensitive to prior treatment of the erythrocytes with pyridoxal phosphate-$NaBH_4$, a potent inhibitor of anion transport in these cells.

G. Gárdos

Anion transport in relation to proteolytic dissection of band 3 protein. S. Grinstein, S. Ship, A. Rothstein (Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada). *Biochim. biophys. Acta (Amst.) 507*, 294 (1978).

Sulfate efflux was measured in inside-out vesicles obtained from human red cells. Inhibition was observed in vesicles derived from cells pretreated with DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonate) or after addition of dipyridamole to the vesicles, both agents being specific and potent inhibitors of anion transport in cells. Trypsinization of the cytoplasmic side of the membrane in order to release a 40,000 dalton fragment from band 3 (the purported anion transport protein) had no effect on sulfate efflux. Further degradation of band 3 to a 17,000 dalton segment, by trypsinization of inside-out vesicles derived from cells that had been pretreated with chymotrypsin, also showed little reduction in transport activity. Furthermore, such vesicles derived from DIDS pretreated cells were inhibited by over 90%. In DIDS-treated cells, the agent is highly localized in band 3. In trypsinized inside-out vesicles, it is largely found in a 55,000 fragment and in trypsinized vesicles derived from cells pretreated with chymotrypsin it is largely located in the 17,000 fragment. The data suggest that both the anion transport and inhibitor binding sites are located in a 17,000 transmembrane segment of band 3.

G. Gárdos

Asymmetry of the chloride transport system in human erythrocyte ghosts. K. F. Schnell, E. Besl, A. Manz (Institut für Physiologie, Universität Regensburg, Regensburg, F.R.G.). *Pflügers Arch. 375*, 87 (1978).

The concentration dependence of the unidirectional chloride flux and the inhibition of the unidirectional chloride flux by sulfate were studied in human red cell ghosts. The concentration dependence of the unidirectional chloride flux and its inhibition by sulfate were asymmetric. The unidirectional chloride flux can be saturated from the inner and from the outer membrane surface. For the inner membrane surface, lower chloride half-saturation constants were obtained than for the outer membrane surface. The inhibition of the unidirectional chloride flux by sulfate is competitive. In contrast to the chloride half-saturation constants, the inhibition constants of sulfate for the inner membrane surface were higher than the inhibition constants of sulfate for the outer membrane sur-

face. Either there are fixed anion binding sites at the inner and at the outer membrane surface which control the access of anions to a pore, or there is a mobile carrier which is in contact with both membrane surfaces. The asymmetry of the concentration response and of the inhibition of the unidirectional chloride flux suggests that the anion binding sites at the inner and at the outer membrane surface differ with respect to their affinities for chloride and for sulfate. Alternatively, the asymmetry of the chloride transport system could indicate an asymmetric distribution of a mobile anion carrier across the erythrocyte membrane.

G. Gárdos

Effect of phloretin on chloride permeability: A structure-activity study. J. L. Cousin, R. Motais (Laboratoire de Physiologie Cellulaire, Faculté des Sciences, Nice Cedex, France). *Biochim. biophys. Acta (Amst.) 507*, 531 (1978).

On the basis of data obtained with thin lipid membranes, phloretin inhibition of chloride, urea and glucose transport in biological membranes has been suggested to be due to the effects of interfacial dipole fields on the translocator of these molecules. From the systematic analysis made in the present paper it effectively appears that the ability of phloretin analogs to inhibit chloride permeability in red-cell membrane depends on their capacity to alter the interfacial dipole potential: from the magnitude of the potential change depending on the dipole moment of the molecule and its membrane concentration, it follows that the inhibitory capacity of a phloretin analog is a function of the dipole moment and the lipid solubility of the compound.

G. Gárdos

Development of hormonal regulation of ion transport in embryonic red cells. M. Wacholtz, L. N. Chan, R. I. Sha'afi (Department of Physiology, University of Connecticut Health Center, Farmington, Conn.). *J. Cell Physiol. 94*, 1 (1978).

Cation transport in red cells from chick embryos is stimulated by epinephrine. The response develops with the maturation of the embryonic definitive cells. Sodium efflux and potassium influx are significantly stimulated (50%) by epinephrine in red cells from embryos incubated 10 days or longer, whereas cation fluxes in erythroid cells from 8- or 9-day embryos are stimulated slightly or not at all.

The effect of epinephrine may be mediated by cyclic AMP as adenylate cyclase activity in membranes isolated from embryonic red cells is only slightly stimulated at 9 days, but the response increases as the cells mature to a maximum of about 180%. The stimulation of cation transport by epinephrine is blocked by propranolol, but not by phentolamine. Although the younger cells respond poorly to epinephrine, cyclic AMP significantly stimulates transport.

Enhancement of cation fluxes by epinephrine or cyclic AMP occurs even in the presence of ouabain. Since both K influx and Na efflux are enhanced by these agents, their action is most likely on some form of the "Na—K" pump which is not ouabain-sensitive, resulting in a significant increase in the maximum velocity of the pump. It is suggested that there are two classes of Na—K pump in these embryonic cells. One pump is similar to that found in many erythrocytes including mammalian cells in that it selectively pumps potassium in and sodium out, is ouabain-sensitive, and is primarily involved in maintaining intracellular cation concentrations. The second pump is enhanced by epinephrine via cyclic AMP, is not inhibited by ouabain, and may have a lower ion selectivity. This hormone-sensitive pump activity is lost as the cells mature, a process which is complete when the animal is fully grown and no longer has significant numbers of embryonic cell in its circulation.

