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Kinetics of Reactions within Single Erythrocytes: Studies by Microspectrophotometry

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Single cell spectroscopy has been applied to red blood cells along three different lines of research: a) distribution of single hemoglobin components among different erythrocytes, b) chemical reaction with ligands and diffusion in the red blood cell, c) dynamics of hemoglobin S aggregation in single red blood cell.

The results reported here show, in general, the great possibilities of using single-cell spectroscopy in investigating complex integrated systems.

Introduction

The classical approach to quantitative studies of biochemical phenomena has involved, generally, the isolation of individual components from cells or tissues and the measurement of their reactions in solutions of known composition. At present, however, advances in various fields of science have promoted attempts to study directly dynamic events occurring in intact cells.

While classical microscopy provides a time independent, static picture of biological structures, combination of high resolution microscopy with spectrophotometry offers a very powerful approach to the study of intracellular phenomena in living cells, and the possibility to follow directly time dependent changes of intracellular components.

The present review summarizes the results recently obtained in the course of a study of single erythrocytes investigated by scanning microspectrophotometry [1–3]. The time constant of the apparatus is low enough to allow measurements of rapid phenomena (half-time of a few milliseconds) and a cross illumination device permits to activate gas exchange reactions making use of the photosensitivity of the carbon monoxide hemoglobin complex. The examples reported below provide basic information on several reactions involving red cells and illustrate the future potentialities of this approach.

Studies on red cells by microspectrophotometry have been conducted, up to now, along three main lines [1–3]:

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i) Investigation of the distribution of hemoglobin components among single erythrocytes in cases in which the hemolysate shows hemoglobin heterogeneity. In the case of trout's blood it was possible to show that the main hemoglobin component is present in the same proportion in different erythrocytes.

ii) Studies on the kinetics of CO and O_2 exchange in single cells. As mentioned above, these studies are feasible due to the photosensitivity of the carbon monoxide hemoglobin complex.

iii) Investigation of changes in the shape of erythrocytes following removal of ligands from intracellular hemoglobin. Also in this case, removal of the ligand (CO) is achieved by a photodissociation method. This approach appears to be specially promising to follow the dynamics of sickling in erythrocytes containing hemoglobin S (HbS).

In what follows a brief description of the apparatus and of the main results obtained along the lines just outlined will be given.

Methods

The measurements on single living erythrocytes were carried out with the aid of a versatile, computer assisted, laboratory instrument based on the original "moving condenser" scanning method, described elsewhere [4, 5]. Figure 1 depicts the main optical parts along with the associated driving electronics and processing equipment.

In this approach, contrasting with the traditional "moving stage" technique, the specimen is maintained stationary since the positional scanning operation is afforded by means of a light condensing objective which is electrodinamically displaced across a plane parallel to the specimen itself (Fig. 1). Studies on living, suspended and degradable materials take significant advantage of this feature which moreover permits much higher speeds and random positioning. "Dual beam" operation and variable resolution area scanning are possible, in this case, while not generally available with other techniques.

The modular and flexible optical set-up can be reconfigured for different microspectroscopic investigations and specific measuring procedures are accordingly programmable under computer control.

Due to the versatility resulting from the cited characteristics, the apparatus was also extensively used in other fields of cellular spectroscopy, ranging from photopigment studies to DNA measurements [6–9].

Among the principal programmable conditions, relevant to the configuration used in absorption microphotometry, are the following:

1) Positional scanning: 500 × 500 μm coverage, 0.25 μm resolution, 7 msec max. Settling time (random) or 5 s typical exploration time (meander scan) for 100 μm × 100 μm area and 100 × 100 points, 1 μm apart;

2) Wavelength scanning: 360–760 nm coverage, 2.5 nm resolution, 10 ms max. settling time (random) or 0.5 s typical exploration time (sweep) for the complete spectrum.

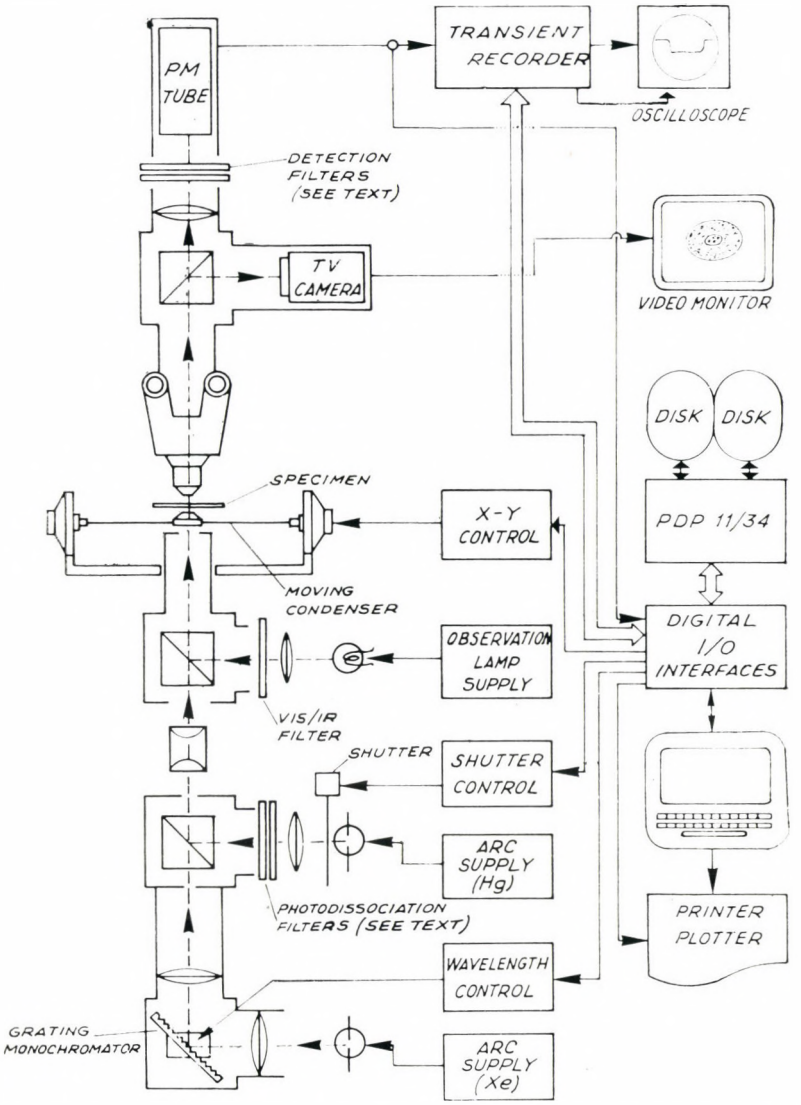

Fig. 1. Configuration of the apparatus as used for the experiments described in this paper. Xenon arc lamp XBO75W2; Absorption filter (Schott, BG38,4 mm), Interference filter (Balzers, λ = 546 nm); Mercury arc lamp, HBO 100 WZ; Photomultiplier tube (EMI 965 88); Interference filter (Balzers FITC 3)

3) Fine electrical focusing: 200 μm coverage approx.

The photometric accuracy, across the useful spectral range, is such to allow signal-to-noise ratios better than 10^3 when measuring optical densities in the range 0 to 1 and using 1 s averaging time. Low specimen irradiation doses may be employed, if needed, due to the efficiency of punctual specimen illumination in the analyzing beam.

Provisions exist to introduce additional source(s) to be used in photoexcitation experiments (such as the photodissociation mode employed with erythrocytes).

The optical set-up is connected "on-line" to the computer real time control, for acquisition and processing, in order to achieve interactive operation and data presentation.

Infra-red/visible closed circuit tv camera and CRT display permit continuous monitoring of specimen conditions, during the measurement, without disturbing to the experiment.

Among the principal operational capabilities, are the following:

1) x; y scanning to produce gray-level, pseudo-color or isometric mapping of spatial optical properties of the specimen;

2) Dual beam measurements, with alternating position of the analysis beam between "test" and "reference" locations, to obtain absorbence information corrected against wavelength dependent source emission, optics transmission and photodetector response (e.g. optical density spectra *vs.* the wavelength);

3) Combination of both 1) and 2);

4) Multicomponent detection (punctual or image), using several selected wavelengths, by means of linear processing procedures;

5) Photoexcitation spectrophotometry for studying the optical properties of the transient species confined in cellular structures, with excitation responses down to 1 ms (conditioned by aperture time of mechanical shutters).

Applications

Distribution of functionally different hemoglobins: The case of trout hemoglobins

A problem which arises in the case of blood containing multiple hemoglobins is concerned with the distribution of these various components among red blood cells [1]. In fact either (i) all the components are represented in each single erythrocyte in the same proportion as in the hemolysate, or (ii) the different hemoglobins are preferentially distributed and, in the limiting case, each erythrocyte contains only one of the components. This problem is relevant to the distribution of abnormal hemoglobins in heterozygotes, also considering that the segregation of some altered hemoglobin may affect the stability and thus the life span of the erythrocyte.

In the case of the blood from trout *Salmo irideus*, which contains four distinct hemoglobin components characterized by different functional and physicochemical properties [10], we have solved this problem by microspectrophotometry

[1]. Thus, the major hemoglobin component from trout blood, i. e. Trout Hb IV, shows a very marked Bohr effect and at pH values below 7 it is only partially saturated in air (Root effect). Taking advantage of this effect which is an exclusive property of Trout Hb IV and carrying out spectroscopic observations on single red blood cells it has been demonstrated that all erythrocytes contain Trout Hb IV in a proportion very similar to that found in the hemolysate. The dependence on pH of the fractional saturation with O_2 of whole blood as observed in air ($pO_2 \sim 155$ mmHg) at 20 °C, indicates that at pH $\simeq$ 6 the overall saturation corresponds to about 50%. This is in reasonable agreement with expectations based on the O_2 binding properties and the relative percentages of the various components (components I and II are fully saturated at every pH value, component IV is only 20% saturated, component III is present only in 1–2%).

The spectral properties of single erythrocytes reported in Figure 2 show that: (i) at pH 7.6, where all the components are fully oxygenated in air, the spectroscopic properties of a single erythrocyte correspond to the well-known spectrum of oxyhemoglobin; (ii) at pH 6, where the whole blood is 50% saturated in air, the pigment within a single cell is only partially saturated.

From these spectra taken on a number of erythrocytes from different preparations it was calculated that, in air at pH 6, the fractional saturation with O_2 of the different individual erythrocytes corresponds, within experimental error, to that of whole blood. This implies unequivocally that each red blood cell contains Trout Hb IV in a proportion very similar to that of the hemolysate.

The results show, in general, the great potentialities of using single cell spectroscopy in investigating this type of problem in other cases where multiple hemoglobins are present in blood.

Kinetics of gas exchange in human erythrocytes
Carbon monoxide binding

Single cell spectroscopy has been applied to reinvestigate the coupled processes of diffusion of gases inside the cell and their combination with hemoglobin [2, 3]. The photosensitivity of the carbon monoxide derivative of hemoglobin allows dynamic spectrophotometric observation of single cells within which reversible dissociation of HbCO is induced by light, as reported in the following scheme:

$$\mathrm{HbCO} \xrightarrow{h\nu} \mathrm{Hb} + \mathrm{CO} \quad (1A)$$

$$\mathrm{Hb} + \mathrm{CO} \xrightarrow{\text{dark}} \mathrm{HbCO} \quad (1B)$$

Photodissociation of intraerythrocytic hemoglobin is obtained using an Hg lamp (546 nm line) as the excitation source, and a suitable filter combination in order to eliminate interferences with the analyzing beam. A number of control experiments, performed by cutting the intensity of the photodissociating beam by known amounts, have demonstrated that the intensity of the stationary light used is sufficient to produce complete photodissociation of the ligand, i.e. to drive reaction 1A completely to the right. It should be remarked that the conditions used for these

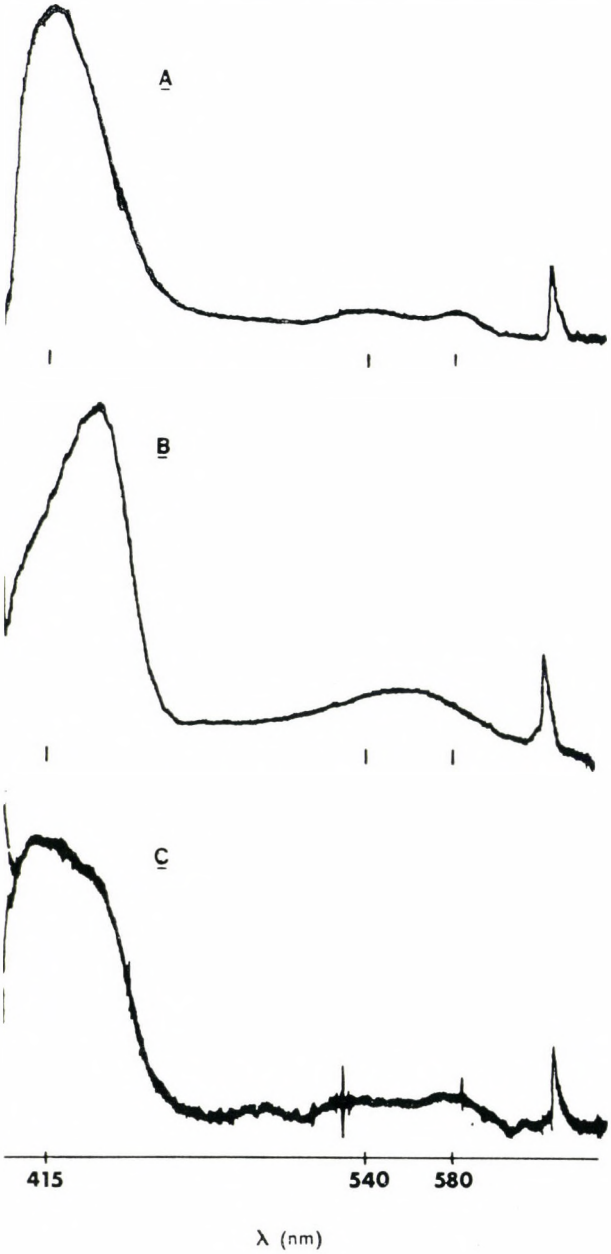

Fig. 2. Absorption spectra of single erythrocytes of trout at: *(a)* pH = 7.6; *(b)* the same + dithionite; *(c)* at pH = 6.0 (from [1] modified)

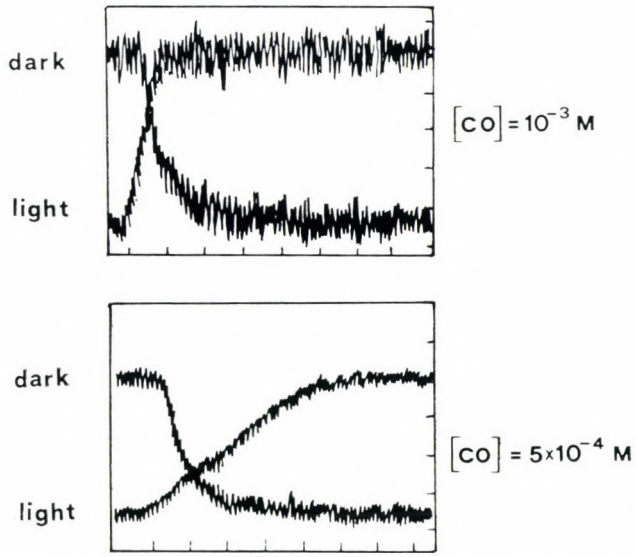

Fig. 3. Relaxation traces of experiments performed at pH = 7.2, λ = 420 nm, temperature 25 °C, sweep = 100 ms/grid division (from [3] modified)

kinetic studies are such that on the average, each erythrocyte is separated from the others by a large distance, i.e. is immersed in a volume of stagnant fluid which is infinite as compared to the volume of the cell.

The kinetics of CO release and uptake going from dark to light (1A) and vice versa (1B) has been studied for a number of erythrocytes as a function of CO concentration, ranging from 10^{-4}–10^{-3} M. Oscillograph traces shown in Figure 3 document the time courses of the two processes.

The main results obtained by these experiments may be summarized as follows:

i) No significant differences in the kinetic properties of several individual cells, and within any single cell among its different spatial regions, were observed.

ii) The approach to the steady state in the light cannot be accounted for by a single exponential process and its half-time is independent of CO concentration (10^{-4}–10^{-3} M) within a factor of two.

iii) The relaxation from the steady state in the light to the equilibrium in the dark corresponds, under all conditions explored, to a zero-order process. The reciprocal of the half-time increases linearly with CO in the same concentration range. This body of results shows that combination of CO with hemoglobin within immobile erythrocytes is about 30-fold slower than that measured with hemoglobin in solution and an order of magnitude smaller than that obtained by stopped flow experiments with red cells [2]. Thus combination of CO with Hb in immobile erythrocyte is rate limited by a diffusive process, as clearly indicated by the zero-order

time course of the observed absorbence change. This finding has been related [2, 3] to the presence around the erythrocyte of an unstirred layer of fluid, whose thickness has been calculated, on the basis of simple diffusion laws, to be between 6–10 μM.

Oxygen binding

The presence around the erythrocyte of an unstirred layer of extracellular fluid which offers a diffusion barrier to the gas exchange should be tested with the physiologically important ligand, i.e. oxygen. In view of the low quantum yield for the photodissociation of HbO_2, experiments have been carried out in the presence of two ligands (O_2 and CO) at different ratios (0.02–0.7 = $[O_2]/[CO]$) [11]. Hence erythrocytes are suspended in isotonic solutions containing both oxygen and carbon monoxide. Under these conditions, in view of the much higher affinity of hemoglobin for CO, the erythrocytes in the dark are fully saturated with carbon monoxide; on the other hand in the presence of a stationary light flux, photodissociation of bound carbon monoxide occurs and combination with oxygen will follow.

An example of this type of experiments is illustrated in Figure 4. Compared to the behaviour of hemoglobin in solution, one essential difference is immediately apparent from examination of the experiment in so far as the approach to the steady state in the light does not yield *directly* oxygenated hemoglobin, *via* an exponential process (see [11]). Instead, irradiation with a steady flux of light is followed by three distinct phases:

a) the formation of deoxyhemoglobin as a transient which is produced with a half-time ($t_1/_2$) of ~ 50 ms (phase a in Figure 4);

b) the combination of oxygen with the photoproduct, i.e. intraerythrocytic deoxyhemoglobin (phase b in Figure 4);

c) the establishment of a steady state corresponding to fully oxygenated hemoglobin (phase c in Figure 4).

When the light is turned off, the relaxation in the dark represents the replacement of oxygen by carbon monoxide and the return to the original absorbence level (phase d in Figure 4).

The overall features of oxygen recombination with the transient intraerythrocytic deoxy-hemoglobin have some similarities with those reported above for the recombination with carbon monoxide. Thus, analysis of the data allows to draw the following conclusions:

a) The time course of the binding with oxygen, which is sucked into the red blood cell from the surroundings follows zero-order kinetics. This indicates that the rate of disappearance of deoxy-hemoglobin from the volume element under observation is rate limited by a diffusional process.

b) the half-time for oxygen binding ($t_1/_2$ = 60 ms) is 600-fold slower than that expected from solution measurement ($t_1/_2$ = 0.1 ms) and also much slower than that observed with a population of erythrocytes in rapid mixing experiments.

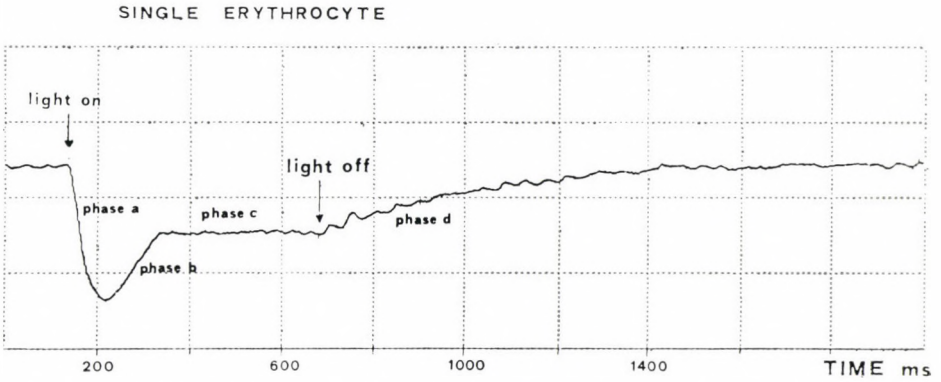

Fig. 4. Oscillograph trace of an experiment performed at pH = 7.2 and T = 25 °C. $[O_2]$ = = 1.7×10^{-5} M, [CO] = 9.6×10^{-4} M. Observation wavelength λ = 440 nm, sweep = 200 ms/grid division (from [11] modified)

Apart from the common features, it should be emphasized that the half-time of oxygen combination is significantly faster (10 times) than that observed in the case of carbon monoxide. Since the diffusion coefficient of the two ($D = 2 \times 10^{-5}$ $cm^2\ s^{-1}$) gases is essentially the same, the faster combination with oxygen may tentatively be related to the process of facilitated oxygen diffusion [12] which is operative from the external boundary to the observation spot. It seems therefore possible that diffusion of oxygen to that volume of deoxyhemoglobin included in the observation beam is considerably faster than the physical diffusion.

Kinetics of shape changes: Observations on sickling erythrocytes

The photochemical approach described above has been employed to investigate the phenomenon of sickling within red blood cells containing over 90% HbS [3, 13]. This is shown in Figure 5 where carbon monoxide dissociation induced by light triggers off the sickling process as it is evident from cell deformation.

Available experiments indicate that the rate of the shape change following removal of the ligand is approx. 4 s in all the cell examined with a range of 3–5 s. Moreover it was found that cells in which sickling was induced several times were always deformed along the same axis. This finding has not been interpreted, but it may be related either to an alteration of the RBC membrane, or to the presence of residual unmelted polymers which may act as templates inducing a preferential direction of sickling.

The photochemical approach described above has also been applied to a preliminary study of possible antisickling effect of chemicals. Microspectrophotometric observations were carried out on single cells of HbS carriers after exposure of the RBC to a reagent specific for SH groups [13], i.e. L-cystine dimethyl ester.

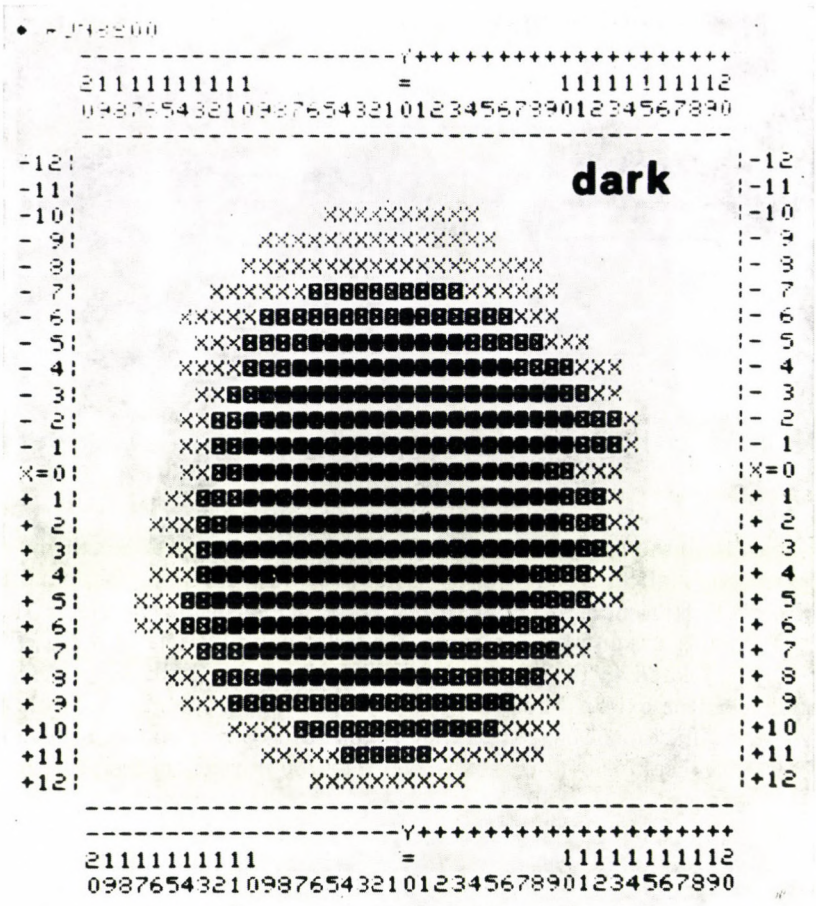

Fig. 5. Absorption maps of a single "ss" erythrocyte in the dark *(a)* and in the light *(b)*. Observation wavelength $\lambda = 430$ nm. The scanning has been carried out over an area of 25 μm × 25 μm

It was possible to demonstrate that this reagent, whose toxicity is relatively low, displays antisickling properties in so far as the morphology of the treated cells shows no deformation after complete photodissociation.

Conclusions and Perspectives

The application of dynamic single-cell microspectrophotometry to the study of the reactions of intraerythrocytic hemoglobin, although just begun, has already proven to be of outmost importance. The examples reported above serve as an in-

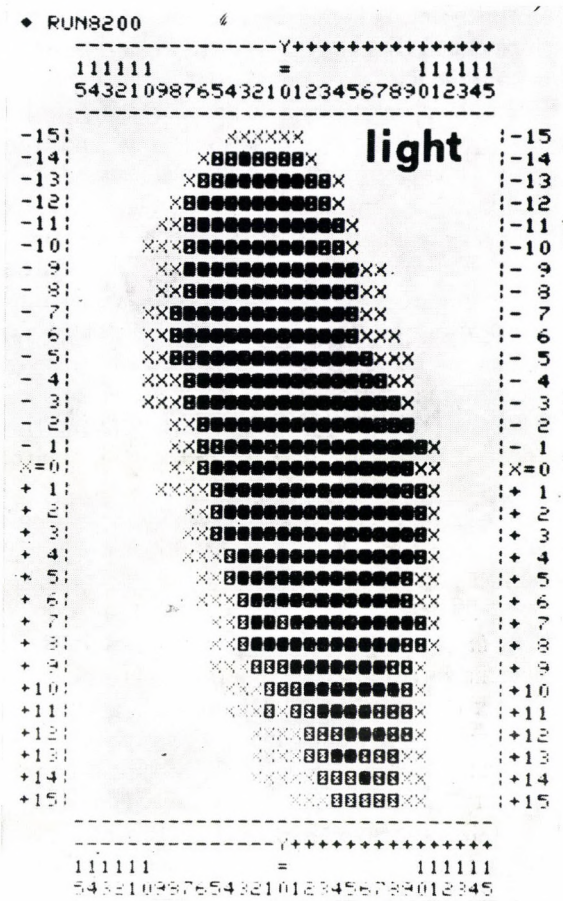

Fig. 5 *(b)*

dication of the potentialities of this approach, which may allow to bridge the gap between the study of phenomena with isolated macromolecules *in vitro* with reactions of the red blood cell as an integrated system. Although the possible applications, even within the restricted boundaries of the red blood cell, are numerous and therefore will not be extensively reviewed, some of the more relevant short-term perspectives are worth discussing.

From the physiological standpoint, understanding of the real significance of the unstirred-solvent layer surrounding the RBC is of high priority. The thickness of this layer as a function of the flow velocity needs to be investigated, since it is known [14] that with turbulent flow the RBC is largely stripped of externally immobilized solvent. Therefore, although the rate-limiting barrier may become insignificant at the blood velocities prevailing in the body, no direct information is

indeed available on this point. It is therefore essential to determine, using the approach originally introduced by us [2, 3, 11], the thickness or consistency of the diffusion barrier around the RBC as a function of flow velocity of the surrounding medium. This information is experimentally accessible and should provide results immediately relevant to the physiology of gas exchange and transport.

Along these lines a second important point is concerned with the understanding of the real physiological significance of the O_2 facilitated diffusion. It is well documented [12] that Hb facilitates O_2 transport by a translational-diffusion contribution, and at the protein concentrations prevailing in the RBC this should be an important contribution to the dynamics of the gas exchange within the cell. The dependence of the observed rate on O_2 concentration, as determined in preliminary experiments on the kinetics in single RBC [11], seems to demand that facilitated diffusion makes a significant contribution. It seems therefore possible to assess the real *in vivo* significance of this phenomenon by performing this type of kinetic measurements and by following *simultaneously* the reaction time course in different volume elements of the RBC.

Finally it may be recalled that the HbS polymerization mechanism still demands quantitative *in vivo* tests. The amount of experimental data on the purified HbS has been sufficient to propose and substantiate a "nucleation-polymerization" mechanism for HbS gelation [15, 16]. A critical test for the applicability of the same mechanism *in vivo* demands the measurement of the delay-time within single RBC cells, and the variability of this parameter within a population of cells varying individually, for example in their Hb content. The photolytic approach is obviously the only one presently applicable to this problem, since the delay-time at the protein concentrations prevailing in the RBC should be very short (10–100 ms). Indeed preliminary results by Eaton and coworkers [17] indicate that the problem is experimentally accessible. The final goal is the characterization of the series of events leading to fiber formation, which should follow a sequential time course involving at least the following steps:

$$\mathrm{HbSCO} \xrightarrow[\mathrm{CO}]{h\nu} \mathrm{HbS} \longrightarrow (\mathrm{HbS})_{\mathrm{nucleous}} \longrightarrow (\mathrm{HbS})_{\mathrm{Fibers}} \longrightarrow \text{Cell deformation.}$$

The effect of antisickling drugs on the various steps of this pathway is of utmost importance for any attempts towards a molecular therapy of sickle cell anemia.

It should be realized that some of these perspective investigations in single RBC microspectrophotometry are already within reach. However some of the more sophisticated and interesting developments, such as simultaneous time and space resolution of Hb reactions, demand considerable improvement and technical development, and therefore substantial efforts in instrument design and engineering. Within this area, foreseeable important steps should be dealing with improvements in handling and control of the sample (such as flow velocity, temperature and eventually pressure control) to vary at will the "boundary conditions" of the cell.

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Aims and Scope of Standardization in Haematology

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In haematology, as in all other aspects of laboratory practice, reliable measurement and interlaboratory comparability depend on standardization. Reference preparations and defined reference materials are essential for calibrating instruments used for tests and for verifying accuracy of assays. WHO is responsible for establishing international biological standards and other forms of standardization for use in medicine in general; at a specialized level in haematology, work on this subject has also been largely undertaken by the International Committee for Standardization in Haematology. ICSH established the International Reference Preparation of Haemiglobincyanide which has resulted in remarkable improvement in haemoglobinometry world wide. In addition, reference preparations are now available (from one or other source) for thromboplastin, various coagulation factors, abnormal haemoglobins, HbA_2 and HbF, erythropoietin, serum iron, vitamin B_{12}, various blood typing sera. Progress is being made in developing blood cell standards, and an ICSH expert panel has recently developed a ferritin standard.

Standardization also refers to establishing *reference methods* with a high level of accuracy and precision, and *selected methods* which are suitable for routine use taking account of the intended scope of the test, economy of labour and materials, and ease of operation. ICSH has been particularly concerned with establishing reference methods. *Inter alia*, these have included haemoglobinometry, prothrombin time testing, identification of abnormal haemoglobins, serum iron assay, Romanowsky staining, ESR, packed cell volume, diagnostic tests with radio-isotopes, antiglobulin test, specimen collection. Standardization of nomenclature is also an important topic to ensure understandable communication and to avoid confusion which may even put patients' lives at risk. To establish a standard requires a collaborative study and consultation with experts from many countries. To ensure effective subsequent use of the standard requires dissemination of relevant information, its acceptance at national level and technical training programmes in which its use is encouraged. International professional bodies, such as ICSH, as well as WHO, have important roles in achieving this.

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Biological standardization came from the need to define adequate potency of antisera used in therapy, and to ensure uniform potency of preparations from different commercial sources. Its scientific basis was established by Paul Ehrlich in 1897 when he published the results of a study on the standardization of diphtheria antiserum which he had undertaken at the request of the German Board of Health [1]. Standardization and control of the potency of pharmaceutical drugs

began 5–10 years later with the work of Elijah Houghton in USA for Parke Davis and Co., and in Britain by Henry Dale who was then working for Burroughs Wellcome [2]. This apparently mundane routine work led to a long line of fundamental scientific discoveries and to an awareness of the need for biomedical standards at an international level.

The mechanism for international biological standardization was established under the aegis of the Health Organization of the League of Nations, and subsequently expanded by the World Health Organization; it has worked harmoniously and effectively and continues to do so [3]. More recently, standardization has been further developed beyond therapeutic substances into the field of diagnostic laboratory tests. The purpose of this type of standardization is to ensure precision and accuracy of analytic measurements. An acceptable level of precision is relatively easy to attain by replicate testing of control materials, and by constant performance of internal quality control procedures. But precision alone can only assure us that results from a laboratory are reproducible within that laboratory. This is not sufficient to serve the needs of present-day practice – patients travel and may require successive tests in different laboratories; interlaboratory collaboration in therapeutic trials requires exchange of patient data between laboratories; reference values should be universally applicable, as also should be published data from clinical research. We, thus, need to ensure that analytic results are sufficiently accurate for interlaboratory comparability to be valid. This can only be achieved by the use of internationally agreed standards. In this context, a standard may have different forms. It may be a reference preparation, a calibration procedure, or it may be a reference method for performing the test. These different aspects of standardization may be described by the following definitions which have, in general, been adopted by various international organizations including WHO:

> *Reference preparation:* A substance which has been characterized by biological, chemical or physical means. *International Biological Standards and International Reference Preparations* are products to which have been assigned values or units of activity as defined by the World Health Organization. These international standards are not intended to be used in laboratory working procedures but serve as materials by means of which national reference preparations and calibrators can be controlled. International reference preparations are usually made available only to national authorities.

> *Reference material:* A substance or physical device, one or more properties of which have been defined by means of a definitive or reference method. It is to be used for the verification of the accuracy of an analytical process (measurement system) used in routine practice. Reference materials should be based on or traceable to a national certified reference material or an international (certified) reference preparation.

Definitive method: A method which after exhaustive investigations is found to have no known source of inaccuracy or ambiguity. It will, however, have a (known) degree of imprecision which should be stated.

Reference method: A clearly and exactly described technique for a particular determination which, in the opinion of a competent authority, provides sufficiently accurate and precise laboratory data for it to be used to assess the validity of other laboratory methods for this determination. The accuracy of the reference method must be established by comparison with a definitive method where one exists.

An international reference method is one that has been established by a competent international authority.

A reference reagent is one which has defined and clearly described properties; it is used with a reference method or procedure, or when appropriate, in conjunction with an international reference preparation.

Selected method: A procedure, the reliability of which has been validated by a collaborative study and which is recommended by a competent authority for routine use in laboratory analysis, having been selected on the grounds of its accuracy and precision, the intended scope of the test, economy of labour and materials, and ease of operation.

Calibrator: A substance or device used to calibrate, graduate or adjust a measurement. It must be traceable to a national or international reference preparation or reference material.

Calibration: The determination of a bias conversion factor of an analytical process under specified conditions, in order to obtain accurate measurement results. The accuracy over the operating range must be established by appropriate use of reference methods, reference materials and/or calibrators.

By universal agreement of national governments, WHO, acting through its Expert Committee on Biological Standardization is responsible for "establishing international biological standards, reference preparations and reference reagents for those biological materials used in prophylaxis, therapy or diagnosis of human (and some animal) diseases that cannot be characterized adequately by chemical and/or physical means alone" [4]. The biological standards and reference preparations are provided primarily to enable the activity of biological preparations to be designated in uniform terms throughout the world; the prime function of the reagents is to check on specificity. The Expert Committee does not itself produce the standards. These can be developed by any group of scientific workers, and the role of the Expert Committee has been to formulate procedural guidelines which take account of many details which must be rigidly adhered to if

a preparation is to be acceptable to WHO. Thus, for example, material must be dispensed from a single homogeneous bulk of material into ampoules made from high quality neutral glass; an international collaborative study must be carried out in an agreed way by two or more laboratories in different countries, and there must then be a collaborative assay by a sufficient number of laboratories in different countries to ensure statistical validity and accuracy of measurement.

When the report on a collaborative assay has been approved by all participants, it is sent for comment and review to the members of the Expert Advisory Panel on Biological Standardization and to other international experts selected by WHO. When any queries and criticisms have been satisfactorily answered, the preparation is ready for recognition by WHO. Clearly, establishing a WHO international reference preparation is a lengthy and onerous task but the end result is a significant contribution to reliable health care, to clinical practice and to medical science in general.

Over the past few years an increasing number of other organizations have also become active in biomedical standardization. A recent meeting under the aegis of WHO identified at least 28 such organizations; they function in all the specialities, each with its own constitution and procedures, albeit all with the same ultimate objectives. Some organizations are concerned only with design and specification of apparatus, others with methodologies, and others again with reference materials. Some have legal authority, whilst others are professional and essentially educative. There is need to have some co-ordination and harmonization but not at the cost of the individuality of the organizations, which contribute, each in its own way, to the science and practice of clinical laboratory standardization. The following is not a comprehensive list but it indicates some organizations well known for their work in standardization, including those referred to in this paper, which are subsequently designated by their initials:

BCR	Community Bureau of Reference (EEC)
BSI	British Standards Institute
CDC	Center for Disease Control (USA)
COWS/WASP	Commission on World Standards of World Association of Societies of Pathology
ECCLS	European Committee for Clinical Laboratory Standards
IABS	International Association of Biological Standardization
ICSH	International Committee for Standardization in Haematology
ICTH	International Committee on Thrombosis and Haemostasis
IFCC	International Federation of Clinical Chemistry
ISO	International Standards Organization
IUIS	International Union of Immunological Standards (USA)
NBS	National Bureau of Standards (USA)
NCCLS	National Committee for Clinical Laboratory Standards (USA)
NIBSC	National Institute of Biological Standards and Control (UK)
WHO	World Health Organization

In haematology, standardization has largely been undertaken by ICSH. The need for it in this speciality was obvious from the confusion which existed in the measurement of haemoglobin. For over one hundred years, haemoglobin has been used in clinical practice and in public health surveys as an index of health. By the beginning of this century, several methods had been developed, each with its own calibration scale, none of which was comparable. Thus, 100% was 13 g/dl by the Haldane method and 17 g/dl by the Sahli method. The need for some form of standardization had been appreciated by Bürker in 1911 [5] when he tried to establish a stable standard solution for the Sahli method, and then by Heilmeyer who, in 1933, established on behalf of the German Society of Internal Medicine a scheme for haemoglobin standardization in Central Europe [6]. During the 1940s and 1950s, there were further developments, *inter alia*, in the UK by King [7], in the USA, by Crosby [8] and by Sunderman and Copeland [9]. In 1958, the USA National Academy of Sciences – National Research Council recommended the use of haemiglobincyanide (HiCN) [10]. But despite these efforts, in an international interlaboratory trial undertaken by the Dutch Institute of Public Health in 1962, participating laboratories, including the most eminent in Europe, reported the haemoglobin of the same blood between 11 and 18 g/dl [11]. This study was reported at a symposium during the 9th Congress of the European Society of Haematology; it was the stimulus which led to the foundation of ICSH and the setting up of an international expert panel on haemoglobinometry [12]. There was general support for adopting HiCN as the reference material. The panel members included W. G. Ziljstra and E. J. van Kampen whose precise and accurate optical density measurements and iron analysis on purified haemoglobin solutions led to the establishment of the (1/4) extinction coefficient of HiCN as 11.0, while the molecular weight of $\alpha_2\beta_2$ haemoglobin was calculated by Braunitzer to be 64 458. On this basis, ICSH established specifications for the haemoglobin reference preparation, and for the haemiglobincyanide method for haemoglobinometry in human blood [13].[1] These recommendations by ICSH were unanimously adopted by the International Society of Haematology, in the USA by the National Academy of Science, by the International Union of Pure and Applied Chemistry (IUPAC) and by other international and national organizations. Subsequently, the World Health Organization adopted the ICSH reference preparation as the "WHO International HiCN Reference Preparation". The panel did not rest on this achievement but has continually re-evaluated the standard and methods of haemoglobinometry, aware of the fact that, as new techniques and further research provide new data, there may be need for revision and modifications [14]. The panel is now studying the need for standards of various haemoglobin derivatives and the difficult problem of developing stable whole blood reference preparations. Control of the international HiCN reference preparation remains the cornerstone of the work of ICSH in this field. It is produced and maintained

[1] This work was supported by a grant from the Public Health Committee of Council of Europe and by the Director General of RIV, Billhoven, The Netherlands.

at the Dutch Institute of Public Health; on application to the ICSH Secretariat, a few ampoules can be provided from this stock to anyone wishing to standardize and control national or locally manufactured preparations.

Blood cell standards

The need for reference preparations for the blood count has become of paramount importance with the use of electronic particle counters, especially automated blood counting systems. These are, in effect, comparators in that they can be adjusted arbitrarily so that to obtain a true measurement on an instrument which will be comparable with that obtained on another instrument or by another method, it is essential to calibrate the instrument; this requires reference preparations with assigned values of defined accuracy. There are serious difficulties in achieving this, and no true reference preparation is yet available. The material should closely resemble blood in its physical characteristics with regard to cell numbers, size, shape, electrical conductivity, homogeneity, viscosity and rheology. Unfortunately, materials which are sufficiently stable to serve as a long-term calibrator do not have the physical properties of blood, while natural blood is not sufficiently stable to be useful for this purpose. Furthermore, an instrument calibrated for counting blood cells of one size may not count cells of another size, if it is not linear in its response, while different instruments may differ in measurements of MCV on the same blood sample, if they use different principles. This has been demonstrated in interlaboratory comparisons of counters which use light scattering (Technicon Hemalog), conductivity (Coulter) and laser light diffraction (Ortho Counter) (Table 1).

Materials which have been tried as potential standards fall into three main groups [15]: (a) artificial materials such as polystyrene latex and other plastic

Table 1
Mean results of MCV measurement by participants in UK National External Quality Assessment Scheme

Method/Equipment	Specimen					
	A	B	C	D	E	F
Fully automated counting system (conductivity principle)	52.1	64.8	85.6	89.5	94.6	96.9
RBC by electronic counter PCV by microhaematocrit	52.6	65.5	85.8	91.3	96.7	96.8
Fully automated system (light scattering and centrifugation)	55.1	67.4	86.1	86.0	89.5	93.2
RBC by haemocytometer PCV by microhaematocrit	61.0	71.1	87.2	95.5	89.8	97.3
Fully automated system (laser light diffraction)	70.0	75.5	—	89.0	93.0	94.0

polymers, (b) biological materials such as pollens, mould spores, yeasts, and (c) natural blood cells which have either been preserved (e.g. in ACD, CPD, or Alsever's solution) or modified by fixation in glutaraldehyde or formaldehyde. The biological materials tend to be too heterogeneous with regard to size, but spherical latex particles have recently become available in a series of defined sizes between 2 and 12 μm in diameter each within a narrow size range. These are undergoing study by an expert group of the Bureau of Reference (BCR) of the European Community and also by the ICSH expert panel [16]. If they are shown to be stable in suspension and sufficiently monosized, it will be possible to establish their volume by physical methods (e.g. mass integration) and by electron microscopic techniques, and they can then be used as a primary reference for platelets (2 μm), red cells (5–6 μm) and leucocytes (10–12 μm).

Blood cells can be permanently stabilized by fixation, especially in glutaraldehyde solution. The fixed cells shrink in size immediately after preparation and the shrinking process continues for 3–4 days. Thereafter the cells remain constant in size and shape, and the preparation gives consistently reproducible measurement of cell numbers and cell size distribution for months or even years. Unfortunately, there is a major disadvantage in using these cells as a reference preparation: in the Coulter counting system, natural (fresh) red blood cells become spherical when diluted in the usual diluent (e.g. Isoton); in addition they are deformed as they pass through the orifice of the cell counter [17], whereas the fixed cells remain biconcave disks and are inflexible so that they cannot be used to calibrate an instrument for subsequent measurement of natural blood. As platelets are less affected in the diluent, glutaraldehyde-fixed platelets provide a useful standard for platelet counting [18]. Leucocytes do not undergo shape changes in diluent; fixed avian (turkey or chicken) red cells provide a simulated leucocyte preparation, which is suitable as a control material (see below) although whether it can also be used as a calibrator is still uncertain.

Red cells collected into ACD, CPD or Alsever's solution are stable for a few days at 20 °C and for 3–4 weeks at 4 °C. With some animal species, especially horse blood in ACD, the RBC, PCV and MCV remain constant for up to 3 months. These materials are useful as *controls* to check the precision and reliable functioning of a cell counting system over relatively short periods of time. This is the basis for an internal quality control procedure and it is also used extensively in interlaboratory quality assessment schemes [19]. The material should not generally be regarded as a *calibrator*; however, it may be used as such and as an intermediate (secondary) reference provided that its value is determined by a reference method using a reference particle counter and cell sizer which have been precalibrated, e.g. by means of latex particles as described above.

Coagulation factors

WHO has established international biological reference materials for several coagulation factors. They include factors VIII and IX, antithrombin III, thrombin, heparin, urokinase, plasmin and ancrod [20]. WHO has been concerned

especially with these materials in context of standardization and control of therapeutic aspects of coagulation. The International Committee on Thrombosis and Haemostasis (ICTH) is also involved with standardization, being concerned with both fundamental and practical problems in blood coagulation; their subcommittees are working on diagnostic assay methods; these include fibrinogen, fibrin and fibrin degradation products, thrombin-like enzymes, factor VIII C Ag, factor IX products, factor XIII, and synthetic substrates [21]. Development of reference thromboplastins and standardization in the control of anticoagulation has been an important task in the WHO programme [20] and also in a collaboration between ICTH and ICSH [22, 23].

In the United Kingdom, BCSH [24] organized a control scheme which has been functioning for many years, as follows: at intervals a batch of thromboplastin, known as British Comparative Thromboplastin (BCT) is manufactured by the National Reference Laboratory for Anticoagulant Control Reagents (Director: Dr. L. Poller). This is a liquid phenol saline extract of human brain. Each batch of BCT corresponds to the previous batch, uniformity between batches being achieved by mass production of saline extract of pooled human brains according to a strictly controlled method which includes screening against a set of coumarin plasmas and lyophilized factor VIII-deficient plasma. Each batch is regularly monitored by a group of expert laboratories in the UK, from whose data the BCT is calibrated. The material is despatched at monthly intervals to all the hospitals in the country which participate in the scheme. The BCT is used by these individual laboratories to calibrate commercial or their own home-made thromboplastins; results on a patient's plasma are expressed as the prothrombin ratio normalized to BCT. At present, it is considered that this ratio should be 2.0–4.0 for satisfactory therapeutic control.

In Europe, the European Community Bureau of Reference in collaboration with ICSH has recently produced three types of reference preparation – human brain, rabbit brain and ox tissue [25]. For each of these, a calibration constant has been obtained, these constants being referable to the original WHO "research standard" (Batch 67/40). The relative sensitivity of any new reagent normalized to the reference preparation is determined in comparative measurements of prothrombin time on batches of plasma as described above for BCT. Thus, it will be possible to control commercial (and home-made) reagents and to achieve comparable results for prothrombin time tests throughout the EEC, irrespective of what reagent is used. The ICSH collaboration should ensure similar comparability beyond the geographic limits of the EEC. The role of reference plasmas in standardizing prothrombin time testing is still under debate [26].

Abnormal haemoglobins

In 1973, ICSH established an expert panel under the chairmanship of Dr. H. Lehmann [27]. The panel has published recommendations for quantitation of HbA_2 [28] and HbF [29], a system for identifying abnormal haemoglobins [30], and specifications for reference materials of HbA_2 [28], HbF [29] and an ab-

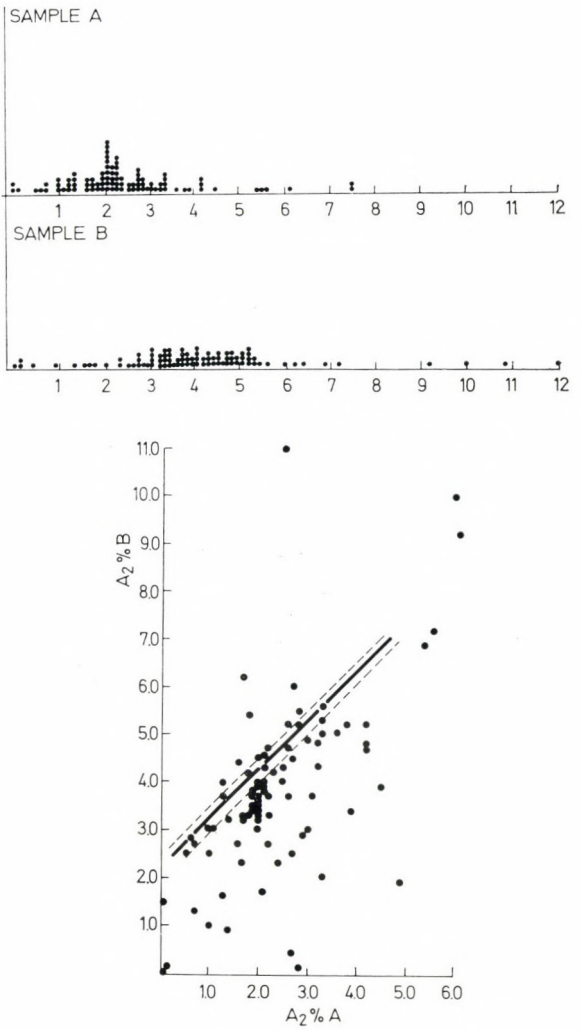

Fig. 1. HbA_2 measurement on two samples (A and B) in an interlaboratory trial. Below, the data are shown in a Youden plot, which illustrates the extent of random variation in the results

normal haemoglobin standard consisting of Hbs-JAFSC [31]. Analytical and stability studies on experimental batches of the various reference materials produced by ICSH have shown that the specifications are practical and that it should be feasible to establish international reference preparations. Interlaboratory trials have highlighted the lack of reliability of HbA_2 assay in routine laboratories [32] (Fig. 1) and it is recognized that a HbA_2 reference preparation is essential for accurate quantification; this need is especially important at the borderline level

of about 3%, in thalassaemia screening programmes. A similar story can be told in the case of HbF assay [32]. Based on the ICSH specifications, BCR has commenced a study of the feasibility of bulk production of HbA_2 and HbF reference materials, as well as a study of their stability and procedures for assigning accurate values.

Vitamin B_{12}

For many years, it has been recognized that determination of serum vitamin B_{12} by microbiological assay is a complicated procedure requiring meticulous attention to technical details [33]. The introduction of radio-dilution assay was a technical advance in so far as the test was simplified, but interlaboratory trials have shown major problems with this procedure in routine practice, with marked variations in analytical results between various commercially available kits [34]. These variations have been ascribed to non-specific binding of inactive analogues of vitamin B_{12} by the R-binder protein which is used instead of pure intrinsic factor in some kits. Accordingly, some kits have been modified by their manufacturers to overcome this defect but as kits continue to proliferate, there is need to ensure their reliability by means of reference method and reference material. Microbiological assay, using *Euglena gracilis*, appears to be the most suitable method for standardization for clinical purposes – in skilled hands, results can be achieved that are accurate (as shown by recovery studies) and reproducible. There is, however, a problem to decide what form the reference material should take and how it should be measured [35]: should it be crystalline B_{12} or should it be natural human plasma or cobalamin in an artificial matrix of B_{12} binders? Should its assigned value be determined by quantitative assay of cobalamin in its pure form, e.g. by isotope dilution mass spectrophotometry? This is arguable as the clinical need is for a reference of biological activity, so that bioassay would be more relevant. This viewpoint has been recommended in the USA by a committee of NCCLS under chairmanship of Dr. R. Schilling [36]. In the UK, the Department of Health in collaboration with NIBSC and BCSH is currently developing biological reference materials of human plasma with "normal" and "low" B_{12} content; values are being assigned to these preparations in a collaborative study by a group of expert laboratories using *Euglena gracilis*, *Lactobacillus leichmannii* and radioisotope dilution methods. Surprisingly, although assay of vitamin B_{12} and other haemopoietic substances plays a major role in extensive WHO nutritional anaemia programmes, vitamin B_{12} does not feature as a WHO International Biological Reference Preparation.