G. Gárdos

Chemically-induced cation permeability in red cell membrane vesicles. The sidedness of the response and the protein involved. S. Grinstein, A. Rothstein (Research Institute, The Hospital for Sick Children, Toronto, Ontario). *Biochim. biophys. Acta (Amst.) 508*, 236 (1978).

Cation fluxes were measured in right-side-out and inside-out vesicles obtained from

human red cells. Rubidium, which is spontaneously released at very slow rates, is rapidly released from both types of vesicle on the addition of valinomycin. P-chloromercuriphenyl sulfonic acid (PCMBS) also increases the cation permeability of the vesicles with reversal to normal after addition of dithiothreitol. The effect of PCMBS is considerably more marked and appears faster in the inside-out than in the right-side-out vesicles, the difference being greater at low temperatures. These data indicate that the SH groups responsible for the changes in cation permeability are more accessible from the inside face of the membrane. The response to PCMBS was not diminished after selective removal of extrinsic proteins by alkaline extraction, and/or after the membranes had been exposed to proteolytic enzymes. The major polypeptide component remaining in the vesicles after both treatments was a 17,000-dalton transmembrane fragment derived from band 3 which might, therefore, be responsible for the permeability response. Addition of Ca^{2+} to either right-side-out or inside-out vesicles, in the presence or absence of ionophore A23187, had no effect on monovalent cation permeability, indicating that the mechanism of Ca^{2+}-induced K^+ permeation was lost or inactivated during the preparation of the vesicles.

B. Sarkadi

Lithium transport pathways in human red blood cells. G. N. Pandey, B. Sarkadi, M. Haas, R. B. Gunn, J. M. Davis, D. C. Tosteson (Illinois State Psychiatric Institute, Chicago, Ill. and Department of Pharmacology and Physiology, University of Chicago, Chicago, Ill.). *J. gen. Physiol. 72*, 233 (1978).

In human red cells, Li is extruded against its own concentration gradient if the external medium contains Na as a dominant cation. This uphill net Li extrusion occurs in the presence of external Na but not K, Rb, Cs, choline, Mg, or Ca, is ouabain-insensitive, inhibited by phloretin, and does not require the presence of cellular ATP. Li influx into human red cells has a ouabain-sensitive and a ouabain-insensitive but phloretin-sensitive component. Ouabain-sensitive Li influx is competitively inhibited by external K and Na and probably involves the site on which the Na—K pump normally transports K into red cells. Ouabain does not inhibit Li efflux from red cells containing Li concentrations below 10 mM in the presence of high internal Na or K, whereas a ouabain-sensitive Li efflux can be measured in cells loaded to contain 140 mM Li in the presence of little or no internal Na or K. Ouabain-insensitive Li efflux is stimulated by external Na and not by K, Rb, Cs, choline, Mg or Ca ions. Na-dependent Li efflux does not require the presence of cellular ATP and is inhibited by phloretin, furosemide, quinine, and quinidine. Experiments carried out in cells loaded in the presence of nystatin to contain either only K or only Na show that the ouabain-insensitive, phloretin-inhibited Li movements into or out of human red cells are stimulated by Na on the *trans* side and inhibited by Na on the *cis* side of the red cell membrane. The characteristics of the Na-dependent unidirectional Li fluxes and uphill Li extrusion are similar, suggesting that they are mediated by the same Na—Li counter-transport system.

G. Gárdos

Kinetics and stoichiometry of Na-dependent Li transport in human red blood cells. B. Sarkadi, J. K. Alifimoff, R. B. Gunn, D. C. Tosteson (Department of Pharmacology and Physiology, University of Chicago, Chicago, Ill.). *J. gen. Physiol. 72*, 249 (1978).

This paper describes the kinetics and stoichiometry of a tightly coupled Na—Li exchange transport system in human red cells. The system is inhibited by phloretin and furosemide but not by ouabain. Li influx by this system increases and saturates with increasing concentrations of external Li and internal Na and is inhibited competitively by external Na. Comparable functions relate Li efflux to internal and external Li and Na concentrations. Analysis of these relations yields the following values for the ion concentrations required to half-maximally activate the transport system: internal Na and Li 9.0 and 0.5 mM, respectively, external Na and Li 25 and 1.5 mM, respectively. The system

performs a 1 : 1 exchange of Na and Li moving in opposite directions across the red cell membrane. There is no evidence for a simultaneous transport of more than one Na and Li by the system. The maximum transport rate of Na-dependent Li transport varied between 0.1 and 0.37 mmol/(liter of cells × h) in the red cells of the five normal male subjects studied. No significant variations between individual subjects were observed for bicarbonate-stimulated Li transport and for the residual Li fluxes which occur in the absence of bicarbonate and in the presence of ouabain plus phloretin.

G. Gárdos

Plasma membrane Ca^{2+} transport: Stimulation by soluble proteins. T. R. Hinds, F. L. Larsen, F. F. Vincenzi (Department of Pharmacology, School of Medicine, University of Washington, Seattle, Washington). *Biochem. biophys. Res. Comm. 81*, 455 (1978).