Erythropoietin

As evidenced by an increasingly large number of articles on erythropoietin in the literature during the past few years, it has become important in diagnostic and experimental haematology. It provides useful information for differentiating

primary and secondary erythrocytosis and it is used in bone marrow culture studies. Originally, one unit of erythropoietin was defined as that amount which had an erythropoietic effect on a fasted rat equal to the effect of 5 μM $CoCl_2$, but due to variability in the fasted rat assay, this was an inadequate specification. An international reference preparation was established by WHO in 1966 [37]. The international unit was defined as the activity contained in 1.48 mg of this preparation, and for working preparations a unit was defined as that amount which has a biological effect equal to that of the International Reference Standard Unit. The original WHO preparation was replaced in 1972 by the second international reference preparation [38]; this was allocated a potency of 10 I.U. per ampoule.

Biological assays are laborious and this has deterred against their more widespread use. Immunological assays for erythropoietin have been developed based on either red cell agglutination by antibody or the formation of radioactive antigen–antibody complexes. In both tests, the antibody is anti-erythropoietin and the erythropoietin content of the test material is estimated from its capacity to compete for the antibody. Reliability of the red cell agglutination (inhibition) test is questionable and most investigators consider the radio-immune assay as the most promising. However, it is not yet sufficiently reliable, because of the lack of either sufficiently purified erythropoietin or proper standardization of the antibody.

Serum iron, transferrin and ferritin

The need for a reliable iron assay method was apparent when an ICSH expert panel was formed in 1966 under the chairmanship of Dr. G. Izak. In a classic collaborative study [39], the members of the panel identified each of the analytical variable, and eventually felt able to propose, as a reference method (and also as a selected routine method), spectrophotometric assay of iron chromogen formed by bathophenanthroline sulfonate. By this study, the panel was also able to define a serum iron reference preparation [40]. Subsequently, the reference method was slightly revised and has now been established as an ICSH Standard [41]. Less progress has been made in the assay of iron-binding capacity. No method could be proposed with sufficient accuracy and reliability to be regarded as a reference method, and the best that the ICSH experts could do in the light of present experience was to recommend the magnesium carbonate adsorption method for assay of total iron binding capacity, as a selected method adequate in general for clinical purposes [42].

In order to evaluate the ICSH iron assay method in routine practice, participants in the UK National External Quality Assessment Scheme were asked to perform serum iron measurements by their usual method and by the ICSH method. Contrary to the findings of the earlier interlaboratory trial by the experts, in these trials there was no evidence that the ICSH method was more reliable in routine laboratories (Table 2) although it must be remembered that the tech-

Table 2

Comparison of coefficient of variation (CV) of methods used for measurement of serum iron in trials from UK National External Quality Assessment Scheme

Trial	Specimen	Automated method	Other routine methods	ICSH methods
1	A	7.5	6.9	15.5
	B	6.8	9.3	11.3
	C*	13.4	23.1	28.7
2	A	6.3	13.9	14.1
	B	5.9	13.2	8.1
	C	8.1	15.5	12.4

* Heparinized plasma; all other specimens were sera.

nicians in these routine laboratories were familiar with their usual methods, which included automated procedures, and few had used the ICSH method previously. This emphasized the fact that it is not sufficient merely to define a selected method, but that it is essential for laboratory technical staff to become accustomed to using it in practice.

The ICSH panel has also been developing a ferritin standard. This is a human spleen derived preparation, in a matrix of human serum albumin. The purity of the ferritin has been checked and its value determined by polyacrylamide electrophoresis, iso-electric focussing and amino acid analysis. A batch of this material has been prepared in UK by NIBSC, and, if the appropriate collaborative studies demonstrate its acceptability in accordance with the WHO guidelines [4], it may be established as an International Reference Preparation.

Immuno-haematology

WHO was responsible for important early developments in this field; in 1950 standards for anti-B and anti-B blood typing serum were established, and maintained at the Danish State Serum Institute. The WHO list of biological substances [43] now also includes anti-C, anti-E, anti-c incomplete blood typing sera and anti-D immunoglobulins, as established international reference preparations.

In 1969, at the International Congress of Blood Transfusion in Moscow, ICSH formed a joint Expert Panel on Serology with the International Society of Blood Transfusion. The panel has concentrated on two main problems: 1. standardization of antiglobulin reagents and 2. standardization of serum albumin used in Rh testing. It is well known that different batches of albumin vary in their efficiency in agglutinating Rh-sensitized red cells. Using serological and physico-clinical methods the panel was able to identify the existence of inhibitors of red cell agglutination in some batches of commercial albumin [44]. The panel is now preparing a protocol for checking routine albumin reagents

and defining the specifications for a reference albumin reagent. Polyspecific antiglobulin has been produced as a candidate international reference reagent. The requirements for standardization of such reagents have been defined [45].

Stains and staining

In the staining of biological material, standardized dyes and standardized staining procedures are essential for consistent results. A wide range of stains has been developed for various purposes, and many modifications have been introduced to try to obtain expected effects. These modifications have often been arbitrary with the addition of "a little of this and a little more of that". But if staining technology is to be a science rather than an art, a more rational approach is required, especially as automation of biological staining methods, computerized pattern analysis and quantitative cytochemistry, become more widely used. Blood cell staining is a good example: the Romanowsky staining effect is obtained by a number of stains, notably, Leishman, Giemsa, Jenner, May–Grunwald, Wright; they produce their effect by the reactions of cellular components to a mixture of cationic thiazine and anionic eosin dyes.

Romanowsky staining has been used extensively and universally for the past ninety years; only recently has there been any serious attempt to identify the factors responsible for the variable staining obtained with the different stains and with different batches of the same stain [46, 47]. The essential requirements are azure B (trimethylthionin) and eosin Y (tetrabromofluorescein). The main cause of capricious staining is the presence of other dyes such as azure A, azure C, methyl violet etc. Some commercial stains contain no less than 10 different dyes (as identified by chromatography) and these in greatly different amounts in sequential batches of what is claimed by the manufacturer to be the same stain [48]. The stains also contain a variable amount of metal salts which influence staining characteristics. Other factors which affect the results are variation of pH in the staining solution, the staining time, the dye concentration, and ratio of azure B to eosin Y. There always has been need for a reliable standardized stain for blood and bone marrow films. This is important when films are sent from one laboratory to another for an opinion or as part of a multicentre study; it is also important when consulting an atlas. It has become essential with the advent of automated differential counting instruments which depend on the recognition of the pattern of stained cells by means of a computer, which has been programmed to identify cells from a training set; obviously, this is reliable only if the staining of the training set of slides and the subsequent routine slide is harmonized. ICSH has demonstrated [49, 50] that a standardized Romanowsky stain with optimal and consistent staining effect can be achieved by a two-component (azure B and eosin Y) stain. To encourage collaboration by commercial manufacturers, BCR is establishing specifications for the standardized stain and a protocol for the physico-chemical analysis of the dyes necessary to ensure their purity. To complement this, what is needed is a reference or control

slide consisting of a matrix of appropriate composition to be able to demonstrate the staining properties to be expected from DNA, RNA, haemoglobin and other constituents of blood cells. The problems are not unique to the Romanowsky stains. There is need to scrutinize various histological staining methods, including, for haematology, reticulocyte stains, reticulin and collagen stains and the Papanicolaou stains – these last are now used extensively for cytological screening, and as this procedure is becoming increasingly automated, the need for standardization is obvious.

Despite the fact that haematologists are aware of the necessity for accurate reticulocyte counting, interlaboratory trials show how unreliable they are in practice, with variances of 100% or more. Apart from distribution errors due to poor films and statistical errors due to inadequate counting, a significant factor is poor staining which may fail to demonstrate late reticulocytes with only sparse ribosomal material or, conversely, may fail to distinguish particles such as Heinz bodies or nuclear remnants. Pure azure B has been said to stain reticulocytes consistently and uniquely [51]. This has not yet been confirmed by others and further investigations are required to identify the optimal dye and the staining specifications for standardization.

Quantitation of bone marrow content of reticulin and collagen is important in diagnosis and management of myelosclerosis and other myeloproliferative disorders. This is usually judged by the extent to which films are stained by silver impregnation. Visual impression can now be supplemented (or at times replaced) by absolute measurement by automated instruments such as the Quantimet Cell Analyzer [52], provided that the stain uptake by the tissue is specific and constant. This principle applies to many cell and tissue stains, and quantitative cytochemistry is a developing science, with enormous potential use in cellular biology; it is, thus, obvious that this calls for standardized stains and reagents and standardized staining procedures. As yet there has been little progress in this area, although ICSH is at present assessing several cytochemical tests used in haematology. The Expert Panel under the chairmanship of Dr. Shibata will shortly publish recommendations on the standardization of methods for leucocyte alkaline phosphatase, peroxidase, acid phosphatase and esterases. The recommendations will include specifications for the chemical reagents to ensure consistent reactions, as well as standardized procedures for preparation of the blood samples and for the tests themselves.

Erythrocyte sedimentation rate (ESR)

Test for which reference materials are not available depend, for their reliability, on standardization of method and, in some cases, the use of standardized equipment. This applies to the erythrocyte sedimentation rate.

The ESR is a complex test and there is no method for its measurement that can ensure that the test is not influenced in misleading ways by variations in PCV and by other unidentified factors. Accordingly, no definitive reference method

is yet available so ICSH has designated, as a selected method, one precisely defined method, based on that of Westergren [53]. This method was selected as it is simple to perform and appears to be reasonably reliable and more reproducible than other methods at present available at levels that are clinically significant. As results are especially affected by variation in the dimensions of the tube in which the test is set up (its bore should be 2.55 $\pm$ 0.15 mm and the bore should be uniform throughout its length, with a variance of less than $\pm$0.05 mm), several national authorities have established standards for ESR tubes which follow the ICSH specifications. These include British Standards Institute (BSI) [55], Germany's DIN and in the United States, NCCLS [54]. Some manufacturers have replaced the traditional glass ESR tubes with plastic tubes. Some plastic materials, e.g. polypropylene and polycarbonate, have been shown to be suitable substitutes for glass but not all plastics have similar properties and before they are acceptable, it is necessary to perform an adequate field trial, comparing ESR as measured in the candidate tubes with results obtained by the ICSH method using the standardized tubes.

Packed cell volume

This is another example of a haematological test which depends largely for its accuracy on the tube which is used. The essential requirement is a constant bore; with the microhaematocrit tubes, it is also necessary to use low melting point glass so as to ensure that they seal flat when heated. To try to ensure that these conditions are met by manufacturers, the various national authorities have established specifications for Wintrobe tubes and for microhaematocrit capillary tubes [56, 57]. This helps to achieve consistency in routine measurement of PCV. Accurate measurement, on the other hand, has become an essential procedure for calibration of blood counting systems and for assigning values to blood cell reference preparations. For this purpose, ICSH has devised a procedure which also takes into account trapped plasma. This has now been proposed as an ICSH Reference Method for PCV [58].

Specimen collection

Specimen collection requires no less stringent standardization than the analytical tests on the collected specimens. The containers should be leak-proof and made of a material which will not influence the investigation either by contamination or by adherence of the analyte to the surface of the tube. If an anticoagulant is used, it must be of a type and concentration which will not influence the blood constituents. Thus, for example, whilst EDTA is a good anticoagulant for blood count specimens, if present at a concentration above 4 mg/ml, this results in an increase in PCV and MCV and a fall in MCH and MCHC. Excess EDTA also affects the platelets – they swell and then disintegrate, causing an artificially high platelet count [59].

ISO and several national standards bodies including BSI, ECCLS and NCCLS, have published specifications for blood specimen bottles and for evacuated container systems with inbuilt collection devices [60–65] (these specifications do not differ substantially one from another). NCCLS has also published standard procedures for skin puncture and collection of capillary blood specimens [64, 65]; more recently, they have provided guidelines for transport and preparation of blood specimens for coagulation testing [66]. These documents might, at first, be considered unnecessary duplication of the information which appears in many textbooks, but the need to try to achieve and maintain good practice and dependable manufacture is well demonstrated by the fact that many of the bottles in general use leak sufficiently badly to constitute a health hazard as well as affecting the reliability of the tests to be performed [67]. Another disturbing observation was that unsiliconized glass tubes with liquid citrate have been marketed by at least one manufacturer labelled "for coagulation studies" – obviously, such tubes might cause inaccurate results of coagulation tests, leading to errors in diagnosis or dangerous instability in the control of anticoagulant therapy [68]. Standardization of the procedure for collecting blood is essential when establishing reference values in a population [69, 70].

Nomenclature

Standardization of nomenclature, and especially the universal adoption of an agreed system for expressing analytical results, is necessary to ensure accurate communication and to avoid confusion which may even put patients lives at risk. The Système International d'Unités (SI) was developed by the Conférence Générale de Poids et Mesures, the intergovernmental body responsible for units of measurement. It is essentially an expanded version of the metric system, based on seven base units, namely, metre (m) for length, kilogram (kg) for weight, second (s) for time, ampere (A) for electric current, kelvin (K) for thermodynamic temperature, candela (cd) for luminous intensity and mole (mol) for amount of substance. In 1977 the World Health Assembly recommended the adoption of SI by the world's medical community [71]. It was already being used fairly extensively in the biomedical field in at least some countries; in 1972, in a joint publication, ICSH, IFCC and WASP [72] made two important recommendations – (1) the litre should be adopted as the preferred unit of volume to be used in the denominator when expressing analyte concentration, (2) the mole (or its submultiples) should be used for substance concentration for all analytes of known molecular weight. Thus, iron, bilirubin, urea, uric acid and most other chemical constituents should be measured in mmol or μmol/l; proteins including fibrinogen, lipoproteins, should be measured in g/l. In the case of haemoglobin, there has been some controversy. As its molecular weight is known, it has been argued by some authorities that it should be expressed in terms of substance concentration, but Hb has a unique place in health care programmes; it is used extensively, as the one essential test, by general medical practitioners, by midwives, by medical assistants in primary health clinics and by epidemiologists and

public health authorities who have been trained to express Hb in g/dl or g/l and to understand its significance for appropriate action. Accordingly, ICSH has recommended that Hb should preferably be reported in mass concentration in g/l. If, however, substance concentration is used (perhaps especially for scientific rather than clinical purposes), this should be based on the monomer – Hb(Fe), MW 16 000. Thus, for example:

Traditional expression	Hb 15.0 g/dl
ICSH recommendation	Hb 150 g/l
Alternative (substance concentration)	Hb 9.4 mmol/l

WHO has recognized a similar practical difficulty with blood pressure. It has been recommended that this should continue to be measured in mm of mercury until there is greater familiarization with the pascal, perhaps after it has been more widely adopted in other fields.

Conclusion

In recent years an awareness of the important role of standardization in the practice of haematology and related disciplines, has stimulated a remarkable increase in activities in developing and establishing standards by national and international organizations and by individual workers, paralleled by an increasing output of scientific papers, reports, recommendations and regulatory documents. In this limited review, it has been necessary to omit reference to many of these, but some indication has been given of the extent of the work which has been done, as well as noting topics which still need attention. The success of standardization is, in reality, the success of collaboration of experts from many countries and its co-ordination. In this WHO plays a vital role by several functions, which include (a) dissemination of relevant information to all governments and to health authorities, (b) ensuring international acceptability of the standards of materials and methods, as they are developed, (c) organization of educational and training programmes, particularly in developing countries, and harmonization of the activities of other organizations working on standardization in the disciplines concerned with biomedical analysis. International professional bodies, such as ICSH, have another important function, namely, to encourage scientific collaboration between the international professional bodies and the scientific experts from industry.

Over the years, ICSH has demonstrated that it also has an important role to ensure that the scientific base is translated into practicality, taking account of the requirements and the available facilities in different countries and at various levels of practice. The haemoglobin project described earlier serves as a model for other standardization programmes in laboratory medicine. In essence, ICSH first demonstrated, by means of interlaboratory trials, that a problem existed and

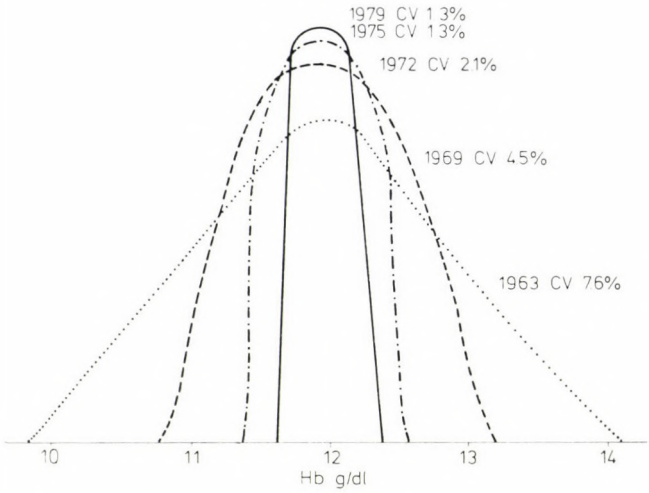

Fig. 2. Reduction in coefficient of variation (CV%) for measurement of haemoglobin in successive years by laboratories in the United Kingdom

defined its extent. A collaborative study by eminent research workers, co-ordinated by ICSH, provided a scientific basis for resolution of the problem. Co-operation of international authorities ensured its recognition by peers throughout the world, and the co-operation of national authorities ensured that secondary preparations derived from the primary reference would be established and maintained at national level. Finally, ICSH, as a professional body, has been able, by journal and textbook publications, symposia and workshops, to encourage the world's scientists, doctors and technicians to use the recommended methods and the reference preparations in their laboratory practice. The effectiveness of this approach is illustrated by the remarkable close interlaboratory agreement in measurement of haemoglobin in trials over the past few years, at least in some countries (Fig. 2).

Finally, it is not sufficient to have a standardized method and standard reference preparations. Both methods and standards must be continually and critically re-evaluated by a competent body of scientists. If necessary, original recommendations must be added to or amended; new techniques for the preparation and checking of standards must be used as soon as they become available and are of proven reliability for this. This will ensure the dynamic standardization which is the essence of scientific progress. ICSH functions on these principles and the ICSH Rules of Procedure have been devised to ensure regular review of all its standards by the original experts, by their critics, by world-wide consultations and also by practising haematologists who, in the end, will be the users of and will benefit from good standards.

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Leukapheresis and Granulocyte Transfusion*

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Newer cell separators can collect more PMN, especially using macromolecular agents and steroid stimulation of donors. Proper indications for PMN transfusion are severe neutropenia and sepsis, or certain localized infections, when prolonged marrow aplasia is expected. Optimal dosage is at least 2×10^{10} PMN per day, probably more. There is a need for more accurate definition of clinical indications for PMN support, for still greater PMN yields from leukapheresis, and for better PMN storage capability.

Introduction

The transfusion of granulocytes (PMN) to neutropenic patients was considered desirable for decades, but impractical without systems for extracting and concentrating the leukocytes from whole blood. This impracticality appeared to be corroborated in occasional experimental trials in which only transitory appearance of transfused leukocytes could be detected in the recipient's circulation [1, 2]. Nevertheless, transfused cells could be observed and did migrate to sites of infection [3].

The technology needed for the separation and concentration of leukocytes from donor blood began with the blood "fractionator" developed by Cohn [4, 5, 6]. His basic concept, far ahead of his time, was that the various components of whole blood should be separated at the time of collection, each stored accord-

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Abbreviations:

CFC	continuous-flow centrifugation
FL	filtration leukapheresis
HES	hydroxyethyl starch
IFC	intermittent-flow centrifugation
MFG	modified fluid gelatin
NCI	National Cancer Institute (USA)
PMN	polymorphonuclear leukocyte, granulocyte
RBC	red blood cell
WBC	white blood cell

ing to its specific needs, and used individually as needed by a patient. Only a few Cohn Fractionators were made, and they were used by various blood research institutes for clinical investigations, primarily in the collection of plasma for fractionation and in the development of procedures for the freezing and thawing of red blood cells for transfusion. Crude by present-day standards, the Cohn Fractionator could nevertheless be used to separate leukocytes and platelets from donor blood [7], and can be considered the ancestor of the modern system of intermittent-flow centrifugation (IFC) leukapheresis and thrombocytapheresis (Haemonetics).

The Cohn Fractionator and its immediate descendants were not, however, directed primarily towards the collection of white blood cells for transfusion. Freireich and his coworkers at the National Cancer Institute (USA) addressed the specific aim of transfusing PMN to victims of leukemia with temporary ablation of the bone marrow due to chemotherapy, since many of those patients were dying of complicating infections. Trial transfusions of white cells collected by plasmapheresis of patients with chronic granulocytic leukemia indicated that transfused PMN could be clinically effective if enough of them were given [8, 9]. In the case of normal donors, the task was difficult. Thanks to the personal interest of an IBM Corporation engineer (Judson) a special continuous-flow centrifuge was developed (1965) [10]. This was called the NCI-IBM blood cell separator, and was able to collect reasonable numbers of granulocytes from normal donors. Thus, studies could begin on the clinical effects of transfusions of normal PMN.

PMN are very close to erythrocytes in density. Consequently, when normal donor blood is anticoagulated with citrate (ACD) and subjected to centrifugation, the resultant buffy coat includes platelets and lymphocytes as well as PMN, in fact lymphocytes usually predominate. The group developing the continuous-flow centrifugation (CFC) procedure had found that they could greatly improve the number of granulocytes in the buffy coat by the addition to the blood of a macromolecular agent [11], which acts apparently by altering the sedimentation characteristics of the red blood cells. The agents used include the Dextrans [12], Hydroxyethyl Starch (HES) [13], and various Modified Fluid Gelatins (MFG) [14]. These are added to the donor blood with the anticoagulant (usually a citrate mixture). Aside from an occasional idiosyncratic reaction, and continued concern over long-term persistence of traces of HES [15], these agents are generally considered harmless to the donor (see below). Some time after the development of the NCI-IBM separator, it was noted that the IFC system, which had been under development for platelet collection and washing RBCs, could also be used to collect PMN with the application of the same macromolecular agents mentioned above [16].

A few years after CFC leukapheresis was developed, an entirely different procedure was discovered by Djerassi [17], based on the reversible adherence of PMNs to nylon fibers in a filter. This continuous-flow procedure is called filtration leukapheresis (FL).

Within the space of about 15 years, therefore, three methods had been developed for the collection and concentration of PMNs from normal donors in sufficient numbers to allow studies of granulocyte transfusions in appropriate clinical circumstances. The purpose of this review is to summarize leukapheresis methodology, considerations of granulocyte storage, donor safety, and the clinical application of PMN transfusions.

Leukapheresis

Macromolecular agents and anticoagulation

As mentioned above, macromolecular agents are used to improve PMN collection by both CFC and IFC methods. They are not necessary for FL, or for platelet collection. Dextrans of lower molecular weights (40 000 to 150 000), HES, and various gelatin formulations (MFG) [12, 14, 18] can apparently be used more or less interchangeably, the choice being largely based on the availability of the agent. HES has been preferred in the USA, MFG and HES in continental Europe, Dextran and HES in Great Britain, and Dextran in Australia. The appropriate anticoagulant must be added to the agent, usually as a concentrate which is mixed with donor blood as it enters the tubing leading to the centrifuge. The anticoagulant is most often trisodium citrate with a small amount of citric acid in the same proportion as in ACD. To prevent clots or aggregates from forming, the anticoagulant must be in the correct proportion to donor blood, either by adjusting the concentration of the anticoagulant itself, or by varying the anticoagulant : blood ratio, depending on the machine being used. Heparin is sometimes used as anticoagulant in CFC or IFC systems, but seems to offer no particular advantage over citrate other than the absence of citrate reactions (see Donor Safety).

Since reversible leukoadhesion to nylon requires calcium ions, citrate anticoagulants cannot be used in FL procedures. For these, the donor must be fully heparinized, and the operator must decide whether or not to neutralize the heparin with protamine after the procedure, and must therefore balance the hazards of bleeding on the part of the donor against those of protamine reactions.

Stimulation of donors

The use of a macromolecular agent greatly improves the efficiency of PMN collection by centrifugal methods, but the number of PMN obtained by leukapheresis is also roughly proportional to the donor's original peripheral granulocyte count [16]. Therefore, if the donor's granulocyte count can be artificially or physiologically elevated before and during leukapheresis, an increased yield usually results. This has been shown with physical exercise [19] and with the administration of various corticosteroids, e.g. hydrocortisone, dexamethasone,

prednisone, methylprednisolone, and etiocholanolone [20]. Even epinephrine has been used (e.g., in the Soviet Union). Epinephrine and physical exercise (the latter, of course, exerts its effect by release of endogenous epinephrine) appear to work primarily by demarginating leukocytes from the endothelial surfaces, and this effect is rapid and transitory. The corticosteroid mechanism is not fully understood, but is probably a combination of demargination, lymphocytic suppression, release of additional granulocytes from the bone marrow reserve, and inhibition of PMN egress from the circulating pool. Different steroids may act by various mechanisms [20].

If corticosteroids are to be effective in stimulating leukocyte donors, they must be given in appropriate dosage, and by such a time schedule as to ensure continuation of leukocytosis during the anticipated period of the leukapheresis. This may require a single dose to be given at least two hours before the start of the procedure. The most effective stimulation seems to be achieved by two or more oral doses of dexamethasone or prednisone given over the 12 to 17 hours preceding leukapheresis, as shown in Table 1 [21, 22].

Table 1

Three schedules of divided dose steroid premedication*

Steroid	Hours before procedure	Total dose, mg	Mean % increase PMN count	Range	N
Dexamethasone [21]					
3 mg/m²	12, 3	8–12	41	−25 to +95	6
Prednisone					
40 mg/dose	17, 12, 2	120	171	118 to 243	6
20 mg/dose	17, 12, 2	60	139	55 to 204	4

* Normal volunteers, same subjects undergoing each schedule, not less than a week apart, in the order given. All counts were done at the same time of day. From Hinckley, M. E., Huestis, D. W.: Premedication for optimal granulocyte collection. *Plas. Ther.* 2, 149 (1981).

Continuous-flow centrifugation (CFC)

The basic design of the NCI-IBM blood cell separator was also used by Aminco in the manufacture of their "Celltrifuge" separator, and both IBM and Aminco versions were widely distributed [23]. Many are still in use. These machines are somewhat cumbersome to use for cell collection, and have the disadvantage of non-disposable bowls, but when used appropriately can provide adequate cell yields. Both have been superseded by newer models with disposable blood pathways. The Celltrifuge was taken over by Fenwal Laboratories and redesigned as the Celltrifuge II, with a disposable bowl. So far, there has been little experience with the new version.

The *IBM 2997 blood cell separator*, as IBM's new model is called, is radically different from the old NCI-IBM machine [24]. Instead of a centrifuge bowl, it has a disposable, hollow, hoop-like, rotating channel, in which the donor blood travels circumferentially, separating a buffy coat as it goes. Collection of the buffy coat is from a collection chamber via a tubing manifold and a rotary ceramic seal. The composition of the buffy coat can be controlled by varying the centrifugal force and flow rate, as well as by the use of a macromolecular agent if granulocytes are wanted as opposed to ACD for platelets. The machine can also be used very effectively for therapeutic plasmapheresis. In addition, IBM manufactures a dual-stage channel for the same machine, to be used for platelet collection. This is a highly eccentric channel, in which platelet-rich plasma is separated in the first half at lower centrifugal force, while the platelet concentrate forms in the wider second half at higher centrifugal force.

An entirely new CFC machine was announced in 1980 by Fenwal Laboratories, called the *Fenwal CS 3000* [23]. This entails two successive centrifugal steps carried out in separated chambers within a single centrifuge. These chambers are plastic bags held in the appropriate geometric conformation by rigid inserts. Buffy-coat-rich plasma is separated in the first chamber, then passes into the second chamber where platelets or leukocytes are separated from the plasma. The CS 3000 can produce platelets by using ACD as anticoagulant, or PMN plus platelets by using special inserts and a macromolecular agent. Two unique features of this machine are its sealless tubing connector, and the fact that it is controlled by a self-contained computer. The computer programming can be varied to accommodate the machine's different modes of operation.

Intermittent-flow centrifugation (IFC)

The only manufacturer of this type of equipment is Haemonetics, whose Model 30 blood processor is a direct descendant of the Cohn Fractionator [4, 5, 6, 23]. It was the first leukapheresis apparatus to have a fully disposable pathway [25]. The centrifuge bowl (Latham bowl) is a rigid, bell-shaped, polycarbonate structure, with central inlet and outlet passing through a rotary seal at the top. The buffy coat is separated from successive batches of donor blood. As the bowl fills and buffy coat forms, plasma is displaced into an exterior collection bag. When the buffy coat reaches the top, it is diverted into a second bag, after which a reverse pump is activated and RBC and plasma are returned to the donor. The usual leukapheresis entails six to ten such cycles. Although two veins are usually used (one for giving, the other for the return), this machine has the advantage of being able to be used with only one needle.

Filtration leukapheresis

The adherence of granulocytes to nylon fibers has long been used as a means of reducing the number of leukocytes in blood intended for transfusion to patients

with leukocyte antibodies. Its use as a means of collecting PMN for transfusion was developed by Djerassi [17], who constructed a mechanical, pumpless device for that purpose. Blood flow was controlled by gravity. Somewhat later, Fenwal Laboratories manufactured a simple pump-operated device for FL, which has been widely distributed. The mechanism of PMN adherence is not well understood, but Ca^{2+} is required, so citrate cannot be used as anticoagulant. The donor must therefore be heparinized. Two filters at a time are commonly used, and after perfusion for two or three hours, the PMN (and some monocytes) are eluted from the filters by ACD plasma, aided by light tapping of the filters. There is evidence that the first layer of cells, stuck directly to the nylon, is damaged, so that gentle elution techniques that do not dislodge already damaged cells produce PMN that are in a better state of integrity and function [26]. Otherwise, functional impairment has often been noted in filtration-collected PMN. By using prolonged procedures and multiple filters, it is possible to get very large numbers of PMN, but such procedures are seldom practical in terms of donor's time involvement. Other problems include increased reactions in donors (e.g., perineal and abdominal symptoms, and two possibly related cases of priapism), and in recipients (febrile reactions, controlled by slow transfusion rates and medication). The problems and standards for this procedure have been summarized by Djerassi et al. [26].

An interesting variant of the FL procedure is that of *repetitive cycle FL*, in which two filters are alternately loaded with PMN and eluted by reverse flow [27]. This can be done repeatedly in a four-hour procedure to produce a high yield of PMN with good functional integrity.

Gravity leukapheresis

Various manual methods (i.e., not using a special machine) for the separation of PMN from donor blood have been devised. The best is probably the gravity system of Djerassi [28], by which platelet concentrates and separate PMN separations are made from successive collections of donor blood alternating with return of RBC and plasma. This method is cumbersome and time-consuming, and requires a large quantity of macromolecular agent. The PMN yields are small [29]. Its big advantage is that only small amounts of the macromolecular agent remain in the donor, and no expensive equipment is needed.

Summary of leukapheresis methods

Except for manual methods, all the CFC, IFC, and FL systems described can provide satisfactory PMN collections in a reasonable period of time. The matter of optimal or minimal acceptable dosage will be discussed later. A general comparison of the different methods is given in Table 2. Table 3 provides a comparison of cell yields with the various procedures.

Table 2

Comparative characteristics of leukapheresis systems

	Haemonetics	IBM 2997	Fenwal CS 3000	Fenwal Filtration
System	IFC	CFC	CFC	FL
Mechanical complexity	Simple	Complex	Complex	Simple
Ease of operation*	3	4	1	2
Maintenance of machine	Easy	Difficult	Difficult	Easy
Extracorporeal blood volume (ml)	450	250	300	240
Relative centrifugal force ($\times g$)	500–1500	60–120**	270	—
PMN collection efficiency	Moderate	High	High	High
Platelet yields in leukapheresis	High	Moderate	High	—
Platelet yields in thrombocytapheresis	High	Moderate	Moderate	—
RBC in average PMN collection (ml)	40	12–15	20–25	30
Portability	Easy	Less easy	Difficult	Easy
Single vein operation	Yes	No	No	No

* On a scale of 1 to 4: 1 = easiest, 4 = difficult.
** In leukapheresis procedures.

In general, the IFC (Haemonetics) system provides the highest yields of platelets (in thrombocytapheresis), but at the cost of heavy contamination with RBC and lymphocytes. In leukapheresis, both IFC and the Fenwal CS 3000 CFC system include equally high platelet yields. This is usually an advantage to the patient, but may be troublesome if it is desired to subject a donor to leukapheresis on successive days (platelet depletion). Consistently higher yields of PMN are obtained by the newer CFC methods (IBM 2997 and Fenwal CS 3000) than by IFC. FL is little used any more in the USA, partly because of fear of reactions in both donor and recipient, and partly because of doubts as to the functional integrity of the PMN so obtained. Furthermore, the newer centrifugal systems are producing as many PMN in a comparable time without functional impairment.

Finally, a number of studies have shown that neither macromolecular agents used in cell collection nor steroid premedication of donors adversely affect the function of either platelets or PMN collected by centrifugal methods [15, 30, 31].

Hazards of leukapheresis to the donor

Reactions during centrifugal leukapheresis are for the most part inconsequential, and caused by fluctuations in the blood volume, the effects of anticoagulants, and chilling [32]. Blood volume depletion tends to cause *vasovagal reactions*, much as in regular blood donation, and treated likewise. They are more likely to occur when there is a large extracorporeal blood aliquot (as in IFC

Table 3

Mean PMN and platelet yields by various leukapheresis systems*

Method	Volume of blood processed l	Leukapheresis				Thrombocytapheresis		
		PMN $\times 10^{10}$		Platelets $\times 10^{11}$	RBC ml	Platelets $\times 10^{11}$	Non-PMN WBC $\times 10^{9}$	RBC ml
		Steroid	No steroid					
Haemonetics IFC	3—4	2.3	1.3	7.0	33	6.6	5.5	60
IBM 2997 Single stage channel	8—10	3.0	1.9	3.5	15			
Dual stage channel	3—4.5					4.6	0.4	0**
Fenwal CS 3000	3.5—4.0					3.8	3.0	0**
	7	3.2	1.5	6.3				
	10	4.0	2.2	6.7	31			
Fenwal Filtration	8—10	2—4	1—3	—	30			

* Except for FL by the Fenwal system, all figures are from the Blood Transfusion Service, University of Arizona.

** Negligible numbers of RBC in these concentrates.

procedures, in which efforts should be made to keep the extracorporeal blood less than 15% of the donor's blood volume). *Citrate* has the effect of decreasing the blood ionized calcium, which may cause increased neuromuscular tension, even overt tetany. Such reactions can be treated by diminishing the blood return flow rate or anticoagulant proportion, or by providing exogenous calcium in the form of oral tablets or milk. *Chilling* is prevented by the use of blood warmers or by warming the donors with blankets. *Bleeding* is a potential complication of heparin (used in FL), and protamine reactions may occur if heparin is neutralized with that drug. Intravenous steroid, which is not recommended, may cause transient perineal itching and burning sensations. Occasional *allergic type* manifestations and even anaphylactoid symptoms have been suspected to be caused by macromolecular agents, e.g., Dextran or HES [15, 33], and a case of persistent lichen planus has been blamed on HES, although the connection has not been proved [34]. Steroids may cause various transient effects, including euphoria, flushing, and insomnia. Any macromolecular agent, especially if used on successive days, may expand the plasma volume [35] enough to produce headaches and even some peripheral edema.

A group of reactions is peculiar to FL, and may be related to complement activation. These include reddening of the eyes and face, sneezing, crampy ab-

dominal pain, and tenesmus (most often in women) [36]. Premedication with steroid and adequate heparinization both appear to have a preventive effect. Two cases of priapism during FL have been reported in the USA [37], which is one reason for the decline of popularity of this procedure.

Possible long-term effects of leukapheresis procedures have aroused concern, although tangible effects have been few. HES is characterized by a long "tail" of persistence in the body [15], which has aroused fears of possible reticuloendothelial blockage and long-term effects, although none have yet been identified. Platelet depletion, particularly in IFC, can produce thrombocytopenia which requires several days to correct itself [32]. Thrombocytapheresis in particular may result in lymphocyte depletion, the possible effects of which are not known. All donors subject to any form of hemapheresis at frequent intervals should be tested to evaluate possible blood cell and plasma protein depletion.

Storage of PMN

Good laboratory assays of PMN function developed over the past few years have been applied to the evaluation of the characteristics of these cells in storage [38, 39]. For the most part, in practice, PMN are stored in the same medium in which they were collected, e.g., citrated donor plasma containing HES. Storage experiments, however, often entail many handling and storage variables that are not always clearly described, and the storage conditions may or may not reflect the circumstances under which transfusable PMN are stored. Therefore, articles on this subject must be read and interpreted with great care. Despite this, it does seem that certain conclusions are valid, coming as they do from a variety of different observations [39, 40, 41]. The most easily preserved properties are those of phagocytosis and dye exclusion. The latter probably represents membrane function and integrity, comparatively primitive functions, easily preserved for three or four days or even longer at either 4 °C or 22 °C. Less primitive functions and more susceptible to deterioration at two or three days of storage are bactericidal and fungicidal capacity, whereas the most highly integrative performance, which begins deteriorating even before 24 hours of storage, is that of chemotaxis. Since the latter is an extremely important cell function in the defense against infection, it is generally thought that, unless there is strong evidence that a particular set of storage conditions improves the preservation of chemotaxis over those so far reported, storage should be limited to about 24 hours.

The latter appears to be almost the only firm conclusion that can be drawn from experiments that have been reported up till now. It appears that storage at room temperature is better than at 4 °C, that agitation is probably undesirable, that freezing and thawing are as yet unsatisfactory, and that FL-collected PMN should probably not be stored at all (although the gentler elution methods may hold more promise) [38]. At present, the conclusion must be that any PMN concentrate should be transfused as soon as possible, although cells collected by centrifugation can be stored for about a day.

PMN Transfusion in Clinical Practice

Granulocyte transfusion has been a singularly difficult form of therapy to evaluate, at least in part for the following reasons.

1. It is used only in desperately ill patients with complex disease processes.
2. Because of the above, it is always used in conjunction with other forms of therapy, e.g., steroids, antibiotics, chemotherapeutic agents.
3. Most transfusions have probably included less than the optimal number of PMN.
4. Cell collection methods differ as to quality as well as quantity of PMN.
5. The importance or lack of importance of immune compatibility is still uncertain.
6. Controlled studies have focused predominantly on Gram-negative sepsis.
7. The number of patients studied in each controlled trial has been small, and there are too many uncontrolled variables between different institutions to allow direct comparisons or pooling of data.

Added to the above difficulties is the fact that, during the time that experience has been gained with PMN transfusion, there have been simultaneous and overlapping changes and improvements in chemotherapy, antibiotic therapy, and general management of leukemia and cancer patients.

Despite these difficulties, most investigators have concluded that PMN transfusions are effective in appropriate clinical circumstances, that the transfused PMN migrate to sites of infection, that they can kill bacteria, and that they favorably influence the clinical outcome. The evidence is still stronger when all the studies are considered as a group, even though their results cannot be statistically pooled. These various studies and the current status of the clinical use of PMN have been critically summarized by Higby [42, 43].

Which patients should receive PMN transfusions?

As stated above, the efficacity of PMN transfusions has been reasonably proved in the case of septicemia with Gram-negative bacteria, but studies relating to other types of infection are few [44]. It certainly seems reasonable to provide PMN to neutropenic patients with other types of bacterial septicemia, or with severe or spreading local infections. The question of fungal infections is less clear. In dogs, PMN transfusions have been found effective in reducing the severity of candidiasis [45]. However, in a large series of neutropenic patients receiving therapeutic PMN transfusions at the University of Arizona, we could not conclude that the transfusions either prevented the onset of fungal infections or affected their usually fatal outcome [46]. Nevertheless, since PMN obtained by leukapheresis avidly ingest and kill *Candida*, this should not be taken as a final answer. Increased dosage or frequency of transfusion may be needed.

In clinical practice, the two basic criteria for PMN transfusions are infection and neutropenia. Most of the patients for whom this therapy is considered are those with cancer or leukemia, having temporary ablation of bone marrow function brought about by chemotherapy. "Neutropenia" is usually defined as a peripheral blood granulocyte count below 500/μl. Counts approaching zero should be regarded even more seriously. In view of the fact that some patients with such PMN counts remain stable and do not suffer significant infection, PMN transfusions are not usually given unless the patient develops sepsis or a significant local infection (e.g., pneumonitis). Even then, since present-day antibiotics are extremely effective, PMN transfusions are usually withheld during a two or three day trial of appropriate antibiotic therapy [43]. If it seems likely that marrow function will return in a few days, PMN transfusions are probably not necessary, although it must be admitted that that judgement is more easily made in retrospect. Bearing in mind the potentially lethal nature of infections in neutropenic patients, and the uncertainty as to the likely duration of aplasia, most physicians would rather start PMN transfusions early than allow an infection to get out of control. Once a patient is started on a course of PMN transfusions, these are usually continued daily until marrow recovery or death, unless death seems inevitable or marrow recovery unlikely. If the patient's infection has apparently cleared but marrow function has not yet recovered, transfusions are best continued for two or three days longer to prevent recurrence of infection.

Prophylactic PMN transfusions

There have been several studies of PMN transfusions in uninfected neutropenic patients undergoing chemotherapy. A trial in France [47] and another in the USA (the latter in bone marrow transplant recipients) [48] both indicated reduction in the incidence and severity of complicating infections, but no improvement in survival. On the other hand, an English study showed no apparent effect, although the patients in that group received transfusions only on alternate days [49]. The likely benefits of prophylaxis are thus equivocal at best. The actual and potential hazards, however, are considerable, primarily relating to reactions and to alloimmunization. The latter is very important clinically, leading to decreased posttransfusion increments, impaired cell migration, diminished response to transfused platelets, and probably shorter survival [50]. With these considerations in mind, it is safe to say that general methods of preventing infections in neutropenic patients are probably more effective than prophylactic PMN transfusions, and certainly safer [43].

Dosage

The normal daily turnover of PMN in uninfected people is about 10^{11}. Based partly on clinical experience and partly on old experiments with transfusions of cells from patients with chronic granulocytic leukemia, the general impression is that an adequate PMN transfusion should contain at least 10^{10}

granulocytes. Experiments in dogs support this [51], but there is good evidence that larger doses are better [52, 53]. However, to obtain a *minimum* of 10^{10} PMN per transfusion, the *mean* yield must be notably higher than that. We have found, for example, that with a mean yield of 1.3 to 1.4 PMN, which is obtainable by either CFC or IFC from normal donors without steroid, about 25% of leukapheresis will have a PMN yield below 10^{10}, and may therefore be considered inadequate by the above standard. The problem is that PMN yields are unpredictable, and some donors have poor yields despite steroid stimulation and optimal techniques. Few investigators have given attention to the problem as it affects the transfused dose of cells. Therefore, to be sure of a *minimum* of 10^{10} PMN per transfusion, the *mean* leukapheresis yield probably needs to be at least 2×10^{10}, a figure not likely to be achieved by CFC or IFC without the use of steroid stimulation. The matter of dosage deserves more emphasis [54]. With the cost, complexity, and hazards (to patient as well as donor) of PMN transfusions, every reasonable means should be taken to assure a maximal PMN yield.

One of the advantages of centrifugal methods is that the collections can include platelets, and, of course, patients with bone marrow ablation usually need platelets as well as PMN. In leukapheresis procedures, both the Haemonetics and the Fenwal CS 3000 machines produce high platelet yields. The IBM 2997, as usually operated, does not produce as many platelets as the other two, but the numbers are nevertheless adequate (Table 3). Obviously, the provision of both platelets and PMN from one donor, when the patient needs both components, minimizes exposure to foreign antigens.

Compatibility

Since all PMN collections include significant numbers of RBC (15 to 50 or more ml), it is better and easier all round if they are ABO-compatible with the recipient's serum [43, 55]. If the donor is not ABO-compatible with the recipient's serum, there is not much evidence that this interferes appreciably with the effectiveness of the transfusion, but the red cells must be removed first. This is done both by modifying the collection technique to include less RBC, and by removing visible remaining ones before transfusion. We have often transfused ABO-incompatible PMN this way, and found them well tolerated.

The Rh type need not usually be considered unless the recipient has Rh antibody, in which case the RBC can be removed as above. Otherwise, only in the cases of Rh-negative girls and younger women who might achieve a long remission of disease is the selection of Rh-negative donors or the use of Rh immune globulin worth consideration. Similar considerations hold in the case of other RBC antibodies of the type likely to have clinical significance.

The matter of HLA compatibility is somewhat more controversial in that some earlier work suggested a direct relationship between posttransfusion PMN recovery and HLA compatibility as well as ABO [56]. Given the many difficulties involved in such conclusions (small doses transfused, very low cell counts in patients, and other variables in disease, treatment, hemorrhage, and other con-

current blood product transfusions), I doubt that they have much significance. In particular, such data have not been analyzed for the effect of crossreacting HLA antigens [57]. The consensus nowadays is that there is no evidence to support HLA-matching for PMN transfusions to unimmunized patients. In the case of alloimmunized recipients, however, e.g., those who already exhibit refractoriness to platelet transfusions, there is some evidence that transfused unmatched PMN may not survive as well, and may cause severe reactions in some patients [43]. Unfortunately, other than platelet refractoriness and the occurrence of reactions, it is difficult to judge whether or not a patient is alloimmunized. The presence or absence of leukocyte antibodies is of little or no help with present methods [58]. If PMN transfusions in good amounts produce no increments, if there is also no platelet increment from PMN collections that include platelets, and if the patient has severe reactions (e.g., febrile, pulmonary), then it may be necessary to select donors by HLA type as is done with platelets, i.e., matching by cross-reactive antigens [57]. Bearing in mind that PMN transfusions must be given at least daily, this could become a severe burden on the blood donor service and should not be undertaken lightly.

Hazards: Reactions and complications

PMN transfusions involve not only granulocytes, but also platelets, mononuclear cells, RBC, and plasma, plus some anticoagulant and, usually, macromolecular agent. Consequently, it is not surprising that any of the reactions commonly associated with blood transfusion should be encountered, particularly *allergic* and *febrile responses*. The usual symptomatic medications may be used either prophylactically or therapeutically. Mild febrile reactions are rather common and need not be taken too seriously. Severe shaking chills and fever, especially with centrifugally prepared cell concentrates, probably indicate alloimmunization. Such reactions are reported about twice as often with FL cells, perhaps related to some cell damage. Reactions of this sort can be prevented or controlled to some extent by slowing the transfusion rate, e.g., to about 10^{10} cells per hour.

Circulatory overload is not uncommon since most of these patients are also receiving other transfusions (e.g., RBC) and other intravenous fluids and medications. *Local infections*, particularly pneumonitis, may appear to be exacerbated because transfused PMN migrate to the lesion and form an exudate. Severe *respiratory reactions* leading to pulmonary edema may occur in alloimmunized recipients, presumably caused by the action of antibodies on the transfused PMN, with sequestration in the lungs. These and other reactions are discussed by Higby and Burnett [43].

Certain *serologic complications* may occur when PMN or platelets are transfused from a donor whose plasma contains an antibody against the recipient's RBC, e.g., an O donor for an A patient. In that case, antibody may attach to the recipient's RBC, causing a positive direct antiglobulin reaction. If enough antibody is present, it may be detectable even in the recipient's plasma, with the

result that crossmatches may appear incompatible with RBC of the patient's own type. While this rarely causes significant hemolysis, it can cause some confusion and delay in the interpretation of laboratory tests. Fortunately, it is easily prevented by removal of plasma from the PMN concentrate before transfusion.

Graft-versus-host disease (GVHD) is always a possibility when any blood component containing live lymphocytes is given to a severely immunodepressed patient. Almost all reported cases have been in children receiving heavily immunosuppressant chemotherapy [59, 60]. In such cases, or in adults thought to be subject to the same hazard, all cellular blood products should be irradiated before transfusion. A dose of 3000 rads is considered adequate, and is not sufficient to damage the cells that are to be transfused.

Some other special complications or possible complications need to be considered. One report found an increased incidence of *lethal pulmonary reactions* in patients given amphotericin B while receiving PMN transfusions [61]. However, when we analyzed our own data at the University of Arizona [62], we found no such association. There must be significant differences between these two series to explain the discrepancy. Despite the findings of the former report, we do not think that amphotericin B should be withheld from neutropenic patients receiving PMN transfusions if they show evidence of fungal infections, although close observation and caution are advised.

We have also studied the significance of *pulmonary infiltrates* developing in patients already receiving PMN transfusions [63]. Localized infiltrates occurring one to three weeks after a series of PMN transfusions is begun are often caused by resistant infectious organisms and seem to represent a failure of transfusion therapy. They require aggressive investigation and treatment. Infiltrates appearing a few days after the onset of PMN transfusion, or more diffuse infiltrates, may well represent an appropriate migration of PMN to a site of infection and seem to have no ominous significance.

Current and future developments

Most transfusionists agree that better clinical outcome of infections in neutropenic patients could be expected if larger transfusions of PMN could be given. The newer blood cell separators are beginning to achieve that (Table 3). Also, bearing in mind the short life of a circulating granulocyte even in normal circumstances, it is reasonable to expect that more frequent transfusions, e.g., two or even three a day, may be needed [43]. In practical terms, that would be very difficult to achieve. It could be possible nonetheless, if PMN transfusions in general could be used in more strictly defined clinical circumstances, i.e., restricted carefully to those patients most likely to benefit.

Improved storage of PMN, both in the liquid and frozen states, is badly needed, and research in this direction is going on. In our laboratory, we are studying the use of synthetic storage media at refrigerator temperatures. Frozen storage of mature PMN would greatly facilitate the logistics of PMN collection and storage, but the results so far are disappointing.

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Health Risks of Leukapheresis Donors

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The increasing use of normal donors for leukapheresis to provide granulocyte concentrates requires careful monitoring of recruitment and operational procedures to minimize donor health risks. Of 18 288 leuka- and leukaplateletpheresis performed in American Red Cross regional blood services during a 12-month period, only 0.27% were discontinued because of acute donor reactions. None of these reactions had serious or long-term consequences for the donors. The potential toxicities of corticosteroids and hydroxyethyl starch, and the theoretical hazards of lymphocyte depletion, raise unanswerable questions concerning long-term safety, although no serious adverse reactions related to these factors have been observed. Critical factors for leukapheresis donor safety are: selective donor recruitment, adherence to operational protocols, and continuous and informed interest of physicians responsible for pheresis programs. Additional data are needed to assess the effects of repeated leukapheresis on donors and to establish rational guidelines for donor recruitment.

Introduction

Donor risk

The development of centrifugal and filtration leukapheresis technologies for the collection of granulocyte concentrates has introduced new adverse reactions and some potential health risks for donors when compared with conventional whole blood donations. If evaluated in terms of the frequency of serious adverse reactions, normal donor leukapheresis is a safe procedure and only rare serious complications have been reported in tens of thousands of procedures during the past 16 years. On the other hand, the majority of contemporary leukapheresis donors are healthy volunteers, unknown and unrelated to the patient-recipients. One can reasonably ask whether any risks are ethically justifiable for these leukapheresis donors, for whom benefits are limited to the fulfillment of altruistic goals.

The following review summarizes known adverse reactions in leukapheresis donors and discusses the potential or theoretical risks inherent in these procedures. The objective of the review is 1. to assist clinicians in assessing the risks that are incurred by donors who provide granulocyte concentrates and 2. to provide information for obtaining informed consent from potential leukapheresis

donors. The present review does not evaluate the effectiveness of granulocyte transfusion therapy since this subject has been summarized elsewhere included in a recent symposium [1–3]. Similarly, the reader is referred to other recently published articles for operational details of current leukapheresis methodologies including continuous-flow centrifugation leukapheresis (Blood Cell Separator, IBM Corporation, Endicott, New York; Aminco Celltrifuge, American Instrument Company, Silver Spring, Maryland; Model CS-3000, Fenwal Laboratories, Morton Grove, Illinois); intermittent-flow centrifuge leukapheresis (Model 30, Haemonetics Corporation, Natick, Massachusetts); and filtration leukapheresis (Filtration Leukopheresis System, Fenwal Laboratories, Morton Grove, Illinois) [4–7].