Inside-out membrane vesicles were prepared from human red blood cells. In the presence of ATP, these vesicles took up $^{45}Ca^{2+}$ against a chemical gradient. The active transport of Ca^{2+} was increased by addition of an activator protein of Ca^{2+} + + Mg^{2+}-ATPase isolated from the membrane-free hemolysate of human red blood cells. A closely related protein, the protein modulator of cyclic AMP phosphodiesterase from bovine brain, also increased the rate of active transport of $^{45}Ca^{2+}$. Addition of the calcium ionophore A23187 caused a rapid efflux of $^{45}Ca^{2+}$ from loaded, inside-out vesicles. When La^{3+} was added to the system in the presence of activator protein, the uptake of $^{45}Ca^{2+}$ was inhibited. Results are compatible with the interpretation that activity of the plasma membrane Ca^{2+} pump may be modulated by certain cytoplasmic proteins.

B. Sarkadi

Erythrocyte membrane potentials determined by hydrogen ion distribution. R. I. Macey, J. S. Adorante, F. W. Orme (Departments of Physiology and Anatomy, University of California, Berkeley, Calif., USA). *Biochim. biophys. Acta (Amst.) 512*, 284 (1978).

If the extracellular fluid is left unbuffered, dynamic membrane potential changes in the red blood cell may be determined from external pH readings. For some types of experiments it is necessary to accelerate H^+ equilibration by adding minute amounts of hydrogen carriers. The method is independent of the hematocrit over a wide range of membrane potential jumps produced by permeability changes or by changes in ionic composition may be measured. The method provides a convenient means of measuring parameters of both the conductive and nonconductive anion pathways in the red cell.

G. Gárdos

From the International Literature of Haematology

Acta Haematologica (Basel) **59** (1978) No. 1

Detection of cytochemical and morphological anomalies in "preleukemia". *Schmalzl, F., Konwalinka, G., Michlmayr, G., Abbrederis, K., Braunsteiner, H.* (Department of Medicine, University of Innsbruck, Innsbruck, Austria), p. 1

Morphological differences in human peripheral blood lymphocytes. A freeze-etching and scanning electron microscopy study. *Renau-Piqueras, J.* (Instituto de Investigaciones Citológicas, Departamento de Citologia Molecular y Cultiva de Tejidos, Amadeo de Saboya, 4, Valencia-10, Spain), p. 19

Unusual lymphocyte morphology in a case of chronic lymphatic leukaemia: Apparent nuclear extrusion. *Newell, D. G., Jayaswal, U., Smith, J., Roath, S.* (Department of Haematology, Faculty of Medicine, South Laboratory and Pathology Block, Southampton General Hospital, Southampton S09 4XY, England), p. 25

Pure red cell aplasia, toxic dermatitis and lymphadenopathy in a patient taking diphenylhydantoin. *Huijgens, P. C., Thijs, L. G., den Ottolander, G. J.* (Department of Internal Medicine, Free University Hospital, Amsterdam, The Netherlands), p. 31

Occurrence of haemoglobin H in leukaemia: a further case of erythroleukaemia. *Beaven, G. H., Coleman, P. N., White, J. C.* (National Institute for Medical Research, London NW7 1AA, England), p. 37

Auto-immune haemolytic anaemia complicating infectious mononucleosis in a patient with hereditary elliptocytosis. *Ho-Yen, D. O.* (Haematology Section, Department of Pathology, Ninewells Hospital and Medical School, Dundee, DD2 IUB, England), p. 45

Defective maturation of granulocytes, retinal cysts and multiple skeletal malformations in a mentally retarded girl. A new syndrome. *Plum, C. M., Warburg, M., Danielsen, J.* (Biochemical Research Laboratory, Kolonien Filadelfia, DK-4293 Dianalung, Denmark), p. 53

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News Items

Joint Meeting of the 18th Congress of the International Society of Hematology and the 16th Congress of the International Society of Blood Transfusion, August 16–22, 1980, Montreal, Canada

All enquiries should be addressed to:

I.S.H./I.S.B.T. Congress – Montreal
772 Sherbrooke Street West

Montreal, Quebec, Canada
H3A 1G1

Telephone: (514) 392–6744

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Jean Julliard Prize

The 7th 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 XVIth International Congress of Blood Transfusion, to be held in Montreal (Canada) from 16 to 22 August 1980.

The prize is reserved for scientists under 40 years of age in recognition of recently completed scientific work on blood transfusion and related subjects.

In order to qualify candidates must forward six (6) copies of an unpublished manuscript or recently published papers including a curriculum vitae, to the Secretary General Professor Ch. Salamon, 53 boulevard Diderot, 75571 Paris Cedex 12, France

before 20 February 1980

The prize will be awarded during the Congress.

The value of the prize is 3 000 Swiss francs.

Further information shall be requested from the Secretary General.

Lady Tata Memorial Trust

International scholarships and fellowships for research on leukaemia and allied conditions

Academic Year 1979–1980

The Trustees of the Lady Tata Memorial Trust invite applications for awards at predoctoral or postdoctoral level for research on leukaemia, in the Academic Year beginning 1st October 1979. In view of the affinity between leukaemia and other forms of neoplastic disease, candidates with programs of re-

search on any aspect of malignant disease which may throw light on problems of leukaemia will be eligible for consideration. The Trustees specially wish to encourage studies of the leukaemogenic viruses in animals, the epidemiology, natural history and immunologic aspects of leukaemia.