Historical note

The precedent for recruiting healthy family members and unrelated volunteer donors to provide granulocyte concentrates by extracorporeal separation of their blood dates to clinical investigations in the 1960's using the continuous-flow cell separator [8, 9]. Prior to these investigations, granulocyte concentrates had been collected from patients with chronic myelogenous leukemia (CML) using multiple plastic packs [10] or centrifugal cell separators [11, 12], or from normal donors by pooling buffy coat preparations obtained from conventional whole blood collections [13, 14]. An earlier precedent for recruitment of healthy donors for extracorporeal blood separation had been established by platelet- and plasmapheresis programs using multiple plastic packs followed by reinfusion of citrated blood [15]. While these manual hemapheresis procedures had certain inherent risks, continuous-flow centrifugation and filtration leukapheresis introduced, among others, the additional hazards of systemic heparinization, erythrocyte sedimentation agents, drugs for granulocyte mobilization, complement activation and removal of other cellular elements involved in immunoregulation and stem cell replication. The publication of early reports of beneficial results in the management of acute leukemia and other malignant diseases using granulocyte transfusions [12, 16] lead to a sequence of decisions to supply granulocyte concentrates by many other investigative and general service facilities. From the initial three NCI-IBM blood cell separators manufactured for clinical trials in 1966 [17], the number of hemapheresis machines in operation increased to several hundred within only a few years.

In 1978 the National Heart, Lung and Blood Institute (NHLBI) conducted an informal survey of 16 of the largest blood centers in the United States revealing that the number of normal donor leukaphereses had doubled between 1976 and 1977 and that the trend was toward increased numbers of procedures being performed on individual donors [18]. By 1979, 280 blood collection facilities in the United States reported to the Food and Drug Administration that they routinely performed normal donor leukapheresis [19]. Of the 190 of these facilities responding to a mail questionnaire, 83% used intermittent-flow centrifugal leuka-

pheresis (Model 30, Haemonetics Corporation), 25% continuous-flow leukapheresis (NCI-IBM, IBM 2997, Aminco), 13% filtration leukapheresis (Fenwal Leukopheresis System), 3% manual methods (plastic packs) and 23% used more than one method. Ninety-six percent of all establishments used 6% hydroxyethyl starch (HES; Volex McGraw Laboratories, Irvine, California).

The American Red Cross experience is consistent with the trends observed in the NHLBI survey. In 1975, 25 American Red Cross regional blood services performed a total of 836 normal donor leukaphereses. By 1980, 42 American Red Cross blood services were performing leukapheresis and the combined total of leukaphereses and leukaplateletphereses had increased to 18 288 [20].

Categories of Health Risks

Clinical reactions and potential risks. The health risks to normal leukapheresis donors may be divided into 1. adverse acute clinical reactions such as citrate toxicity, hypotension, hemorrhage, hypervolemia and infection and 2. potential risks such as the theoretical consequences of HES deposition in donor tissues and lymphocyte depletion. Adverse clinical reactions are readily identifiable. They can be quantitated and preventative measures can be instituted. The potential or theoretical risks, on the other hand, are more troublesome and persist as nagging concerns because of the lack of adequate data to support or to refute them.

Adverse donor reactions

Citrate toxicity. The most frequent adverse reactions associated with leukapheresis donations are the symptoms of acute hypocalcemia resulting from rapid reinfusion of citrated donor blood and typically manifested as tingling around the mouth and fingers. Occasionally, these reactions are magnified by donor hyperventilation. Since these early symptoms are promptly reversed by slowing the reinfusion rate of citrated blood, overt tetany and other serious complications of acute hypocalcemia are rarely encountered. Concern that anticoagulation with citrate alone was not adequate to prevent platelet clumping has lead to the use of combined citrate-heparin anticoagulation in some pheresis programs, but this is done infrequently. The reversal of signs of acute citrate-induced hypocalcemia with an injection of 10% calcium gluconate has been recommended [21]. However, the risks of iatrogenic acute hypercalcemia to the donor may be at least as serious [22], and such an approach is not recommended.

Hypotension. As in conventional blood donation, the leukapheresis donor is at risk for syncopal and other hypotensive reactions secondary to acute hypovolemia, particularly with discontinuous-flow centrifugation when as much as 550–650 ml of blood may be in the extracorporeal circuit. Most patients respond to rapid infusion of 0.9% sodium chloride and/or return of their own blood with

prompt elevation of blood pressure and restoration of well-being. An occasional donor may experience persistent hypotension, weakness and diaphoresis despite adequate correction of impovolemia. This prolonged donor reaction may be due to an exaggerated vasovagal reaction or to anxiety. One author (SGS) recalls a healthy-appearing 45-year-old male who experienced a massive myocardial infarction while in the donor room following a routine 450 ml whole blood donation. Clearly, a similar reaction precipitated by the mild hypovolemia of leukapheresis could occur in a predisposed leukapheresis donor and, therefore, all hypotensive reactions not responsive to fluid replacement should be evaluated for other causes of hypotension. Some hemapheresis programs consider routine electrocardiograms on all persons over 40 years old to be advisable [21], but the effectiveness of this approach for detecting donors at risk for cardiovascular complications is unproven.

Hemorrhage. Donors receiving heparin during filtration leukapheresis are at risk of unexpected bleeding, but such complications have been rare. Also, in the United States in the past few years, the use of filtration leukapheresis has decreased markedly. Donor risks of bleeding are minimized by taking careful medical histories to exclude menstruating females and all persons with past histories of peptic ulcers and other diseases likely to increase the risk of local or generalized bleeding. Some investigators believe that prothrombin and partial thromboplastin times are essential for all pheresis donors receiving heparin for the first time but a careful medical history will usually suffice. When performing a series of closely scheduled procedures using heparin, such monitoring of the coagulation system is indicated, however. The combination of thrombocytopenia and heparinization significantly increases the risk of bleeding and, therefore, platelet counts or estimates should be performed on all donors scheduled for heparin anticoagulation and multiple leukaphereses.

Any person receiving heparin is at risk for the known adverse and idiosyncratic reactions of this drug which include hypersensitivity, thrombocytopenia, alopecia and priapism. Neutralization of heparin's anticoagulant effects with protamine sulfate may be desirable in certain circumstances but increases the risk of additional complications, including hypotension, bradycardia, dyspnea and anaphylaxis.

Because of evidence in animals suggesting an HES-induced bleeding tendency [22], coagulation and platelet function have been studied in leukapheresis donors receiving HES [23]. Partial thromboplastin and prothrombin times were prolonged and accompanied by decreased activities of factors V and VIII and fibrinogen. Levels of factor IX, antithrombin III, fibrin monomers and fibrin degradation products remained normal. These laboratory results, although statistically significant, were slight and are not considered to reflect a clinically relevant tendency to bleeding or thrombosis [23].

Hypervolemia. Headache, swollen fingers, peripheral edema and other symptoms of acute plasma volume expansion are recognized risks of infusing 6% HES in normal leukapheresis donors. In a study designed to simulate serial

leukapheresis of a single donor, 500 ml of 6% HES were administered intravenously at a constant rate over a two-hour period for each of four consecutive days [24]. The recipients experienced an average accumulation of 850 ml and an average 37% increase in plasma volume. While normal healthy adults will tolerate physiologic alternatives of this order, the rare predisposed donor with borderline cardiac function is a increased risk and may experience overt congestive heart failure.

Equipment failures. With 250–600 ml of blood *ex vivo* for 2–4 hours, the leukapheresis donor is at risk of a wide range of complications resulting from mechanical failures. Isolated episodes of hemoglobinuria have been attributed to kinking of the tubing and mechanical hemolysis of donor erythrocytes by pumping devices [25, 26], but these events are less common following technical improvements. At least one donor has been observed to have experienced recurrent hemolytic episodes with no obvious mechanical cause or morphologic defect of his erythrocytes [26]. Clotting in the needle or tubing may prevent return of the donor's blood and, rarely, massive extracorporeal clotting has occurred due to layering of the HES-sodium citrate mixture resulting in inadequate dispensing of the anticoagulant [27].

Infection. A case of Gram-negative bacteremic shock in a healthy donor has been reported developing minutes after terminating discontinuous-flow leukapheresis with 6% HES and trisodium citrate [28]. Cultures of the HES-trisodium citrate solution, the granulocyte concentrate and all blood specimens from the donor subsequently yielded *Enterobacter cloacae* [28]. The portal of entry was a hairline crack in the infusion bottle which had not been detected by the staff. This report in a normal leukapheresis donor, as well as other reports of contaminated intravenous solutions in different settings [29–33], have alerted hemapheresis staffs to the serious risks of contaminated intravenous solutions. The possibility of lifethreatening donor infections from contaminated solutions remains a constant, although unlikely, donor risk.

Chills. The cooling of large volumes of donor blood extracorporally may result in "chills" which are usually controlled by covering the donor with warm blankets. Increasing the ambient temperature in the donor room is useful but adding in-line warming units to the return tubing to minimize this discomfort increases the complexity of the procedure and is not recommended.

"*Anaphylactoid reactions.*" Acute cutaneous reactions characterized by urticaria, pruritus, flush and, occasionally, angioedema have been reported in leukapheresis donors receiving 6% HES, as well as in plateletpheresis donors who have not received HES. The cause of these acute reactions is unknown and it is possible that several different pathophysiological mechanisms are involved.

Although antibodies to HES have been produced in rabbits by immunization with a protein-HES conjugate [34], antibodies to HES have not been demonstrated convincingly in humans. The observation that recipients of HES-containing granulocyte transfusions do not become sensitized to HES is consistent with the lack of immunogenicity anticipated for this glycogen derivative

and suggests that HES-antibody-antigen mediated hypersensitivity is an unlikely pathogenic factor. The possible roles of histamine, prostaglandins, bradykinin, serotonin, complement, anaphylatoxins and IgE is speculative, but intriguing similarities with clinical syndromes associated with these mediators may be noted [35–37]. While activation of complement by nylon fibers in the filtration leukapheresis procedure is well documented [38–40] and complement activation occurs during other extracorporeal procedures [41–43], the minor changes in complement components during intermittent-flow centrifugation platelet-leukapheresis are not thought to result from complement activation [23].

Chronic cutaneous reactions. A donor who had received 6% HES for leukapheresis on only one previous occasion developed lichen planus in the anticubital fossa near the injection site only a few days after leukapheresis [44]. While there is no conclusive proof of a direct causative relationship, the temporal association between the onset of initial symptoms and HES infusions prompted the authors reporting the case to conclude that the findings strongly supported this conclusion. Aside from this single case report, whose relevance to donor risks of leukapheresis remains a matter of opinion, no other similar reports have appeared in the medical literature.

Abdominal pain. An unusual donor reaction characterized primarily by lower abdominal and/or perineal pain occurs rarely during filtration leukapheresis and almost exclusively in females [45–48]. The pathogenesis of this reaction is unknown, but it has been suggested to be a possible "angio-neurotic edema bowel syndrome" resulting from nylon fiber-induced complement activation [45].

Adverse Donor Reactions – American Red Cross Experience (1980)

During 1980, 42 of 57 American Red Cross regional blood services operated normal donor leukapheresis programs. During this 12-month period a total of 41 356 cytapheresis procedures were performed, consisting of 2919 leukaphereses, 15 369 combined leukaplateletphereses and 23 068 plateletphereses [49]. Thirty-eight regional blood services reported moderate-severe donor reactions which required discontinuation of the pheresis procedure. The overall rate of adverse donor reactions requiring discontinuation of leuka- and leukaplateletpheresis was 0.27% (Table 1).

Severe reactions

The most common of these severe reactions was acute hypotension (Table 1). Twenty-two donors experienced anxiety, profound weakness and diaphoresis. Ten donors lost consciousness and had mild tonic-clonic seizures. While mild citrate toxicity, manifested by occasional perioral or peripheral paresthesias, were common and probably incompletely reported, severe symptoms resulting in discontinuation of procedures occurred in 10 donors. Other donor reactions resulting

Table 1

Adverse reactions causing discontinuation during 18 288 normal donor leukaphereses; American Red Cross Blood Services (January 1–December 31, 1980)

Reaction	Number*
Hypotension/Vasovagal	
Pallor, severe weakness, diaphoresis, anxiety, nausea (22) Syncope or seizure (10)	32
Citrate toxicity	
Paresthesis, perioral numbness, pressure sensation in head/chest, leg muscle spasm	10
Blood loss	
Clotting in system (200 ml)	1
Equipment malfunction (695 ml)	1
Miscellaneous	
Painful needle site	1
Rash and subcutaneous nodules (urticaria)	2
Asthma attack (known past history)	1
Severe cough	1
Severe febrile reaction	1
	50

* Rate of severe reactions for 50 reactions per 18 288 total leuka- and combined leuka-plateletphereses was 0.27%.

in discontinuation of phereses included protracted cough (without signs of congestive failure), urticaria, painful needle sites, hematomas and an unexplained severe febrile reaction.

"*Anaphylactoid reactions*"

Ten cases of acute "anaphylactoid" reactions were reported consisting of urticaria and/or pruritus, periorbital edema, erythema ("flush"), focal swelling of the upper lip and sensation of pressure in the head. All symptoms resolved within 2–24 hours. There was a subjective impression report that those donors who received diphenhydramine (Benadryl) had beneficial clinical responses to the medication.

Potential Risks

In addition to the observed adverse donor reactions, evidence of additional physiological alterations raises concerns that leukapheresis may be inducing other, as yet undetected, adverse effects in normal donors. These theoretical or potential

risks have been the subject of symposia addressing both the clinical [50, 51] and the ethical issues [52, 53]. The principal concerns may be categorized as 1. drug toxicity and 2. lymphocyte depletion.

Drug toxicity – HES. The primary concern related to HES is that it is given in massive dose and its prolonged catabolism and potential accumulation in the recipient's reticuloendothelial system may have consequences requiring years of observations to detect. HES, or amylopectin, is a starch molecule manufactured from waxy sorghum (maize) and treated with ethylene oxide to attach hydroxyethyl groups to individual glucose residues. Commercial preparations of HES are heterogenous compounds with molecular weights ranging from 10 000–2.5 million daltons [54]. Ironically, these commercial HES preparations which are used as sedimenting agents in leukapheresis were designed as plasma volume expanders and, therefore, were selected for their properties of delayed catabolism and prolonged intravascular effect. The elimination of intravenous infusions of 6% HES in humans is complex, although virtually all injected material is eventually recovered in the urine and feces [54–56]. Approximately 50% of HES administered by rapid intravenous infusion is eliminated from the blood and excreted by the kidneys within 72 hours [55]. The smaller HES molecules (< 50 000 daltons) are rapidly cleared by the kidneys, whereas a portion of the larger molecules which are susceptible to prompt degradation by alpha amylase are hydrolyzed before excretion. This second phase of protracted elimination of HES has been variously measured after infusions of HES, revealing at least 1% plasma residual at 17 weeks [55]. After multiple infusions of HES, complete elimination from the circulation requires several months [56]. Presumably, these residual molecules are too large for glomerular filtration and are structurally resistent to catabolism by alpha amylase, but precise characterization of the slowly metabolized HES molecules has not been performed. Serial infusions of HES may result in intravascular accumulation of HES and has been detected, transiently in both parenchymal and reticuloendothelial cells of several organs [57–59].

Aside from the discomfort and hazards of plasma volume expansion in some donors, the significance of persistent intravascular or tissue HES in leukapheresis donors is unknown. Based on the absence of reported complications in tens of thousands of leukapheresis donors, these potential risks would appear to minimal, if any. It is possible that alternative HES preparations could be manufactured which would have the same sedimentation properties but which are catabolized more rapidly than current preparations. These would be more suitable for normal donor leukapheresis and promise to increase the procedure's efficiency while reducing the potential risks associated with current preparations [54].

Drug toxicity – Corticosteroids. To increase the efficiency of leukapheresis collections by raising the donor's white blood cell count, donors have been pretreated with etiocholanolone [60] or one of the various corticosteroid preparations. While the discomfort of painful injection sites, fevers and malaise have resulted in reduced use, if not discontinuation, of etiocholanolone as a stimulus for leukocytosis, the 1980 NHLBI survey reported that 48% of the reporting

facilities used corticosteroids to induce leukocytosis. Dexamethasone in an average dose of 7 mg per donor (4 mg per square meter body surface area) administered 10–12 hours prior to donation accounted for 79% of the corticosteroid usage. In the remaining 21% prednisone was used in an average dose of 40 mg per donor (1.7 mg per kilogram body weight) orally approximately 8–12 hours prior to leukapheresis [19].

Single doses of dexamethasone may evoke headache, fever, tiredness, sweating and changes in mood and performance [61]. All the above signs and symptoms may occur in leukapheresis donors but are minimal hazards. Other recognized side-effects of corticosteroids include exacerbation of diabetes mellitus, peptic ulcer, tuberculosis and hypertension. These complications must be rare, if they occur, since all leukapheresis donors are carefully screened and no reports of these complications have been published nor are known to the authors. The theoretical risk that corticosteroid administration prior to each in a series of repeated leukaphereses could suppress adrenal function has been addressed and considered improbable [62].

Lymphocyte depletion. While the number of lymphocytes removed during a single centrifugal leukapheresis ($2–4 \times 10^9$) is only about twice the number of lymphocytes in an ordinary whole blood donation [48], the practice of using a single "matched" donor for multiple leukaphereses introduces theoretical risks of lymphocyte depletion and immunoincompetence. This theoretical concern has not been observed, although hundreds of family members and other selected normal donors have participated in intensive multiple leukapheresis programs.

Since the total body pool of lymphocytes is at least 1×10^{12}, a single leukapheresis will remove only 0.1% of these and is unlikely to cause lymphocyte depletion and measurable immunosuppression [4]. The removal of $10–200 \times 10^9$ lymphocytes, which reportedly will cause lymphopenia and clinical immunosuppression, has been estimated to require 60 daily leukaphereses by conventional methods [4]. Nevertheless, these figures are only gross estimates and the actual number of lymphocytes removed by a leukapheresis will vary widely, depending on the individual donor and the procedure. For example, in a study of rheumatoid arthritis patients the removal of approximately 3.5×10^9 lymphocytes 2–3 times per week for 5–7 weeks using a technique deliberately selected for maximal depletion of lymphocytes resulted in absolute lymphocyte depletion comparable to that reported for chronic thoracic duct drainage [63]. The greatest changes in peripheral blood lymphocyte numbers occurred after only 3–4 leukaphereses. While the absolute lymphocyte counts returned to at least 50% of normal in some patients over days-to-weeks, other patients were followed for more than one year with lymphocyte counts remaining below 50% of preleukapheresis levels. In addition, there was a selective depletion of T lymphocytes, loss of phytohemagglutinin mitogenic responsiveness and decreases in IgG, IgA and IgM levels. There are at present no comparable studies on chronic leukapheresis donors, but lymphopenia and selective depletion of B lymphocytes have been noted in frequent plateletpheresis donors [64].

The problem of estimating long-term clinical risks from these changes in peripheral lymphocyte measurements is complex. The present authors are unaware of any adverse reactions in leukapheresis donors that could be attributed to lymphocyte depletion. With due reservations for the lack of relevant data, we concur with the viewpoint that the consequences of lymphocyte depletion from individual leukaphereses are unlikely to differ from those of whole blood donations [4, 65] and these procedures as currently performed are safe and without risk for normal donors. However, there may be only a narrow margin of safety between the effects of these seemingly inconsequential individual donations and the more profound and longer lasting effects of multiple leukaphereses. Clearly, more data are needed to assess the long-term effects of multiple leukaphereses and establish rational donor criteria and standards.

Conclusion

Leukapheresis procedures, while not without risk, have been associated with few serious donor reactions. Undoubtedly, this record is the result of an awareness among blood center personnel performing leukapheresis procedures of possible complications and their ability to maintain operational protocols and strategies which minimize donor risks. It is our view that there are three critical factors for increasing donor safety of leukapheresis procedures: 1. selective donor recruitment; 2. adherence to operational protocols delineating donation frequency and other safeguards; and 3. continuous and informed interest of physicians responsible for leukapheresis programs. While certain potential hazards of leukapheresis procedures remain to be evaluated, particularly in the area of potential delayed complications, such hazards would also be minimized by adherence to these principles.

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The Enzymatic Status of Circulating Lymphocytes as an Index of the Regenerative Process in the Lungs under Stimulation with Pyrimidine and Purine Derivatives
A Clinical Experimental Study

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Enzyme activities in the circulating lymphocytes permit to judge the character of the regenerative process in the lung, and the severity of the patient's condition; it helps in estimating the prognosis and in evaluating the efficacy of the applied therapy.

Potassium orotate and Riboxin intensify the regenerative process in the lung undergoing a compensatory-hypertrophic rearrangement, and enhance the functional activity of circulating lymphocytes as participants of the regenerative process and stimulate the oxidation-reduction processes in lymphocytes.

Introduction

Removal of a lung or part thereof in mammals including man and many different pulmonary diseases accompanied by increased functional loading lead to regenerative or compensatory hypertrophy in the remaining pulmonary tissue due to increased proliferation of epithelial cells, synthesis of nucleic acids and protein [1–5].

Subsequently, the compensatory-hypertrophic processes as a rule develop into sclero-atrophic changes with resulting pneumosclerosis and emphysema. Hence, there is pressing need for finding ways and means of stimulation of regenerative processes in the lung and for prophylaxis of sclero-atrophic alterations.

During the last three decades clinical studies have been conducted on pyrimidine and purine derivatives (methyluracil, potassium orotate and inosine), which stimulate intracellular and cellular regeneration [3, 6–14].

There exist numerous morphological tests allowing to judge the course of the regenerative processes in the lung and the effect produced on them by pharmacological substances. Since these tests being based on examination of the pulmonary tissue cannot be used in clinical practice, the search for more informative and acceptable laboratory methods, permitting assessment of the restorative process in the lung and the efficacy of drugs stimulating regeneration, has become of paramount importance.

In recent years attention of a broad circle of investigators has again been attracted by the circulating lymphocytes which play an important part in immunogenesis, preservation of genetic characters of the cells, in control of the growth

of proliferating tissue, being at the same time lymphoid elements convenient for use in research and reflecting the status of metabolic processes in internal organs whose direct morphological study is impossible [15–17].

Our intention is to give examples of the value of lymphocyte assays partly in animal experiments (compensatory hypertrophy in the rat), partly in certain clinical states like purulent inflammation. In the following we report on our findings obtained in these two fields.

Experiments in Rats

The influence of the pyrimidine derivative potassium orotate and of the Soviet-made purine derivative Riboxin on the dynamics of the enzymatic status of circulating lymphocytes, as well as the processes of synthesis and proliferation in the regenerating lung during its compensatory-hypertrophic rearrangement was studied in 340 adult male rats. The animals were subjected to left-side pulmonectomy under ether anaesthesia, and were administered 100 mg/kg of potassium orotate or 25 mg/kg body weight of Riboxin daily. Control animals received an equivolume amount of 2% starch mucilage. Prior to the operation, and 24, 48 hours and 5, 7, 10, 20, 30 and 60 days after it the peripheral blood lymphocytes were studied by Nartsissov's cytochemical method [18] and the proliferative activity of the interalveolar septum cells by counting the number of mitoses in histological paraffin sections. DNA synthesis by the regenerating lung cells was studied with the use of ^{3}H-thymidine.

The study of lymphocytes included determination of lactate dehydrogenase (L-lactate NAD-oxidoreductase, 1.1.1.27, LDH); succinate dehydrogenase (succinate oxidoreductase, 1.3.99.1, SDH); hyaloplasmatic alpha-glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD-oxidoreductase, 1.1.1.8, α-GPD(H), and mitochondrial L-glycerol-3-phosphate: acceptor-oxidoreductase, 1.1.99.5, α-GPD(M).

Computerized mathematical processing of the data obtained was performed with the computer Nairi 3-I (by M. L. Nikolaev).

The findings obtained in these studies (Table 1) have convincingly shown that potassium orotate and Riboxin could stimulate the compensatory-hypertrophic rearrangement in the remaining lung. The process manifested itself with a considerable increase in mitotic activity (MA) and an increased index of ^{3}H-thymidine-labelled nuclei (ILN) of the cells of the interalveolar septa. The stimulatory effect was most pronounced in the periods of pseudo- and true hypertrophy, during the first 10 days, whereas in the period of completion and stabilization of the rearrangement the intensity of the proliferative and synthetic processes decreased. Riboxin and potassium orotate not only stimulated regeneration (compensatory hypertrophy) of the lung, but produced a marked effect on the functioning of circulating lymphocytes. In the periods of pseudo- and true hypertrophy they increased the activity of SDH, α-GPD(H), and α-GPD(M),

slowed down that of LDH, ensuring simultaneously an intensification of the Krebs cycle and of the glycerophosphate shuttle, creating thereby favourable conditions for energy generation in the cell. Moreover, exposure to Riboxin contributed to the intensification of aerobic metabolism of lymphocytes ensuring the economical use of alpha glycerophosphate, an ingredient of phospholipid synthesis utilized for restoration of lost structures. In the period of stabilization and completion, the activity of all dehydrogenases under study decreased. The same was observed in the controls, but the process was most pronounced in the treated animals. The regenerative process in this period may have been ensured among others by lymphocytes with a low level of oxido-reductive processes.

The compensatory-hypertrophic rearrangement of the lung brings about not only changes in the activity of lymphocytic enzymes, but also a rearrangement of the cell population with growing heterogeneity of lymphocytes with regard to hydrogenase activity. The effect of both regeneration stimulation had one feature in common, in that they both increased the enzymatic activity of lymphocytes and decreased their heterogeneity.

The initial hypothesis that enzymatic activity of circulating lymphocytes reflects, with a sufficient degree of accuracy, the course of the regenerative process in the lung has been confirmed by regressive analysis, which could be judged by the level of mitotic activity of the interalveolar septum cells. Using regressive equations, an algorhythm was created, permitting the assessment of the intensity of regenerative processes in the lung on the basis of the enzymatic activity in lymphocytes. The correlation of MA with the activity of the enzymes under study is

$$MA = K_1 \cdot \alpha\text{-GPD(H)} + K_2 \cdot \alpha\text{-GPD(M)} + K_3 \cdot SDH + K_4 \cdot LDH + K_0$$

At different periods of the compensatory-hypertrophic rearrangement, coefficients $K_0 \ldots K_4$ varied but the general pattern of the interconnection between enzymes in lymphocytes and MA of the interalveolar septum cells remained unchanged throughout the investigation period.

The study of dynamics of the regeneration process in the lung and in the circulating lymphocytes using kinetic parameters on theoretical curves has made it possible to establish a number of important regularities (Table 2). The peak increase of enzyme activity in lymphocytes exceeded by 19–26 hours the maximum mitotic activity of the interalveolar septum cells, and the incorporation of labelled ^{3}H-thymidine by 31–36 hours. Theoretically, a higher IMN and enzyme activity was expected than that observed.

A favourable effect of the preparations could be achieved in two ways: by enhancing the lymphoid tissue response and the proliferative activity of the interalveolar septum cells and their Riboxin-induced DNA synthesis, or by lengthening the process by means of potassium orotate. As judged by the intensity of regeneration, the former mechanism was the more effective.

Table 1

Enzyme activities in circulating lymphocytes; mytotic activity and index of labelled nuclei of DNA-synthesizing cells of interalveolar septa

Indicator	Group	Hours $\bar{x} \pm m$		Days, after operation $\bar{x} \pm m$					
		24	48	5	7	10	20	30	60
In circulating lymphocytes									
α-GPD(H)-activity	healthy rat	7.71 0.11							
	C	7.19 0.24	18.21^{x} 0.54	10.18 0.25	11.78 0.79	7.26 0.28	7.34 0.31	7.87 0.29	5.49^{+x} 0.25
	PO	7.26 0.30	15.26 0.50	11.15^{x} 0.28	12.31 0.38	7.17 0.29	8.42 0.28	7.70 0.26	4.27 0.16
	R	10.07^{+o} 0.30	17.11^{o} 0.65	11.03^{+} 0.28	12.62 0.53	8.79^{+o} 0.27	8.23 0.29	7.47 0.31	4.36 0.18
α-GPD(M)-activity	healthy rat	7.96 0.22							
	C	8.46 0.32	17.45^{+} 0.53	10.69 0.25	10.83^{+} 0.38	8.14 0.54	6.70 0.25	6.20^{+} 0.26	6.89^{+x} 0.25
	PO	7.79 0.27	17.38^{o} 0.66	12.38^{ox} 0.31	10.89^{o} 0.40	8.87 0.34	$12.32^{x;}$ 0.37	6.36^{o} 0.21	5.37 0.18
	R	9.56^{+o} 0.31	10.97 0.49	10.58 0.31	9.51 0.41	10.36^{o+} 0.46	8.02^{+} 0.25	5.24 0.25	5.16 0.21
SDH-activity	healthy rat	13.39 0.27							
	C	9.67 0.32	19.54 0.58	14.09^{+} 0.37	15.22 0.54	9.99 0.31	10.68 0.36	10.57 0.31	9.35^{+x} 0.27
	PO	8.89 0.37	19.68 0.53	14.08^{o} 0.29	16.69^{x} 0.65	11.20^{x} 0.30	11.79^{x} 0.33	10.28 0.32	5.65 0.18
	R	11.65^{+o} 0.30	19.49 0.67	12.72 0.29	16.48^{+} 0.71	12.50^{o+} 0.34	13.09^{o+} 0.33	10.64 0.37	7.12 0.26

LDH-activity	healthy rat	15.09 0.34							
	C	14.27^{x+} 0.40	23.96^{x+} 0.70	15.11^{+} 0.49	17.59^{+} 0.65	14.03^{x} 0.38	20.23^{x+} 0.51	10.08 0.33	13.89^{x+} 0.36
	PO	13.27 0.37	21.82^{0} 0.59	15.34^{0} 0.47	19.57^{x0} 0.87	12.56 0.40	18.02 0.35	12.35^{x} 0.40	11.03^{0} 0.34
	R	13.21 0.38	18.91 0.47	13.76 0.41	15.44 0.95	17.05^{0+} 0.46	17.21 0.42	12.03^{+} 0.66	8.97 0.27
MA (light)	healthy rat	0.79 0.06							
	C	0.42 0.02	1.05 0.13	1.70 0.08	1.03 0.09	0.86 0.13	0.47 0.01	0.43 0.03	0.43 0.03
	PO	0.77^{x} 0.06	1.09 0.09	2.21^{x} 0.11	1.34^{x} 0.04	1.03 0.07	0.92^{x} 0.06	0.74^{x} 0.07	0.89^{x0} 0.13
	R	2.34^{+0} 0.01	3.38^{+0} 0.53	2.68^{+0} 0.13	1.84^{+0} 0.13	2.63^{+0} 0.19	1.03^{+} 0.09	0.92^{+} 0.06	0.68^{+} 0.08
ILN (light)	healthy rat	4.42 0.12							
	C	3.13 0.24	7.89 0.23	9.16 0.21	5.79 0.26	9.90 1.37	10.85^{x} 1.14	5.16 0.70	3.70 0.48
	PO	4.47^{x} 0.21	10.13 1.35	15.30^{x} 1.11	9.34^{x} 0.47	8.08 1.20	7.31 0.97	11.07^{x0} 0.32	3.86 0.65
	R	12.47^{+0} 0.98	22.53^{0+} 4.90	20.91^{0+} 0.50	14.84^{0+} 0.52	20.63^{+0} 0.79	9.06 2.84	6.85 0.51	4.06 0.18

Notes: C — Control, PO — Potassium orotate, R — Riboxin.
MA (light) — mytotic acitivity of interalveolar pulmonary cells;
ILN (light) — index of labelled nuclei of DNA-synthesizing cells of interalveolar pulmonary cells.

$^{+}$ — significant difference between C and R ($p < 0.02$; $p < 0.01$);
^{0} — significant difference between R and PO ($p < 0.02$; $p < 0.01$);
^{x} — significant difference between C and PO ($p < 0.02$; $p < 0.01$).

Table 2

Parameters of theoretical curves

Indicator	Group	t_{R+1} days	Q_{max}	t_{max} days	S	F	V
In circulating lymphocytes							
SDH-activity	C	5.1	21.26	2.8	87.96	106.24	12.59
	PO	5.3	20.82	2.9	86.11	106.67	12.13
	R	4.3	21.83	2.6	76.35	99.44	13.82
α-GPD(M)-activity	C	4.6	17.85	2.7	65.83	84.15	10.97
	PO	5.3	18.29	2.9	75.62	93.67	10.65
	R	4.9	12.83	2.8	55.65	91.11	5.61
LDH-activity	C	4.2	26.54	2.6	91.06	119.47	16.74
	PO	4.5	25.19	2.6	91.39	117.61	15.58
	R	4.1	18.73	2.5	67.90	120.75	8.20
α-GPD(H)-activity	C	4.6	20.87	2.7	70.27	91.05	13.35
	PO	5.2	16.49	2.8	67.25	83.82	9.66
	R	4.3	19.02	2.6	66.46	127.06	11.92
ILN (light)	C	8.0	10.12	3.6	59.39	66.59	4.74
	PO	8.9	15.10	3.9	93.88	106.42	6.58
	R	6.7	22.52	3.3	128.83	193.14	23.33
MA (light)	C	9.5	1.59	4.1	10.42	11.75	0.65
	PO	7.5	3.59	3.5	15.05	15.62	1.18
	R	4.7	3.42	2.7	14.26	23.64	1.50

Notes: t_{R+1} — time of normalization;
Q_{max} — maximum indicator;
t_{max} — time of maximum beginning;
S — "mean of regulation";
F — "square of regulation;
V — speed.

A correlation analysis of the empirical data allowed to establish a pattern of interrelationship between the activity of dehydrogenase in circulating lymphocytes and the indices characterizing the intensity of restorative processes in the lung as well as of the relationships between the enzymes.

The organization of enzyme systems in lymphocytes undergoes changes at different times following pulmonectomy which manifests itself in a rearrangement of correlation pleiads. Exposure to these preparations leads to their reorganization. Riboxin increases a number of bonds between MA and ILN of the interalveolar septum cells and the lymphocyte dehydrogenases. Potassium orotate acts in a similar way in the period of true hypertrophy.

Reorganization of pleiads under the effect of drugs opens prospects for a new approach to studies into the mechanism of action and to assessment of

therapeutic potency of drugs. Generally, pathological effects and potency of therapeutic action are assessed by the magnitude of the parameter deviation; our findings convincingly show feasibility of evaluation of the harmony and interconnections of parameters.

Clinical Investigations

Experimental results strongly support the advisability of using pyrimidine derivatives (potassium orotate and methyluracil) in patients with inflammatory suppurative diseases of the lungs and pleura. The enzymatic status of circulating lymphocytes and neutrophils was studied in 53 patients with pulmonary abscesses and gangrene, empyema, bronchiectasia and in 90 donors. Patients of the control group underwent a conservative or surgical complex treatment. The patients in the main group, in addition to conventional treatment, received orally 1.5 g potassium orotate daily, and 250 ml of 0.5% methyluracil solution by intravenous drip 4–5 times every other day prior to and after the operation.

Cytochemical investigation, in addition to the assessment of SDH and α-GPD(M) activity in circulating lymphocytes, included an evaluation of the activity of acid phosphatase (phosphohydrolases of monoethers of phosphoric acid 3.0.3.2), acid phosphatase and alkaline phosphatase (phosphohydrolases of phosphoric acid monoether, 3.13.1), alkaline phosphatase by the method of Goldberg and Barka [19] in the modification of Nartsissov [20]; myeloperoxidase (donor: H_2O_2-oxidoreductase 1.2.1.7), PO by the method of Sato (1928) in the modification of Quaglino [21], as well as phospholipids (PL) in circulating neutrophils by the method of Sheehan and Storey [22].

An analysis of clinical data revealed (Table 3) that at admission patients with inflammatory suppurative pulmonary diseases showed considerable changes in the enzymatic status of leukocytes as compared to healthy individuals. There was a considerable depression of the dehydrogenase activity in lymphocytes, PO in neutrophils (the cytochemical coefficient was as low as 40%), activation of hydrolases (activity of acid phosphatase and that of alkaline phosphatase increased 2 and 4 times, respectively), and a drop in the PL content down to 32% in neutrophils was noted. Coefficients of variation, excess, and asymmetry characterizing heterogeneity of lymphocytes with regard to dehydrogenase activity, exceeded normal values almost twice. This indicates a high cell heterogeneity and a change in lymphocyte distribution in patients with inflammatory pulmonary diseases.

The findings obtained in these studies indicate a definite relationship between the activity of intracellular enzymes of leukocytes and the clinical course of the pathological process in the lungs. The more severe the patient's condition, the lower the activity of SDH and α-GPD(M) in lymphocytes, the activity of PO and the PL content in neutrophils, and the higher the activity of acid phosphatase and alkaline phosphatase in neutrophils. During therapy, normaliza-

Table 3

Influence of pyrimidine derivatives on dynamics of the enzyme activities in circulating leukocytes in patients with inflammatory suppurative diseases of the lungs and pleura

Groups and dates of analysis	SDH	α-GPD(M)	Acid phosphatase
	$\overline{X} \pm m$	$\overline{X} \pm m$	$K \pm m$
Healthy men	$14.12 + 0.12$	$9.39 + 0.11$	0.82 ± 0.12
Control			
1–2 days	8.99 ± 0.25^x	6.81 ± 0.20^x	1.60 ± 0.12^x
9	8.83 ± 0.31^x	7.15 ± 0.25^x	1.39 ± 0.13^x
21	10.93 ± 0.47^x	7.99 ± 0.36^x	1.21 ± 0.09^x
33	11.21 ± 0.27^x	8.16 ± 0.31^x	1.26 ± 0.11^x
Principal			
1–2 days	8.49 ± 0.23^x	6.98 ± 0.19^x	1.56 ± 0.11^x
9	11.68 ± 0.23^x	8.92 ± 0.19	1.17 ± 0.12^x
21	13.23 ± 0.25^x	9.97 ± 0.21	0.95 ± 0.15
33	13.42 ± 0.30	10.66 ± 0.34^x	0.89 ± 0.16
45	16.66 ± 0.29^x	13.00 ± 0.58^x	0.81 ± 0.16

Groups and dates of analysis	Alkaline phosphatase	Myeloperoxidase	Phospholipids
	$K \pm m$	$K \pm m$	$K \pm m$
Healthy men	0.38 ± 0.16	2.16 ± 0.13	2.31 ± 0.11
Control			
1–2 days	1.57 ± 0.08^x	1.30 ± 0.07^x	1.61 ± 0.13^x
9	1.37 ± 0.12^x	1.42 ± 0.12^x	1.72 ± 0.06^x
21	1.16 ± 0.16^x	1.58 ± 0.14^x	1.80 ± 0.13^x
33	1.02 ± 0.21^x	1.69 ± 0.21^x	1.87 ± 0.21^x
Principal			
1–2 days	1.68 ± 0.10^x	1.28 ± 0.12^x	1.58 ± 0.08^x
9	1.16 ± 0.11^x	1.70 ± 0.06^x	1.89 ± 0.12^x
21	0.90 ± 0.12^x	2.02 ± 0.06	2.10 ± 0.14
33	0.74 ± 0.16^x	1.90 ± 0.14	2.29 ± 0.18
45	0.63 ± 0.18^x	2.15 ± 0.06	2.41 ± 0.18

Note: K — average cytochemical coefficient [23].

tion of leukocyte enzyme activities proceeded slowly, and one month treatment failed to normalize the values. Addition of pyrimidine derivatives to the drugs considerably accelerated normalization of the leukocyte enzymes; an improvement could be observed by the 9th day and within a month all values except that for alkaline phosphatase were normal.

Dynamics of the activity of SDH, α-GPD(M), PO, and PL contents in the process of treatment was described by the logarithmic function, $Q = Q +$

$+ K \cdot \lg t$; acid phosphatase and alkaline phosphatase by the exponential, $Q = Q_0 \cdot e^{-kt}$, where Q is the index of activity, *t* the time in days, Q_0 and K are constants calculated on the basis of experimental data, e, base of natural logarithm. Having calculated the constants of the normalization rate of enzyme activity ("sanation" constants) in the two groups of patients, we have obtained an objective cytochemical criterion of the efficacy of the preparations. In patients treated with pyrimidine derivatives the process of normalization of the SDH and PO activities and of the PL content was 2.8 times, the rate of activity of α-GPD(M) two times and that of acid phosphatase and alkaline phosphatase 1.5 times more rapid than in the patients on conventional complex therapy.

The results suggest that the leukocyte enzyme activities allowed to estimate the severity of the patient's condition and to prognosticate the further course of the disease. Thus, the lowest dehydrogenase activity was observed in patients with a protracted sluggish pulmonary process. Particularly informative from the point of view of prognosis was the hydrogenase activity. An analysis of transgression of cytochemical activity distributions showed that a lymphocyte dehydrogenase activity below 6.5 was prognostically unfavourable and called for immediate urgent measures. A persistent decrease in the SDH and α-GPD(M) activities below the critical level of 3.5 suggested a fatal outcome.

Our findings allowed to conclude that a surgical intervention on the lung and pleura may be performed only when SDH activity is not lower than 9, and that of α-GPD(M) is not lower than 7.5. Naturally, the interpretation of cytochemical data should be correlated with analyses of the clinical condition and of laboratory tests.

In patients with inflammatory processes in the lungs and pleura prognostically the most informative are the SDH and α-GPD(M) activities in lymphocytes, and those of acid phosphatase and alkaline phosphatase and PO, and the PL content in neutrophils. The higher the hydrolase activity, the more extended and protracted the pathological process in the lungs. A considerable reduction in the activity of acid phosphatase and alkaline phosphatase of neutrophils associated with a marked depression of lymphocyte dehydrogenase should be considered an unfavourable prognostic sign. A drastic drop in the activity of PO and a low level of PL in neutrophils point to a severe and protracted course of the disease.

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Operation of the Ca-Dependent K(Rb)-Transport in Human Lymphocytes

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The operation of a Ca-dependent, quinine-, chlorobutanol-, and chlorpromazine-sensitive K-transport (the so-called Gárdos-effect) is demonstrated in human lymphocytes. During stimulation this process presumably contributes to the activation of the Na, K-pump and to the acceleration of Ca-uptake.

Introduction

Kaplan et al. [1, 2] and Averdunk [3] reported the activation of the Na, K-pump of lymphocytes on concanavalin A and phytohaemagglutinin stimulation. Negendank and Shaller [4] demonstrated that the K-level of concanavalin A stimulated lymphocytes sank to 83–85% of the controls, whereas the Na-level remained practically unchanged. The K-loss is not due to cell agglutination and does not need complement. The authors suggest that K-loss elevates K-concentration in the surrounding of the cell and this may contribute to the activation of the Na, K-pump.

In 1979 Freedman [5] demonstrated in spleen lymphocytes of the mouse that on concanavalin A impact a rapid Ca-influx develops which is increased by preincubation with dbcGMP and is reduced by preincubation with dbcAMP.

We intended to study the eventual causal relationship between Ca-uptake and selective K-loss, in other words, whether or not lymphocytes possess the K-transport mechanism activated by the elevation of intracellular Ca, the so-called Gárdos-effect.

Abbreviations:

cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
CPZ	chlorpromazine
dbcAMP	dibutyrylcAMP
dbcGMP	dibutyrylcGMP
EGTA	ethyleneglycol-bis(2-aminoethylether)-N,N'-tetraacetate
MEM	minimum essential medium

Materials and Methods

Lymphocytes were isolated from 100–400 ml of fresh human blood. 10 g/dl dextran T 250 was added to the blood at a 1 : 20 ratio, erythrocytes were sedimented and the cells of the supernatant centrifuged at 1000 rpm for 15 min. The pellet was suspended in Hanks solution containing 0.5 g/dl albumin and incubated with ~ 0.1 g iron powder at 37 °C for 30 min. Then suspensions were centrifuged at 2500 rpm for 15 min through a Ficoll-Uromiro gradient (sp.g. 1.075). Erythrocytes and phagocytes pelleted, and lymphocytes with platelets were in the layer between the two phases. This layer was collected and lymphocytes were separated from platelets by 3 washings in Hanks solution at low speed centrifugation (1000 rpm for 15 min). More than 96% of the lymphocytes proved to be viable by the trypan blue extrusion test. A modification of the "minimum essential medium" (5 mM glucose, 4 mM 1-glutamine, 0.2–1.8 mM $CaCl_2$, Tris-HCl pH 7.4 in 0.155 M NaCl) was used as suspension medium, supplemented, during ^{86}Rb loading experiments, with 100 U/ml penicillin G and 100 μg/ml streptomycin.

In ^{86}Rb and ^{45}Ca-uptake experiments lymphocytes were separated from the medium by (*a*) centrifuging through an 0.7 M sucrose cushion dissolved in 0.16 M KCl; the sedimented cells were homogenized in concentrated formic acid before radioactivity measurements; (*b*) filtration through Sartorius cellulose-acetate filter membrane (pore diameter: 0.6 μm), and three washings with cold isotonic washing solutions. The radioactivity of the cells on the filter was measured in a scintillation liquid. The filtration technique proved to be superior. In ^{86}Rb-efflux experiments both the radioactivity of the supernatant and the cell sediment separated by centrifugation was measured in a well-type scintillation crystal (Biogamma, Beckman).

Results and Discussion

In previous work with erythrocytes [6, 7] we have shown that the Ca-dependent K-transport is bidirectional and in the early phase of the incubation, when adding ^{86}Rb to the medium, a rapid isotope uptake can be measured. In lymphocytes we inhibited the activity and activation of the Na, K-pump by ouabain, in order to abolish K-accumulation by the K-pump. Figure 1 shows that in the first 6 (–10) minutes the Ca-ionophore A23187, as well as 1 μM acetylcholine in the presence of the cholinesterase inhibitor neostigmine, significantly increased Rb-uptake. This phenomenon can be abolished by chelating Ca in the medium. A concomitant increase in ^{22}Na-uptake (in media where 0.155 M NaCl was substituted by 0.155 M choline chloride) was not observed. In such cases electroneutrality usually is maintained by chloride (+ water) loss with volume reduction of the cells as a consequence [6, 7]. Figure 2 shows the volume reduction of the lymphocytes as measured in a Coulter-type counter (Medicor, Hungary)

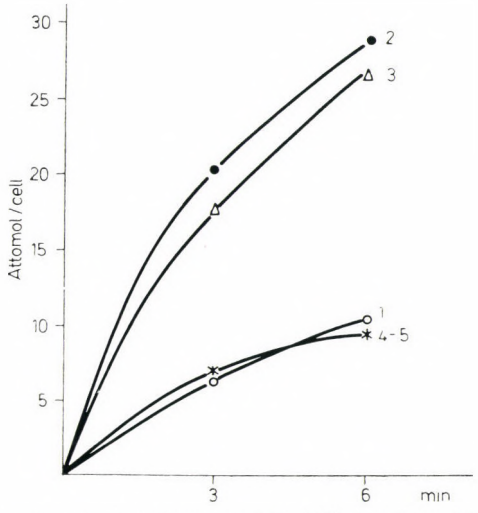

Fig. 1. Rb-uptake into human lymphocytes. $15 \cdot 10^6$/ml cell in MEM. $[Ca]_0 = 0.2$ mM, $[Rb]_0 = 1.5$ mM, ouabain = 0.05 mM, t = 37 °C. 1: Control, 2: 5 μM A23187, 3: 1 μM acetylcholine + 75 μM neostigmine, 4: *2* + 1 mM EGTA, 5: *3* + 1 mM EGTA

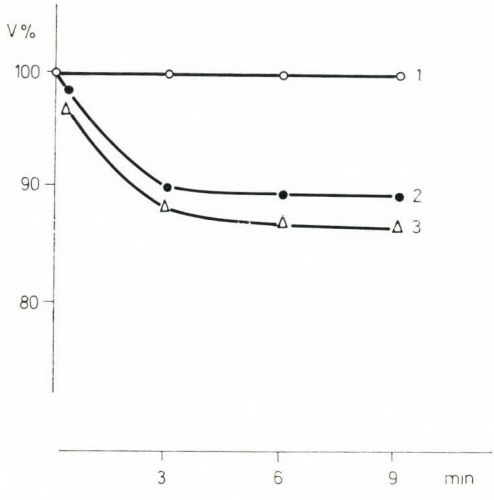

Fig. 2. Volume changes of lymphocytes stimulated by A23187 or acetylcholine. $4 \cdot 10^6$/ml cell in MEM. $[Ca]_0 = 0.2$ mM, $[K]_0 = 0$, t = 37 °C. Initial lymphocyte volume: 138 fl. 1: Control, 2: 5 μM A23187, 3: 1 μM acetylcholine + 75 μM neostigmine

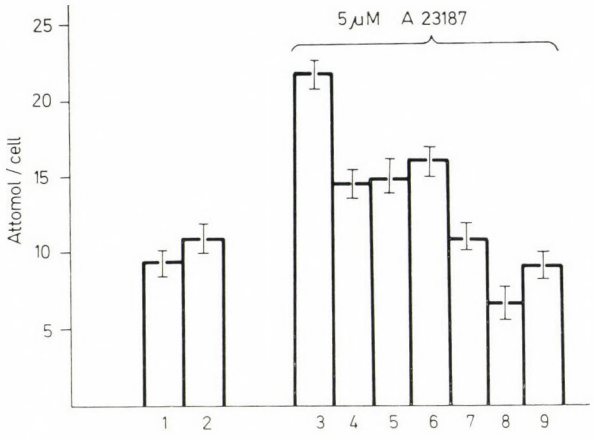

Fig. 3. Effect of A23187 + Ca on Rb-uptake in lymphocytes. $15 \cdot 10^6$/ml cell in MEM. $[Ca]_0 = 0.5$ mM, $[Rb]_0 = 1.5$ mM, ouabain = 0.1 mM. Incubation time = 5 min, t = 37 °C. 1: Control, 2: 250 μM CPZ, 3–9: 5 μM A23187 + in tube 4: 0.5 mM quinine, 5: 3 mM chlorobutanol, 6: 50 μM CPZ, 7: 150 μM CPZ, 8: 250 μM CPZ, 9: 1 mM EGTA

using a cetrimide haemolyzing solution to eliminate red cell contamination. The time scale of volume reduction correlated with the transport phenomena. Cells reacted in an inhomogeneous way, there was a shift in the distribution of cells among the various size compartments of the instrument.

Figure 3 demonstrates that the drug sensitivity of the A23187-induced, ouabain insensitive Rb-influx is very similar to that of the Gárdos-effect in the red cells [7], but cell damage occurs at lower CPZ concentrations (> 50 μM). In short term experiments external Ca is indispensable, the Ca-ionophore does not reach the intracellular Ca-stores and does not mobilize Ca from them, not even at a relatively high, 5 μM ionophore concentration.

In ^{86}Rb-loaded lymphocytes the effect of A23187 on K/Rb permeability could be demonstrated in the increased Rb-efflux, especially when the reaccumulation was inhibited by ouabain (Fig. 4). 0.5 mM quinine abolished this Rb-efflux. Hence it is conceivable that the increased pericellular K-concentration caused by the Ca-dependent K-efflux contribute to the activation of the Na, K-pump.

Hadden et al. [8] demonstrated the stimulation of murine lymphocytes and spleen cells by low concentrations of acetylcholine. Figure 5 shows that in human lymphocytes 1–100 μM acetylcholine increased Rb-transport in a Ca-dependent manner. The effect of acetylcholine was significantly potentiated by the cholinesterase inhibitor neostigmine. 100 μM acetylcholine itself was less effective. 1 μM norepinephrine conspicuously reduced Rb-transport.