The awards offered by the Trust are open to suitably qualified investigators of any nationality, working either in their own institutions or in other centres abroad.

Awards are offered at rates appropriate to age, seniority, and experience of the candidates, and to the corresponding stipends in the Institution at which the work will be done. They are tenable for 1 year in the first instance, renewable up to a maximum of 3 years. University fees and superannuations can be taken into account if necessary.

Forms of application may be obtained by writing to Mrs. Miglierina, Secretary for continental Europe of the Lady Tata Memorial Trust, Laboratory of Professor M. Seligmann, Bâtiment INSERM, Hôpital Saint-Louis, 2, place du Dr. Fournier. 75475 Paris Cedex 10, France (tel.: 209.33.50 ext. 642). Applications must be submitted before March 31st, 1979 and awards will be announced by the Trustees in June.

"Le Prix du Jeune Chercheur"

A l'occasion du VIII° Congrès Européen de Cardiologie qui se tiendra à Paris du 22 au 26 juin 1980, la Société Européenne de Cardiologie remettra "Le Prix du Jeune Chercheur" d'un montant de F. 100.000.– dont le financement est assuré par la Fondation Claude-Adolphe Nativelle.

Ce prix récompensera un travail personnel ou de groupe contribuant à l'évolution de la cardiologie sous tous ses aspects: recherche en biologie fondamentale, physique, chimie, application de problèmes cardiologiques en techniques d'investigation, méthodes de traitement médical et chirurgical, recherche clinique etc ...

Les candidats devront être européens et âgés de moins de 35 ans.

Les dossiers de candidature devront comprendre un mémoire ne dépassant pas 100 pages dactylographiées, accompagné de toutes informations utiles sur la réalisation de cette recherche.

Les projets seront soumis à des experts européens compétents dans les domaines concernées et à un jury de classement constitué par les présidents de séance du Congrès Européen de Cardiologie.

Ce jury déterminera 4 projets qui seront soumis à présentation publique en 15 minutes au plus pour chacun des 4 candidats retenus.

A l'issue de l'exposé, chaque projet sera discuté publiquement.

Un vote désignera le candidat retenu pour le prix.

Les candidatures doivent être déclarées avant le ler février 1980 et le réglement détaillé peut être demandé au Secrétariat Général du Congrès: P.M.V. Boîte Postale 246 – 92205 Neuilly s/ Seine (France).

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.

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IMMUNOLOGY 1978

Proceedings of the Fourth European Immunology Meeting Budapest, Hungary, 12—14 April 1978

Ed by J. GERGELY, G. A. MEDGYESI, S. R. HOLLÁN

This is the first time that the proceedings of a European Immunology Meeting are published. The volume comprises the lectures delivered at the symposia together with the invited talks of the Fourth European Immunology Meeting in Budapest, 12—14 April 1978. Up-to-date information is to be found on the most formidable problems of immunology as well as views of leading authorities on these.

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Recommended to all concerned with theoretical and clinical immunology, haematology, biochemistry, and genetics.

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AKADÉMIAI KIADÓ, Budapest
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Beobachtungen zur Epidemiologie und zur Genetik der Haemophilie im Bezirk Magdeburg

Von OMR Dr. med. GEORG v. KNORRE, Chefarzt der Klinik, Oschersleben, ASTRID SCHLOTE und HELGA BODE

(Nova Acta Leopoldina. Neue Folge. Nr. 232. Bd. 49)

1978. 84 Seiten, 46 Abbildungen, 9 Tabellen
Broschiert 16,— M

Die Angaben über die Häufigkeit des Vorkommens der Hämophilie in der Weltbevölkerung sind noch recht unterschiedlich. Das Anliegen dieser Schrift ist eine exakte Erfassung aller Bluter in einem geographisch — administrativ begrenzten Raum, dem Bezirk Magdeburg. Hier wohnten 1972 1 311 866 Menschen, davon 609 240 Männer. In dieser Bevölkerung fanden sich 57 Bluter. Mit den verstorbenen Hämophilen aus den gleichen Familien und außerhalb des Bezirkes wohnenden waren es 141 Bluter, über die berichtet wird. Geschildert wird die Verteilung der beiden Hämophilieformen A und B. Die genealogischen und genetischen Zusammenhänge werden ausführlich erläutert und in Tafeln dargestellt.

JOHANN AMBROSIUS BARTH LEIPZIG

Card Index

E. Clark, P. Lake, N. A. Mitchison, G. Winchester

New ideas about self-tolerance and autoimmunity. Haematologia *12*, 7 (1978/1979).

Recent advances in our understanding of B cell ontogeny strengthen the theory that self-tolerance is generated principally by clonal deletion. B cells pass through a stage: the "baby B cell", during which they are particularly susceptible to receptor cross-linking. Further evidence of clonal deletion comes from studies on the maintenance of tolerance of the liver differentiation alloantigen F. In spite of this accumulating evidence room can still be found for the concept of immunological silence: it is a prediction of the dual recognition theory of T cell receptors that differentiation macromolecules which occur only on the surface of Ia negative cells cannot be seen by T helper cells.

D. Givol, M. Gavish, R. Zakut, Y. Ben-Neriah, P. Lonai

Combining site and antigenic determinants of the V regions. Haematologia *12*, 15 (1978/1979).

Preparation of Fv fragment and its peptide chains V_L and V_H is described. The renaturation of completely unfolded V_L and V_H to generate an active Fv indicates that the variable domain, which is only a part of the peptide chains (L or H) contains all the information for its folding and generation of antibody combining site. Model building studies (together with Drs. Davies and Padlan, NIH) were attempted on the basis of known coordinates of other immunoglobulin V regions. The model was found to be compatible with the affinity labeling of protein 315 and with other studies on mapping the combining site. This mapping indicates that Trp 93L is the major residue interacting with the DNP hapten. Indeed V_L was found to contain the subsite for the DNP ring and its affinity to DNPOH is similar to that of Fv ($V_L + V_H$). On the other hand DNP haptens with longer side chains bind much better to Fv suggesting that the side chain of the hapten interacts predominantly with V_H. V_L exists as a dimer which can undergo conformational transition near pH 7. One conformer binds two haptens per dimer whereas the other binds only one hapten. Thus the combining site made up of two V_H chains is more flexible than that made up of $V_L + V_H$. Antibodies were prepared against V_L and V_H. These antibodies react with many different V regions on L chains (anti V_H) or H chains (anti V_H). Anti V_H reacts with isolated H chains much better than with intact Ig. These antibodies were used to detect V regions on T cells by employing inhibition of antigen binding to T cells. It was found that the inhibition by anti V_H and anti V_H affects different T cell populations. Anti-V_H inhibit the binding to Lyt-1^+ cells known also to be T helper cells whereas anti-V_L inhibit antigen binding to Lyt-2^+3^+ cells known to be suppressor cells. These results suggest that the expression of V regions on T cells is different from that on B cells. Probably in T cells there is unlinked expression of V_L and V_H and they are selectively expressed on different T cell subpopulations.

G. Klein

Cancer, viruses and environmental factors. Haematologia *12*, 25 (1978/1979).

The genesis of Burkitt's lymphoma is visualized as proceeding in three steps:
I. Primary EBV infection affects the young child, probably at a relatively high multiplicity. It immortalizes a certain number of B-lymphocytes *in vivo*.
II. This is followed by the impact of an environmental promoting agent, perhaps chronic holoendemic malaria, providing a chronic stimulus to the proliferation of the EBV-carrying preneoplastic cells.
III. Chromosomally abnormal variants appear in the stimulated tissue by chance. After certain types of changes, particularly the 8 to 14 translocation that leads to the 14q+ marker, the affected B lymphocyte would no longer obey the negative feedback controls that would otherwise restrict its proliferation *in vivo*.

G. Möller, L. Hammarström, E. Möller, U. Persson, E. Smith, D. Waterfield, E. Waterfield

Lymphocyte activation by Con A. Haematologia *12*, 37 (1978/1979).

Con A can activate cytotoxic effector cells, helper cells and suppressor cells. In all cases Con A activates polyclonally and thus the entire T cell repertoir can be revealed by Con A activation. Nylon purified T cells cannot be activated by Con A. However, in the presence of serum from several species, Con A activates purified T cells. The serum factors that are responsible for Con A activation of purified T cells are present in the albumin fraction. It is suggested that Ia molecules may be responsible for the ability of Con A to activate purified T cells and a general hypothesis for T cell activation is outlined.

D. R. Stanworth

Immune complexes and triggering of cellular mechanisms. Haematologia *12*, 47 (1978/1979).

Although there is growing awareness of the involvement of immune complexes in many diseases, little is known yet about their mode of interaction at the cellular level. Inevitably, therefore, the emphasis in this paper is on theoretical aspects of this fascinating topic; although, the ideas discussed are based on the one hand on a growing understanding of the molecular basis of mast cell triggering resulting from anaphylactical antibody-antigen interaction; and on the other from work in progress in my laboratory on the role of the antibody in the *in vitro* triggering of macrophages by antigen-antibody complexes. Crucial questions which are discussed in considering possible mechanisms of interaction between immune complexes and target cells include the possibility of distinguishing between receptors for monomer and aggregated antibody and between aggregate binding and target cell triggering functions. In other words, are certain types of antigen-antibody complexes capable of directly triggering non-sensitized target cells *via* a distinctive activation site; or is the Fc receptor multi-functional, with triggering merely involving an increase in association brought about by the cross-linking of antibody molecules by antigen?

J. Gergely

The biological role of immune complexes. Haematologia *12*, 55 (1978/1979).

The biological role of immune complexes (IC) is reviewed. The mechanism of IC elimination, the influence of IC composition on their biological properties as well as on their elimination are discussed. The regulatory effect of IC in immune phenomena is briefly summarized.

S. R. Hollán

Clinical significance of circulating immune complexes. Effect of plasmapheresis. Haematologia *12*, 69 (1978/1979).

1. The tissue damage in immune complex diseases is most probably caused by actively deposited immune complexes. But even in this disease entity circulating immune complexes may lack the capacity to trigger inflammatory reaction.

2. Potentially pathogenic immune complexes may have beneficial effect.

3. In many clinical disorders the high incidence of circulating immune complexes is most probably secondary to the underlying disease. In sarcoidosis, Crohn's disease and in some other chronic diseases the tissue damage together with an impaired function of the mononuclear phagocytic system is responsible for the circulating immune complexes.