Since the increased K-efflux makes the membrane potential more negative due to the slower efflux of the accompanying anions, it is to be expected that this

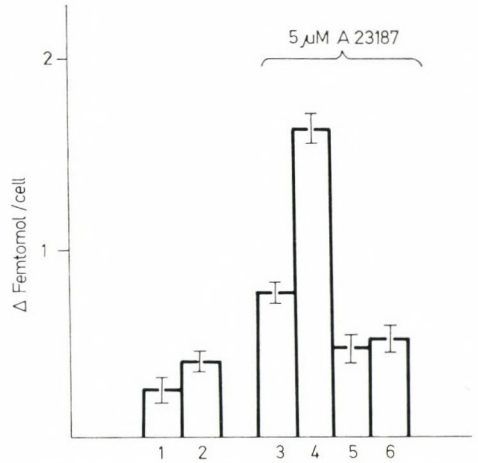

Fig. 4. Effect of A23187 + Ca on ^{86}Rb-efflux in lymphocytes. 5 · 10^6/ml cell in MEM. Initial $[Rb]_i$ = 9.5 femtomole/cell, $[Ca]_0$ = 0.2 mM. Incubation time = 15 min, t = 37 °C. 1: Control, 2: 0.1 mM ouabain, 3–6: 5 μM A23187 + in tube 4: 0.1 mM ouabain, 5: 0.5 mM quinine, 6: 0.1 mM ouabain + 0.5 mM quinine

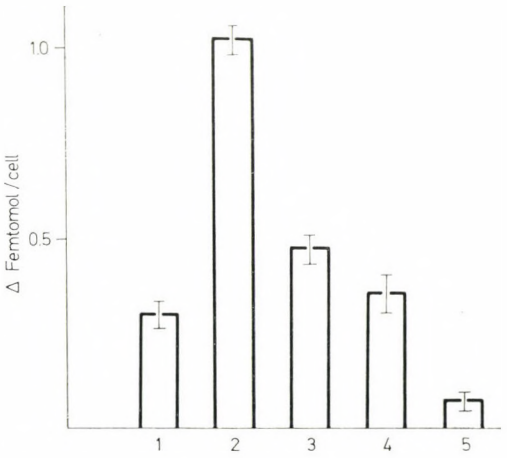

Fig. 5. Effect of various stimuli on the ^{86}Rb-efflux in lymphocytes in the presence of 0.1 mM ouabain. 5 · 10^6/ml cell in MEM. Initial $[Rb]_i$ = 8.6 femtomole/cell $[Ca]_0$ = 0.2 mM. Incubation time = 15 min, t = 37 °C. 1: Control, 2: 1 μM acetylcholine + 75 μM neostigmine, 3: 1 μM acetylcholine + 75 μM neostigmine + 0.5 mM quinine, 4: 0.1 mM acetylcholine, 5: 1 μM norepinephrine

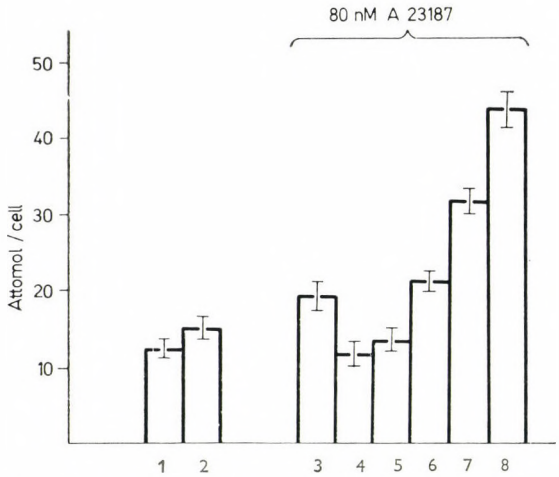

Fig. 6. Effect of drugs inhibiting the Gárdos-effect on the A23187 induced Ca-uptake of lymphocytes. $5 \cdot 10^6$/ml cell, $[Ca]_0 = 0.5$ mM. Incubation time = 30 min, t = 37 °C. 1: Control, 2: 250 μM CPZ, 3-8: 80 nM A23187 + in tube 4: 0.5 mM quinine, 5: 3 mM chlorobutanol, 6: 50 μM CPZ, 7: 150 μM CPZ, 8: 250 μM CPZ

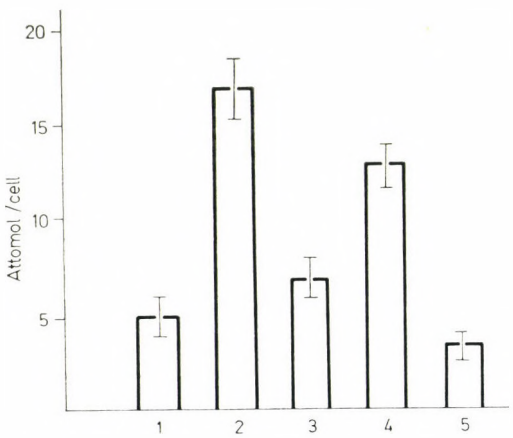

Fig. 7. Effect of various stimuli on the Ca-uptake of lymphocytes. $15 \cdot 10^6$/ml cell in MEM. $[Ca]_0 = 0.5$ mM, Incubation time = 5 min, t = 37 °C. 1: Control, 2: 5 μM A23187, 3: 1 μM acetylcholine, 4: 1 μM acetylcholine + 75 μM neostigmine, 5: 1 μM norepinephrine

effect promotes the uptake of cations including calcium. This assumption was verified by the results presented in Fig. 6. The inhibition of K/Rb efflux by quinine and chlorobutanol prevents hyperpolarization and accordingly Ca-uptake is reduced. From among the inhibitors of the Gárdos-effect, CPZ does not exert this effect. This compound is a well-known calmodulin antagonist. Thereby it

probably inhibits the activation of the Ca-pump, and increases the cellular Ca-level. (Higher CPZ concentrations damage the cells.) The fact that the rate of Ca-uptake is modulated by the Ca-dependent K-transport suggests that one effect of the latter phenomenon may be in fact the amplification of the stimulus-induced "early Ca-influx".

In systems containing acetylcholine or norepinephrine, Ca-uptake correlated with the K/Rb permeability changes demonstrated above (Fig. 7.) Acetylcholine did not stimulate Ca-uptake more than did the inhibition of the Ca-pump by 0.2 mM $LaCl_3$. Hence the question arises, whether the cholinergic-β-adrenergic agents alter K/Rb permeability *via* the cAMP-cGMP dependent Ca-pump regulation.

It is to be mentioned that if lymphocytes are prepared from "buffy coats" (side-products of some blood preparations) stored for 24 h under sterile conditions, the quinine and chlorobutanol sensitive K-transport could be demonstrated without the addition of A23187 or acetylcholine. In these cells the Ca liberated from intracellular stores probably increased the calcium concentration of the cell plasma and thereby activated the specific K-transport pathways of the plasma membrane.

*

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Freeze-fracture Study of the Red Cells and Red Cell Precursors of a Patient with Congenital Inclusion Body Anaemia

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Comparison of thin sections and freeze fracture replicas of red cells and red cell precursors from the peripheral blood of a patient suffering from congenital inclusion body anaemia provided new morphological information on Heinz body substructure, Heinz body-membrane attachment, intracellular membrane formation, as well as on membrane fusion and orientation of intracellular membranes. It is suggested that the fusion of autophagic vacuoles with the plasma membrane may contribute to the increased haemolysis of red cells in inclusion body anaemia.

Introduction

In the past years many papers have been published concerning the ultrastructure of erythrocytes and their precursors in inclusion body anaemia of different origin [1, 2]. In addition to conventional electron microscopic studies, freeze-fracture investigations have been carried out to study the membrane changes in haemoglobinopathies by *in vitro* manipulations on the red cells [3, 4]. No freeze-fracture studies have been published so far which would have given an insight into the *in vivo* formation of inclusion bodies in these diseases. This paper describes the results of freeze-fracture investigations on the erythroid cells of a patient with congenital inclusion body anaemia.

Case report

The patient's data have been summarized in detail elsewhere [5]. Since his first admission in 1968, he has regularly been treated with blood transfusions in our Institute.

Material and Methods

Peripheral blood was taken by venipuncture into isotonic sodium chloride containing 1‰ heparin in a period when the patient received no transfusion. Red cells were washed with isotonic phosphate buffered saline pH 7.4 and processed for thin section and freeze-fracture electron microscopy. Thin section electron microscopy was carried out according to standard procedures [6]. For

freeze-fracture electron microscopy, washed, unfixed red cells were incubated in isotonic phosphate buffered saline containing 10% glycerol at 37 °C for 30 minutes. After incubation the cells were quenched in Freon 12 cooled by liquid nitrogen. Freeze-fracture was carried out in a Balzers 510 type freeze-etch apparatus. Replicas were cleaned with household bleach, washed with distilled water, mounted on uncoated 300 mesh grids and investigated in a Philips EM 300 electron microscope.

Results

A significant number of nucleated red cell precursors and reticulocytes were seen in the patient's peripheral blood with the same ultrastructural alterations as in the bone marrow [5]. Both the erythroblasts and the reticulocytes contained inclusion bodies. While these inclusions showed an electron dense, homogeneous structure in thin sections (Fig. 1a), freeze-fracture revealed a granular structure and this was significantly more coarse than that of the surrounding cytoplasm (Fig. 1b). In many cases mitochondria, tubular structures and vesicles were seen around the inclusions (Figs 1 and 2). The vesicles contained only a few intramembrane particles and sometimes were closely attached to the inclusion bodies (Fig. 2). Inclusion bodies associated with the plasma membrane were rarely observed. Autophagic vacuoles containing inclusions, iron-laden mitochondria, myelin figures and demarcated cytoplasmic areas were frequently seen in both erythroblasts and reticulocytes (Fig. 3a). By freeze-fracture many intramembrane particles were seen on the vacuolar membrane (Fig. 3b). The myelin figures revealed by thin sectioning showed no intramembrane particles in the freeze-fracture preparations (Fig. 3b). The vacuolar membrane was often attached to the plasma membrane (Fig. 3b), sometimes apparent fusion could be seen (Fig. 4). In some cases the membrane of the autophagic vacuoles contained no intramembrane particles (Fig. 4). Demarcation of cytoplasmic areas containing neither inclusion bodies nor cytoplasmic organelles were occasionally seen in the erythroid cells (Fig. 5a). The freeze-fracture image of the membrane of these structures suggested an inverted orientation relative to the plasma membrane (Fig. 5b). The plasma membrane of the pathological erythroid cells showed no intramembrane particle aggregation (Fig. 5b). Concentric lamellar structures were seen in several erythroid cells (Fig. 6a), the membranes of these structures showed few intramembrane particles (Fig. 6b). In some of the circulating erythroblasts, nuclear abnormalities, penetration of inclusion bodies into the karyoplasm were seen (Fig. 7a). Freeze-fracture images of these nuclei showed strong intramembrane particle aggregation on both the inner (Fig. 7b) and the outer (Fig. 8) nuclear membrane.

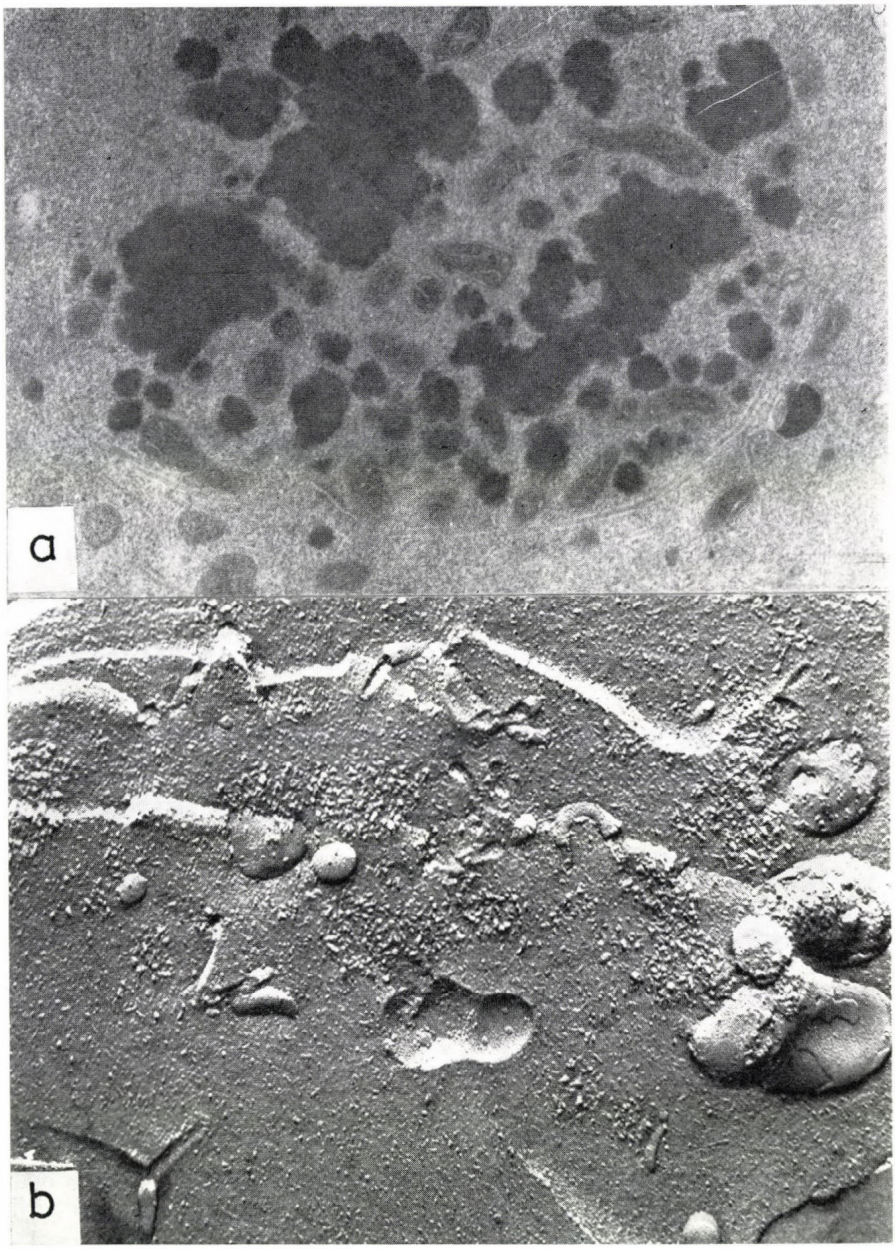

Fig. 1. (a) Electron micrograph of a thin section of an erythroid cell containing inclusion bodies. Note the precipitation of haemoglobin surrounded by mitochondria and tubular structure. × 32 000

(b) Freeze-fracturing reveals a coarse granular substructure of the inclusion bodies. Around the inclusions mitochondria, vesicles and tubular structures are seen. × 54 000

Fig. 2. Freeze-fracture image of an inclusion body surrounded by small vesicles containing a few intramembrane particles. Note the close relationship between the precipitated haemoglobin molecules and the vesicles. × 80 000

Discussion

Since no freeze-fracture studies have been carried out on the red cells of patients with *in vivo* occurring inclusion bodies, our aim was to make such investigations and compare the results to those obtained by thin section electron microscopy.

While in thin sections the inclusions had a homogeneous appearance, the freeze-fracture replicas showed clearly that the Heinz bodies have a granular structure (Fig. 1b). A similar structure has already been described by Lessin et

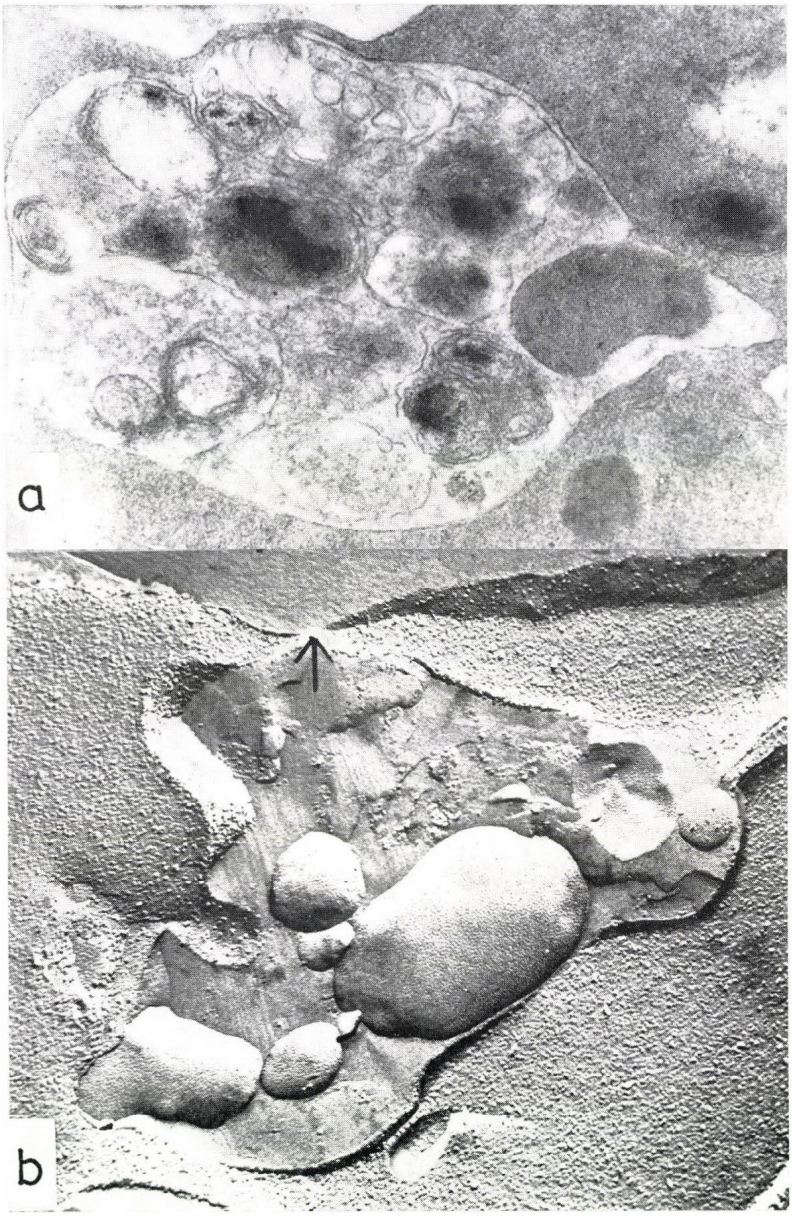

Fig. 3. (a) Large autophagic vacuole in a reticulocyte containing iron-laden mitochondria, demarcated parts of the cytoplasm and myelin figures. × 50 000
(b) Freeze-fracture image shows mainly particle free vesicles and granulated cytoplasmic remnants inside the vacuole. Note the attachment to the plasma membrane (arrow) and the membrane of the vacuole containing many intramembrane particles. × 54 000

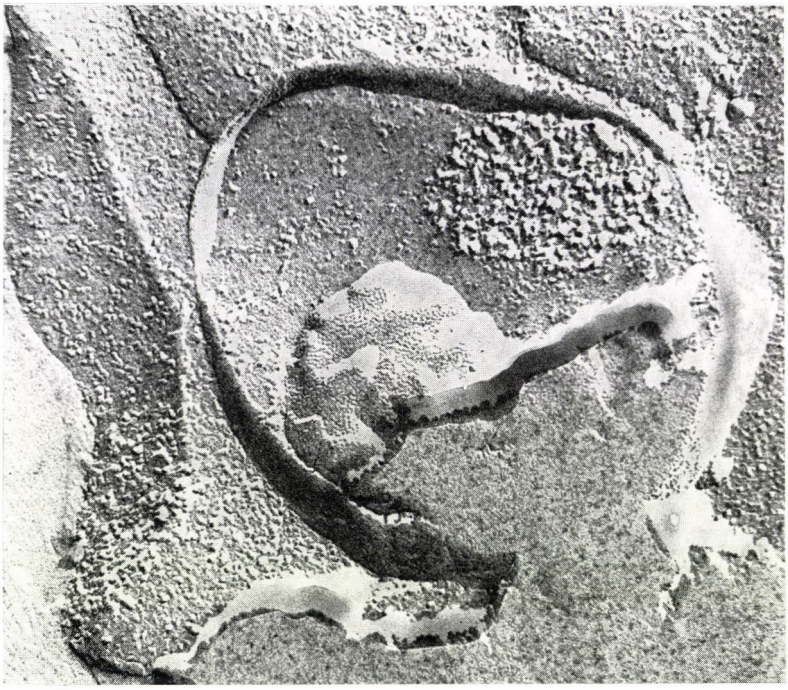

Fig. 4. Freeze-fracture image of a Heinz body containing autophagic vacuole. The content of the vacuole is expelled out of the cell. In contrast with the plasma membrane, the membrane of the vacuole does not seem to contain intramembrane particles. × 82 500

al. [3, 4] in α-thalassaemic red cells exposed to redox dyes, which caused *in vitro* Heinz body formation in these cells. The coarse granular structure of the inclusion bodies in the red cells of our patient represents denatured and precipitated haemoglobin molecules forming Heinz bodies different in size.

In contrast with the Heinz bodies seen in α-thalassaemia [3, 4] and after phenylhydrazine treatment of normal red cells [7], the inclusions in our case showed no attachment to the plasma membrane. Instead, they tended to attach to intracellular membranes of vesicular structures. In most cases these vesicles contained few intramembrane particles (Fig. 2). According to Jacob [8], phenylhydrazine-induced Heinz bodies are attached to the sulfhydryl groups of plasma membrane proteins by disulfide linkage. Since in our case the intracellular vesicles contained few intramembrane particles which probably represent membrane proteins, some haemoglobin–lipid interaction may also be involved in the Heinz body–vesicular membrane bonding, but this remains to be verified biochemically.

Large autophagic vacuoles containing inclusions, cytoplasmic debris and iron laden mitochondria were frequently seen in thin sections (Fig. 3a). While

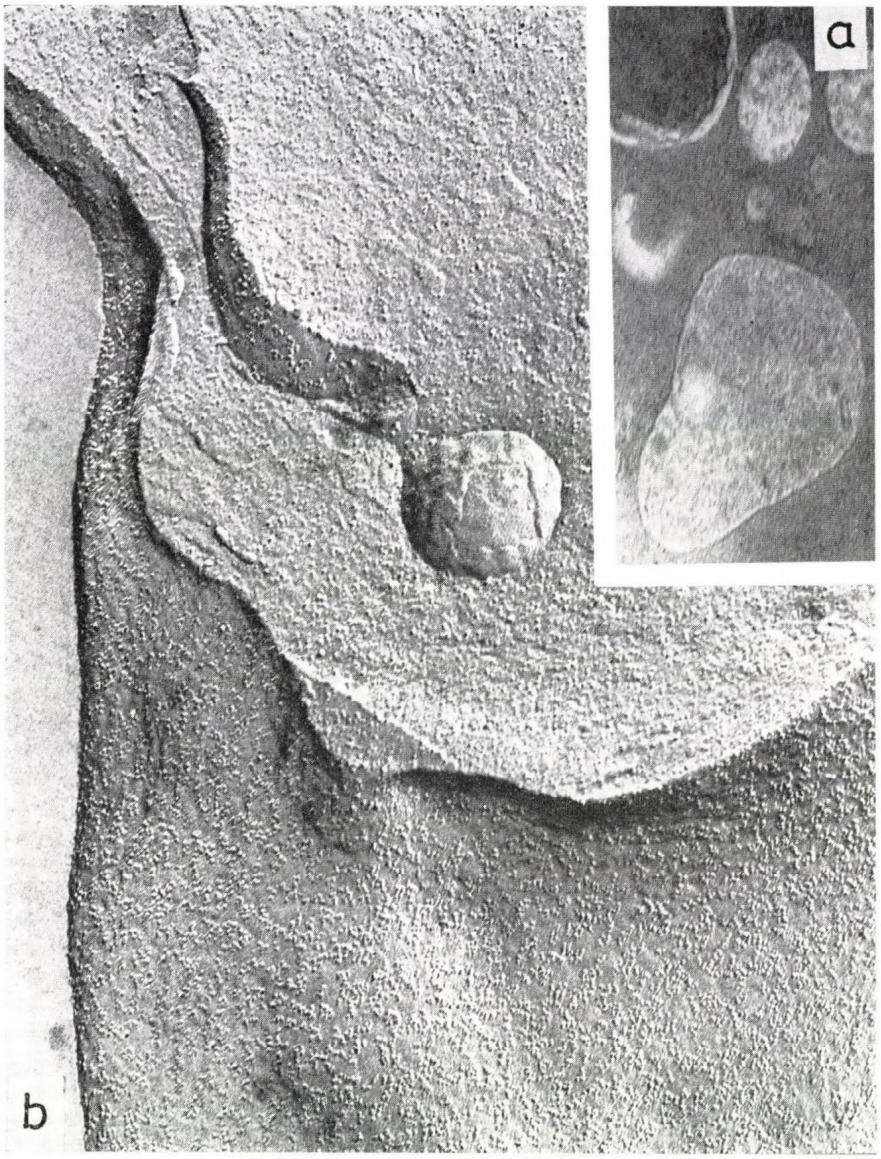

Fig. 5. (a) Demarcation of cytoplasmic areas containing no inclusions and cell organelles. × 60 000
(b) Freeze-fracture image suggests inverted orientation of the vacuolar membrane. No particle aggregation can be seen in the P face of the plasma membrane. × 60 000

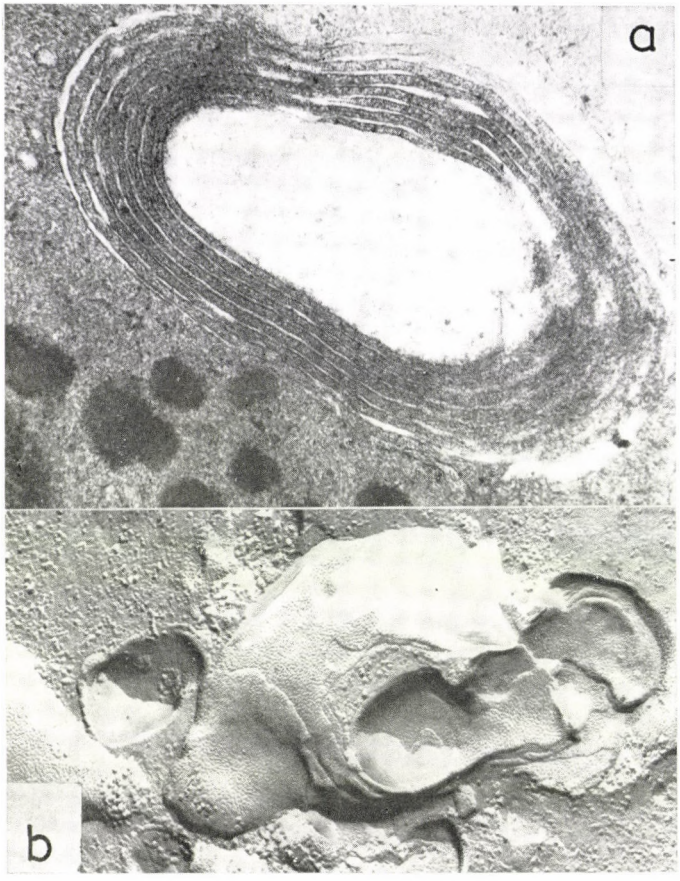

Fig. 6. (a) Electron micrograph of a concentric lamellar structure in a reticulocyte. × 50 000 (b) Freeze-fracture image of a lamellar structure. The membrane system is mainly particle free, only few layers contain intramembrane particles. × 66 000

in freeze-fracture preparations the membrane of most vacuoles showed numerous intramembrane particles (Fig. 3b), in some cases the vacuolar membrane seemed to be completely free of particles (Fig. 4). Both these autophagic vacuoles and those having intramembrane particles had fused with the plasma membrane (Fig. 4). The release of proteases and other biologically active substances from the autophagic vacuoles, and the presumably increased susceptibility of these areas to mechanical injury may contribute to the haemolysis and rapid elimination of Heinz body containing cells [5, 9].