4. The incidence of circulating immune complexes is high in different malignancies. The size and composition of the immune complexes change in the course of the disease. The presence of circulating immune complexes in malignancy is most probably due to their impaired elimination.

5. Methods applied for the detection of circulating immune complexes are all indirect assays and thus reveal *presumed* immune complexes. Direct techniques of measuring and characterizing immune complexes and methods giving better insight into their *in vivo* activity are badly needed.

6. Intensive plasmapheresis can be successfully applied in the treatment of some fulminant immune complex diseases and in disseminated melanoma. The dramatic response to plasmapheresis may be due, apart from the removal of circulating immune complexes, to the simultaneous reduction of other plasma constituents with high biologic activity and/or to the replacement of low affinity Ab-s with their high affinity counterparts.

G. Füst

The biological role of the complement system and the clinical importance of complement measurements. Haematologia *12*, 85 (1978/1979).

Properties of serum proteins belonging to the complement system, two pathways of the complement activation (classical and alternative pathway) as well as the physiological role of the complement system are discussed. Complement has essential importance in some physiological processes: In the induction of the humoral immune response, in the elimination of immune complexes and in the protection against bacterial and viral infections. After a short discussion of the genetics of the complement system, the principle and possibilities of clinical applications of the complement measurements are described. Finally, different approaches to the therapeutic manipulation of the complement system are discussed.

E. Klein, F. Vánky, B. M. Vose

Natural killer and tumor recognizing lymphocyte activity in tumor patients. Haematologia *12*, 107 (1978/1979).

Several mechanisms can lead to the killing of a target cell by lymphocytes. In order to study immunologically specific phenomena it has to be ensured that the natural killer (NK) effect does not operate in the system. Using targets which are sensitive to NK, the effector populations have to be depleted of lymphocytes with such potential. The blood lymphocytes of tumor carrying patients often have reduced NK activity. In two assay systems anti-tumor autoimmune reactivity has been demonstrated (the majority of tested patients had lung carcinomas or osteosarcomas). The tests were: 1. Induction of blastogenesis in blood lymphocytes by in vitro confrontation with autologous biopsy cells. 2. Lymphocyte mediated killing of autologous biopsy cells in short term in vitro assay. Cross reactivity between patients was rare which indicates either that the putative tumor antigens are individual specific or their recognition is restricted by histocompatibility.

T. Bakács, E. Klein, M. Steinitz, P. Gergely

Surface markers and functional characteristics of human blood lymphocytes residing in the operational "null" subset. Haematologia *12*, 113 (1978/1979).

After removal of nylon wool-adherent B and E rosetting T cells from the human blood lymphocyte population, a small fraction remains, often denoted as "null" subset. This fraction contains lymphocytes which are highly efficient in antibody-dependent and non-selective lymphocyte cytotoxicity. These cells possess markers. About half of them carry Fc receptors, and the majority has low avidity E and C3 receptors. Removal of low avidity E receptor-carrying cells did not abolish cytotoxicity to K-562 cells. The majority of cells carry receptors for Epstein-Barr virus but cannot be infected. The cells in mixed lymphocyte culture have a low reactivity. They are stimulated by PHA and ConA to a similar extent and in that they differ from the cells which readily sediment as E rosettes; these respond stronger to PHA. The "null" fraction may contain stem cells common for both T and B lineages.

G. Astaldi, Ü. Özger Topuz, M. A. Brunelli, A. Çavdar, G. P. Bagnara, J. Erten, U. Ertem

Acute leukaemia: cell markers and thymic factor on E-rosette frequency. Haematologia *12*, 127 (1978/1979).

At least theoretically, the so-called "null cell" acute leukaemias (AL-s) could form clones from either the uncommitted stem cell, or the differently committed stem cells, including the T cell progenitor or the T_0 lymphocyte. An attempt to single out different forms among the miscellaneous groups of the above-mentioned AL-s, as well as to schematize both hemic and lymphatic cell ontogeny has been made either with personal experiments by incubating peripheral blood and bone marrow cells from AL-s with human serum thymic factor, or on the basis of the recent informations derived by membrane phenotyping, assaying of specific enzyme markers, testing cell reaction to antisera raised against given cell types, etc. Results of the personal assay showed that incubation of AL cells with human serum thymic factor (which induces maturation of committed precursors to T cells), caused a clear-cut increase of E^+ cells in 6 out of 36 patients for the peripheral blood mononuclears, and in 3 out of 11 patients for the bone marrow lymphoid cells. It results that significant percentages of cells interpreted as "null cells" were rather quite immature, not yet E-rosetting T cells. The latter have been shifted to a more mature (E-rosetting) stage by the thymic factors. These results seem to substantiate the interpretation that the T cell precursor (or the T_0 cell) may undergo leukaemic clonal expansion, or that AL cases may be accompanied by a contemporaneous growth, and peripheral blood traffic of quite immature T cells.

P. Hernández, C. Cruz, N. L. Fernández, R. León, J. M. Ballester

T lymphocyte number and functions in paroxysmal nocturnal haemoglobinuria (PNH). A preliminary report. Haematologia *12*, 135 (1978/1979).

T-lymphocyte counts in the peripheral blood, lymphocyte response to phytohaemagglutinin (PHA) and delayed hypersensitivity reactions were studied in ten patients with paroxysmal nocturnal haemoglobinuria (PNH). Delayed hypersensitivity was abnormal in most patients and in vitro studies revealed impaired lymphocyte transformation to PHA in 50 percent of the cases. These tests gave evidence of a functional alteration of lymphocytes in some PNH patients. The hypothesis of a disorder originated in a pluripotent lymphohaematopoietic stem cell is suggested.

V. Stenszky, L. Kozma, I. Ambró, L. Karmazsin

Calculation of disease susceptibility gene frequency in insulin-dependent diabetes mellitus. Haematologia *12*, 141 (1978/1979).

An analysis of HLA-linked genetical factors conferring susceptibility to IDDM is reported. On the basis of population and family studies a recessive mode of inheritance of disease susceptibility provided by an assumption HLA-B8-linked DS gene was observed. The characteristic component of the immunogenetical background was the high frequency of HLA-B8 (0.208) and the HLA-A1, B8 haplotype (0.134) (linkage disequilibrium D = 0.1031), reminiscent of that found also in other disorders with autoimmune features, such as Graves disease, SLE, etc. Considering the HLA-B8 and IDDM association, the DS gene frequency (pD = 0.25) was estimated and the gametic association between HLA-B8 and DS gene was calculated. The low value of penetrancy (4.8%) revealed the important role of non-HLA-linked genetical and environmental factors. The HLA-linked genetical factors in question might be responsible for an inclination to several kinds of autoimmune disorders.

A. D. Pavlov, E. F. Morshchakova, N. M. Kalacheva

RNA synthesis in rabbit and rat kidney under experimental changes of erythropoiesis. Haematologia *12*, 149 (1978/1979).

RNA synthesis in rabbit and rat kidneys and erythropoietin level in plasma were studied under hypoxic stimuli (bleeding, phenylhydrazine and cobalt treatment, exposure to simulated altitude of 6000 m). An increase in renal RNA synthesis and in plasma erythropoietin activity was found. By using the "endocrine" kidney it was shown that 4-hr hypoxia produced a considerable increase in RNA synthesis both in the intact and in the "endocrine" kidney. The increase was correlated with the increase in the plasma titre of erythropoietin. The plasma titre of erythropoietin in rats having a single endocrine kidney also increased after 4-hr hypoxia. It is suggested that the renal biogenesis of erythropoietin under hypoxic stimuli involves, apparently, the mechanism of depression of specific RNA synthesis.

H. A. B. Al-Mondhiry

Aplastic anaemia in Iraq. A prospective study. Haematologia *12*, 159 (1978/1979).

The problem of aplastic anaemia (AA) in Iraq has not been previously investigated. This paper presents on 60 patients evaluated prospectively at the University of Baghdad Teaching Hospital during the period 1975—1978. Criteria for the diagnosis included pancytopenia and hypocellular or acellular bone marrow. A surprising finding at variance with published reports about the disease from other parts of the world was a 3 : 1 preponderance of males over females. A discernible aetiology of drug or chemical exposure was detected in half of the patients. Chloramphenicol, alone or in combination with other antibiotics, was thought to be responsible for marrow injury in 12 patients. The occurrence of AA seems to be on the rise in Iraq, probably as a result of the increased use of potentially toxic therapeutic agents and chemicals, and of the growing environmental pollution.

K. Worowski, S. Głowinski, Z. Skrzydlewski

The effect of basic proteins on the haemostatic system of the dog. Haematologia *12*, 165 (1978/1979).

Intravenous administration of histones or protamine to dogs resulted in a fall of the number of blood platelets, a lowered prothrombin consumption, a fall in the fibrinogen level, activation of the fibrinolytic system and deterioration of blood clot retraction.

O. Matsuo, T. Kawaguchi, T. Kosugi, H. Mihara

Role of the kidney in the inactivation rate constant of urokinase activity. Haematologia *12*, 169 (1978/1979).

The role of the kidney in the inactivation rate constant of UK activity (ke) was examined experimentally. The results obtained were as follows. There was a remarkable divergence in FA between the abdominal aorta and renal vein. When the bilateral renal arteries and veins were ligated, ke was 0.38, in contrast to 0.79 in the normal state. When UK was infused into rabbits with ligated renal vessels, the FA lasted for an increased period after the termination of UK infusion. These findings suggest that the kidney functions as an inactivator of UK.

A. Spinelli, J. Kunz

Amniotic fluid and blood coagulation. Haematologia *12*, 175 (1978/1979).

A study was made of the incidence and activity of coagulation factors in normal amniotic fluids. According to the results, two groups of amniotic fluid can be distinguished. To group A belong those that are transparent, showing an activity similar to Russell's viper venom. To group B belong the fluids that are milky and translucent. In this second category, factor VIII seems to be a necessary component in order to obtain maximal coagulation activity. The clinical consequences of these facts are discussed.

G. M. Janković, S. Janošević

Correction by factor VIII of decreased neutrophil alkaline phosphatase activity in classic haemophilia and von Willebrand's disease. Haematologia *12*, 183 (1978/1979).

Neutrophil alkaline phosphatase (NAP) score was estimated in ten patients with hereditary disorders in which antihaemophilic factor (factor VIII) is functionally deficient. NAP scores were estimated immediately before cryoprecipitate was administered, and following correction of patients' clinical condition and factor VIII activity in their plasma. While lower than normal NAP scores were observed before the treatment has been started, normal NAP score estimates were obtained following the successful correction of decreased procoagulant factor VIII ($F_{VIII}C$) activity in plasma of patients with classic haemophilia and von Willebrand's disease (vWd).

T. Sylwestrowicz, K. Sokołowska

Serum thrombocytopoietic activity in thrombocytopenia. Haematologia *12*, 189 (1978/1979).

By means of a bioassay performed in rats utilizing ^{75}Se-selenomethionine as a platelet marker *in vivo*, serum thrombocytopoietic activity (STA) was determined in 9 healthy volunteers and in 82 patients with thrombocytopenia, viz. 27 cases of ITP (9 acute and 18 chronic), 19 of aplastic anaemia, 19 of acute leukaemia and 17 of chronic proliferative disorders. STA was the highest in the ITP groups ($p < 0.01$), particularly in the cases of short duration ($p < 0.001$). In acute leukaemia, the STA was remarkably elevated ($p < 0.02$) and a negative correlation could be demonstrated. Such a negative correlation of the platelets with ITP, thus with the duration of thrombocytopenia, was also found. No significant increase of STA was observed in patients with ITP after corticosteroid administration. In 5 out of 7 cases who responded satisfactorily to splenectomy a significant fall of STA was demonstrated after surgery. A slight, but statistically significant, rise of the STA value was found in patients with aplastic anaemia ($p < 0.05$). No STA elevation was observed in the cases of chronic proliferative disorders with thrombocytopenia.

P. Szerze, Cs. Balázs, J. Csongor, J. Nagy, A. Leövey

Investigation of the chemiluminescence associated with phagocytotic and intracellular killing activity of human polymorphonuclear leucocytes and monocytes. Haematologia *12*, 199 (1978/1979).

The chemiluminescence associated with phagocytotic and intracellular killing activity of human polymorphonuclear leucocytes and monocytes was investigated. A significant, opsonization-dependent increase in photon emission of polymorphonuclear leucocytes and monocytes was observed induced by engulfment of opsonized, heat-killed and living fungi *(Saccharomyces cerevisiae)*, aggregated gamma globulin, latex and India ink particles. Mononuclear cells displayed only minimal enhancement of photon emission after incubation of various particles. It is suggested that the method can be used in clinical practice as a test of phagocytotic and intracellular killing function.

J. M. Mishler IV

The uptake of ^{3}H-dexamethasone during phagocytosis of Staphylococcus aureus by human neutrophils. Haematologia *12*, 209 (1978/1979).

In the normal human neutrophil under basal *resting* conditions, cell-associated levels of ^{3}H-Dexamethasome (^{3}H-DXM) increased in a linear fashion following addition of the drug. However, during phagocytosis of ^{14}C-labelled *Staphylococcus aureus (Staph. aureus)*, the intracellular ^{3}H-DXM levels rose above basal concentrations in direct relation to the quantity of ingested organisms. The observation that the cell-associated activity of ^{3}H-DXM rises in a parallel fashion to the quantity of ingested *Staph. aureus* must be recognised and taken into consideration as a variable, when assessing the results between *resting* and *activated* phases of neutrophil function in the presence of glucocorticoids.

J. Janele, V. Brychnáč, V. Lodrová

Sea-blue histiocyte syndrome with bone anomalies. Haematologia *12*, 215 (1978/1979).

Two sisters, now 29 and 24 years old, are described. They presented a congenital storage of, most probably, phospholipids in the histiocytes of the sea-blue type or blue pigmentophages. The granules of these cells showed a PAS positivity and strong positivity for acid phosphatase, but they were negative also for non-specific esterase, naphthol-AS-D-chloroacetate esterase and iron acid also for urine mucopolysaccharide. The two cases differed from all the described cases in the absence of hepatosplenomegaly and in the presence of bone changes resembling late spondyloepiphyseal dysplasia or atypical dysostosis multiplex.

E. Fagiolo, G. Tosato

IgM plasmacytoma. Report of a case and review of the literature. Haematologia *12*, 221 (1978/1979).

IgM paraproteinaemia is described in a patient with diffuse plasmacytoma involving the pleura, lymph nodes, and kidneys. Sixteen cases of pure plasmacellular tumour (diffuse or solitary plasmacytoma and plasmacellular leukaemia) associated with IgM paraproteinaemia have only been reported. These cases and experimental data indicate that neoplastic plasma cells can synthetize IgM as well as other immunoglobulins and that this particular M component is not associated solely with Waldenström's macroglobulinaemia.

E. Heilmann, B. Schreck

Prognosis of chronic lymphatic leukaemia. Haematologia *12*, 231 (1978/1979).

In a retrospective study 117 patients with chronic lymphatic leukaemia (CLL) were classified according to Rai and analyzed with regard to their survival time. With increasing severity of the stage of the disease the prognosis becomes worse. A change of the stage was always in the sense of progredience of CLL. Untreated patients had the longest survival time. Among the various forms of treatment, monotherapy with corticosteroids had the worst, local irradiation therapy the best prognosis.

E. Heilmann, B. Schreck

Chronic lymphatic leukaemia and malignant tumours. Haematologia *12*, 247 (1978/1979).

Ten of 117 patients with chronic lymphatic leukaemia (CLL) showed malignant tumours. In 2 cases a double carcinoma was diagnosed. The frequency of carcinoma increased with the progredience of the stage of disease classified according to Rai et al. The observations of the authors are compared with papers published in the literature.

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