The limiting membrane of the demarcated areas containing neither inclusions nor cytoplasmic organelles (Fig. 5a) contained numerous intramembrane

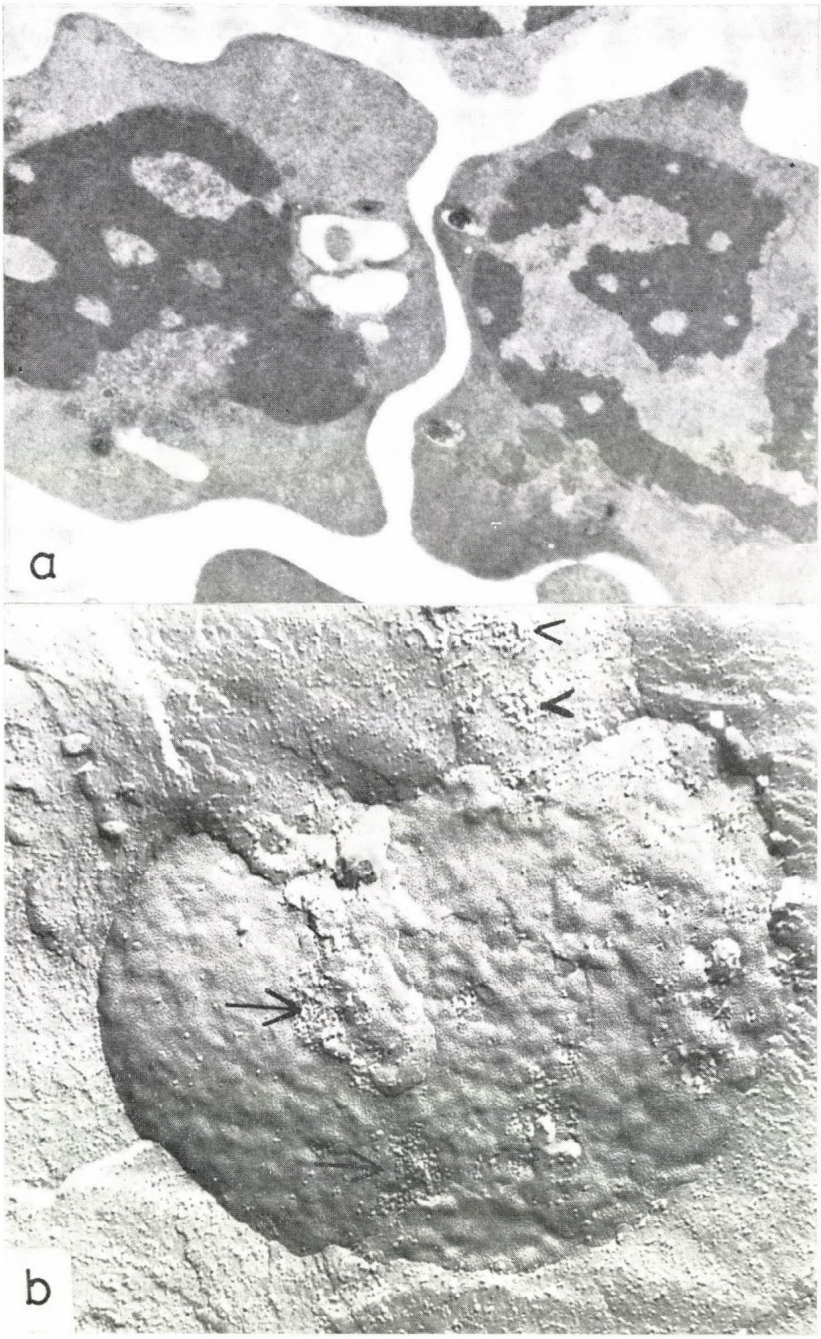

Fig. 7. (a) Electron micrograph of two erythroblasts containing inclusion bodies inside their nuclei. × 30 500
(b) Freeze-fracture image of a damaged nucleus showing strong particle aggregation on the outer face of the inner nuclear membrane (arrows). Note the small inclusions near the nuclear envelope (arrowheads). × 54 000

Fig. 8. Freeze-fracture image of the outer nuclear membrane of an erythroblast. Arrows indicate the site where inclusions penetrate into the karyoplasm through the nuclear envelope. Note aggregation of the intramembrane particles. × 54 000

particles and showed an inverted orientation relative to the plasma membrane (Fig. 5b). The role of these structures is not known, but they may contain abnormal haemoglobin molecules not yet precipitated to form visible Heinz bodies. The inverted orientation of these membranes suggests that they may be capable of fusion with the plasma membrane. The plasma membrane of the abnormal red cells showed no significant intramembrane particle aggregation (Fig. 5b). This makes it unlikely that particle clustering should play a role in the elimination of red cells containing inclusion bodies.

The circular-lamellar structures (Fig. 6a) observed in several red cells were composed of numerous layers containing few intramembrane particles (Fig. 6b). The origin and function of these structures are not known, but we think that they are composed mainly of lipids. In addition to the low number of particles, the coexistence of such structures with a significant elevation of the red cell lipid content [10] supports this hypothesis.

The characteristic nuclear abnormality observed in different haemoglobinopathies [1, 5, 10] was found in the patient's circulating erythroblasts, too, i.e. a penetration of inclusion bodies into the karyoplasm (Fig. 7a). Freeze-fracture images showed very significant particle aggregation on both nuclear membranes (Figs 7b and 8). The cause and significance of the aggregation are not known; it might be connected with the disintegration of the nuclear envelope.

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The Treatment of Childhood Leukaemia in Hungary

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Children suffering from leukaemia in Hungary are treated according to uniform therapeutic protocols in the framework of a national multi-centre study. Their most important clinical data are stored in the central registry and are analyzed by computerized methods. Since January, 1971, 846 new patients were entered in the registry. Initially treatment results were very poor but showed gradual improvement during the past few years, somewhat parallel to more intensive chemotherapy. The latest treatment protocol includes medium-dose MTX and the combination of ARA-C and VM-26. Preliminary data are encouraging.

*

Among the more impressive advances of medicine during the past decade the treatment of childhood leukaemia takes an important place. The introduction of multi-agent chemotherapy led to a vast improvement in the rate of initial remissions and maintenance chemotherapy helped to prolong these remissions. At the beginning the main obstacle to long-term disease-free survival was the frequent occurrence of meningeal relapse. Cranial irradiation as introduced by Pinkel and coworkers [6] at St. Jude Children's Research Hospital resulted in a dramatic improvement and the incidence of meningeal relapse dropped to less than 10%. With this approach some 40–50% of the ALL patients in the best paediatric-oncological centres had a chance to recover. With improved prognosis a late complication was brought to the lime-light: testicular relapse occurred in 5–15% of the boys with ALL [2, 3]. This often happened during the year following cessation of therapy.

Moe and coworkers [4] prompted by preceding ALGB studies have introduced MDMtx (500 mg/m^2) in the treatment of ALL. In their series the incidence of testicular relapse was nil and meningeal leukaemia seemed less frequent.

* *Principal investigators for the Working Party:* P. Cholnoky, S. Eckhardt, S. R. Hollán, L. István, P. Kajtár, P. Kassai, P. Kemény, R. Koós, I. Kovács, G. Nyerges, Á. Petróczky, I. Rényi, L. Velkey, I. Virág, I. Zimonyi.

Abbreviations: VCR: vincristine, DR: daunorubicine, Pred: prednisolone, Asp: asparaginase, CP: cyclophosphamide, 6-MP: 6-mercaptopurine, MTX: methotrexate, MDMtx: medium-dose methotrexate, ARA-C: cytosine arabinoside, VM-26: podophylotoxin, NHL: non-Hodgkin lymphoma, ALL: acute lymphoblastic leukaemia, CNS: central nervous system, CCR: cumulative complete remission.

A dogma fairly widely held in paediatric oncology was that 'more treatment does not necessarily mean more cures'. Riehm et al. [8] in the BFM Group have demonstrated in the past few years that this dogma too has to be dropped. With their highly intensive combination of cytostatic agents they achieved very encouraging results in ALL.

The poor treatment results in Hungary in the early 70-s prompted paediatricians to form a Working Party and start a national program for the treatment of childhood leukaemia. In this paper we report some of the problems and therapeutic results and present preliminary data obtained with the use of new protocols.

Patients and Methods

Patients

All children diagnosed to have leukaemia in Hungary are transferred to one of the ten centres actively participating in the national Working Party on Childhood Leukaemia. Bone marrow smears are then sent to the National Institute of Paediatrics where two haematologists review the slides and evaluate the cytochemical reactions. Children up to the age of 16 years are treated in the above centres, older patients are referred to internal medicine departments.

Treatment protocols

At the time of its establishment in 1971 the Leukaemia Working Party introduced a very simple induction scheme, followed by maintenance therapy. As from 1973, cranial irradiation and i.thec.MTX was introduced for the prevention of meningeal relapse. During 1975–76 we investigated the role of cyclic treatment pulses in the eventual outcome of ALL. This approach was based on the positive results obtained with the LSA_2L_2 protocol in NHL [13]. The preliminary results failed, however, to show any improvement and a new protocol was started in 1978 and run for 2 years (Fig. 1). Induction was achieved with 'standard' VCR-Pred-DR therapy, followed by cranial irradiation plus i.thec.MTX. Patients were allocated to 2 prognostic groups on the basis of the criteria given in Table 1. High-risk patients were given intensification using

Table 1

Criteria for 'high-risk' prognosis in ALL (1975–80)

1. Initial WBC count over 50 G/l
2. Presence of mediastinal mass
3. Initial meningeal involvement
4. Age: under 1 year
   over 12 years
5. T-cell character of blast cells
6. Failure to remit after 4 weeks

Patients were considered 'high-risk' if any one of the above criteria was found.

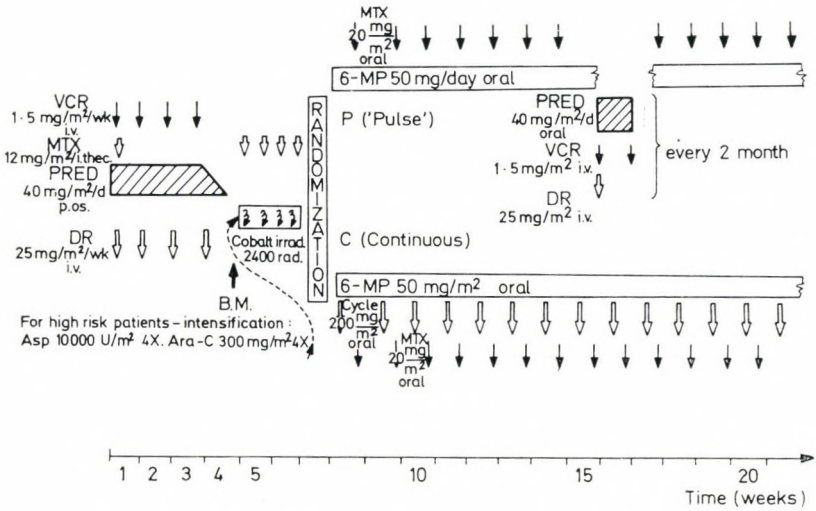

Fig. 1. ALL treatment protocol 1978

Ara-C and Asp. for 2 weeks. In order to investigate the role of regular re-induction pulses, patients entering remission were randomized by a card-envelope system to 2 forms of maintenance therapy: one with pulses, the other with a 3-drug (6-MP, MTX and CP) continuous maintenance therapy.

Since the results were still unsatisfactory, the Working Party decided to intensify therapy for ALL patients in 1980. The new protocol (Fig. 2) included some of the elements introduced by the BFM group in Germany, including a longer course of Asp injections daily. Because relevant literary data indicated an equal effectiveness, the cranial irradiation dose was lowered to 18 Gy (1800 rads). High-risk patients received a 4-week intensification with Ara-C and 6-MP.

In a pilot study half of the boys were given medium-dose MTX 3 times at 2 weekly intervals with leukovorin rescue, in order to study if extra-medullary relapses can be prevented through this approach at a permissible degree of toxicity.

The latest, 1981 protocol (Fig. 3) is based on our previous treatment schedules but incorporates the use of MDMtx and cranial irradiation. On the basis of favourable observations in relapsed patients, the combination of Ara-C and VM-26 as suggested by Rivera et al. [10] was introduced for high-risk patients as an intensification phase of therapy. Maintenance remained the 'classical' 6-MP and MTX combination with bi-monthly VCR-Pred pulses.

Collection and analysis of data

Important clinical data at diagnosis are collected from all the patients and stored on magnetic discs. At the end of each year these data are up-dated, the

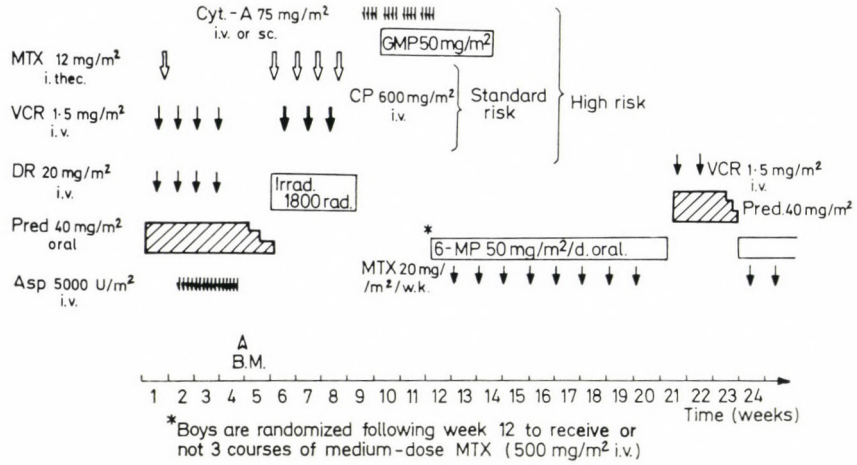

Fig. 2. ALL treatment protocol 1980

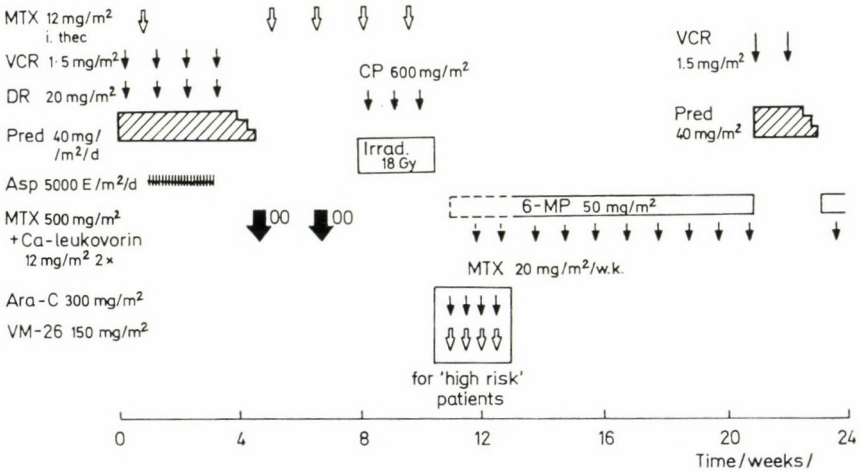

Fig. 3. ALL treatment protocol 1981

length of remissions, relapses and complications etc., are registered. Since 1971, 846 new patients were registered in the country and their data were recorded in a computer.

Analyses were carried out using the Biomedical Data Programs of the UCLA and the logrank test [1, 5]. Through the former group of programs the chosen variables can be computed in the form of histograms and their distribution

investigated in various sub-groups of patients. The logrank survival program, kindly made available by Dr. P. G. Smith of the Oxford Cancer Unit, is used for survival analysis in the chosen group of patients.

Results

During the first two years of its existence the Working Party on Childhood Leukaemia established uniform diagnostic criteria and treatment models for all the participating centres. At that time not more than 70% of the children with ALL achieved remission and only 18% of the patients remained well and disease-free 8–10 years after diagnosis.

Later protocols led to higher remission rates and longer survival, resulting in approximately 35% of the patients achieving 5 years survival. Right from the beginning one of the most important variables affecting survival was the place of treatment: some small centres with few patients, a lesser degree of expertise and sometimes poorer facilities showed significantly inferior results than others.

The 1978 ALL protocol introduced a phase of intensification for the high-risk patients. Despite this fact, however, only 75% of these patients entered remission as compared to 91.5% of the standard-risk patients. Of the 55 leukaemic children diagnosed in 1978, 2 were lost during induction due to therapeutic side

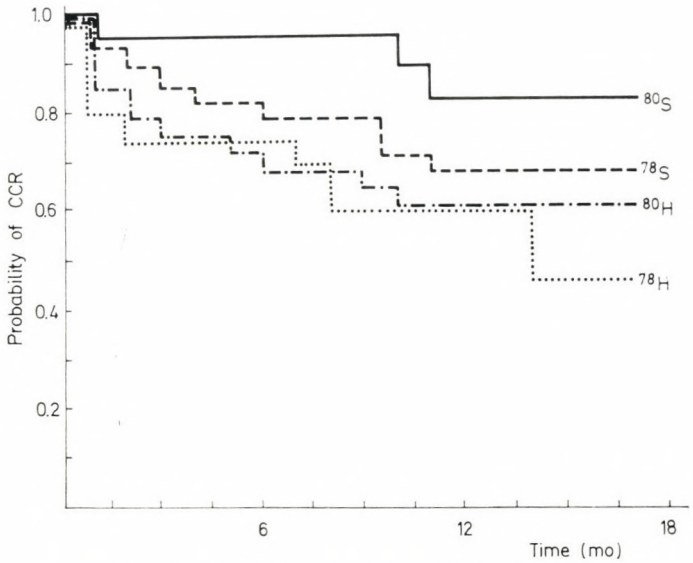

Fig. 4. Cumulative complete remission in ALL

78 S: Standard risk patients (n = 35)
78 H: High risk patients (n = 20)
} diagnosed in 1978.

80 S: Standard risk patients (n = 26)
80 H: High risk patients (n = 29)
} diagnosed in 1980.

effects. The life table estimate of the probability of CCR in the first 18 months following diagnosis is shown in Fig. 4, as compared to the values of patients diagnosed and treated in 1980. So far, no difference exists between the 'pulse' and 'continuous' maintenance group of the 1978 patients (not shown in Fig. 4).

Fifty-five patients were entered on the 1980 protocol. Of these, 29 were considered high-risk and 26 standard-risk patients. Remission rates were 79 and 95%, respectively. The life table estimate of CCR in the past 18 months shows improvement as compared to the values of patients diagnosed in 1978, but at present the difference is not significant statistically. There were 2 patients in this group too, who died during induction therapy due to therapeutic complications (acute pancreatitis, renal failure).

Discussion

The introduction of multi-agent chemotherapy and prophylactic CNS irradiation led to a considerable improvement in the disease-free survival of leukaemic children. More and more patients achieve remission and remain well over 5 years thus aspiring for a complete cure of the disease [11].

The recognition of risk factors led to the stratification of patients into prognostic groups all over the world. Up to the present time, however, no generally acceptable prognostic classification exists. Perhaps the most important initial predictive factors are initial blast cell count and hepato-splenomegaly [9]. Stratification and differential treatment of our patients failed to abolish the difference between the results of high-risk and standard-risk patients and remains an unsolved problem [7].

Despite uniform diagnostic criteria and treatment protocols some centres in a national study yield superior results to others. This fact should also be kept in mind when comparing therapeutic results from a large oncological centre to those of a multi-centre trial. Eventually these differences should disappear as the level of expertise improves in all the centres involved in a national program.

The duration of remission should also increase with the use of more intensive chemotherapy. This is partly the message from the results obtained by Riehm and his coworkers in the BFM group. In our last two protocols induction therapy is supplemented by a 3-week continuous Asp course and CP is given in a medium dose during CNS irradiation. Toxicity seems less of a problem than anticipated; 2 patients were lost during induction due to toxic complications.

Preliminary analysis of the life table estimates for the 1978 and 1980 ALL patients shows an improvement in the ratio of disease-free survival in the patients of the latter group (Fig. 4). Although the difference at present is not significant statistically, clearly both the standard- and high-risk patients fare better with the more intensive treatment protocol.

In a pilot study accompanying the 1980 protocol, 11 boys were given MD-Mtx following remission induction. No clinical or laboratory evidence of neuro-

toxicity was recorded, despite the fact that the drug was given after the course of cranial irradiation. Still, partly because of warning observations made at St. Jude Children's Hospital [12], partly because of the low number of patients eligible for the study, this project was stopped and a new approach to MDMtx therapy was sought.

The 1981 ALL protocol includes 2 doses of MDMtx *prior* to cranial irradiation and with 2 leukovorin rescues. This approach seems quite novel and it is hoped that this form of consolidation will both help to lengthen haematological remissions and lower the incidence of extra-medullary relapses. Data will be analyzed sequentially in comparison to those of the 1980 patients. A close survey of neurological signs is being conducted along with chemotherapy and irradiation in order to monitor early signs of CNS toxicity.

Since the report of Rivera et al. [10] on the combination of Ara-C and VM-26, these drugs have successfully been used in relapse patients. The impressive effect has prompted us to incorporate this drug combination in the consolidation phase of the *initial* therapy for high-risk patients. Since the beginning of this year about 20 children were given this mode of treatment and it is well tolerated even after a full induction and prophylactic CNS therapy. Whether the addition of MDMtx and VM-26–Ara-C combination will basically alter the life expectancy of high-risk patients, remains to be established. The tolerable rate of initial complications and the previously inferior results seem to justify this approach. We are aware that more intensive chemotherapy can only be accomplished with more intensive substitution haemotherapy and closer observation of patients. In a national, multi-centre study this needs the closest collaboration of all participating centres and professions, and constant discussion of possible complications.

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Immunological Monitoring in Lung Cancer: Use of the Leukocyte Migration Inhibition Assay

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The agarose microdroplet leukocyte migration inhibition assay has been applied to the management of patients with lung cancer.

Sera of patients with various stages of the disease were assayed in the indirect LMI system for their influence on the LIF production of normal leukocytes. A probably cell-mediated immunity blocking activity could be detected both in the serum of patients with inoperable tumours and in that of operable patients before and two weeks after surgery. The activity, however, disappeared in two thirds of the cases in about 6 months after surgery.

The immune competence of patients with inoperable tumours was investigated before and during combined chemotherapy. A positive correlation was established between the tuberculin skin reactions and the leukocyte migration inhibition obtained in the direct LMI assay against PPD, but only in the presence of autologous serum. Sera of patients were also tested for putative *in vivo* LIF activity by their effect on the migration of autologous leukocytes. A significant correlation between anergy to PHA, as evaluated by skin testing, and the migration inhibition caused by sera was found. When, however, sera from healthy persons and from patients with chronic bronchitis or lung cancer were compared for their effect on the migration of normal leukocytes, no difference could be detected among the three groups. Single doses of the drugs temporarily weakened the skin reactions to PHA and the migration inhibition to PPD in autologous serum, but activity was restored when the drug influence had ceased.

Introduction

The central question for a tumour immunologist is, whether, from an immunological standpoint, a cancer patient is detectably different from a normal individual. One field where differences may exist is the general immune status of the patient, as tumour growth can produce a secondary immunosuppression. Anomalies of immune competence and reactivity can have diagnostic implications and can serve as a prognostic guide [1–3]. In a given patient the spectrum of immunologic abnormalities varies greatly. As a consequence, by employing a variety of laboratory techniques, the immunological profile of the patient may be established. Immunological procedures are thus potentially important tools in the diagnosis and the management of cancer.

Any test which is thought to be applicable to human cancer has to be evaluated in a carefully designed study on a number of patients with known disease.

Leukocyte migration inhibition (LMI) by antigens is considered to be an *in vitro* correlate of cell-mediated immunity (CMI) [4–6]. The LMI assay is also widely used to measure specific anti-tumour immune reactions to tumour antigens [7–10]. We utilized the technique, however, as a tool for investigating general immune competence.

The aim of this study was to evaluate the usefulness of the highly sensitive, simple and very economical agarose microdroplet LMI assay of McCoy et al. [11] in the *in vitro* monitoring of our patients with lung cancer before, during and after therapy.

Patients and Methods

Serum testing for CMI blocking activity

Leukocytes. The donors were healthy O Rh_+ individuals with positive skin tests to tuberculin. Blood was taken by venipuncture in the presence of 3.3% sodium citrate. Mononuclear cells for LIF production and polymorphonuclear leukocytes (PMN) as indicator cells in the indirect LMI test were prepared according to Böyum [12].

Medium. In all the experiments *in vitro*, cells were washed and cultured in Tissue Culture Minimal Medium Eagle (DIFCO, USA) completed with glutamine, sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin and occasionally with serum.

Serum samples. The sera of 21 blood bank donors or laboratory personnel, as normal controls, 4 patients with chronic bronchitis, 12 patients with inoperable and 25 with operable lung cancer were compared. The patients had not been given steroids or cytostatics for at least blood sampling. Sera from 20 patients subjected to surgery were obtained on the day of the operation and at two weeks and about six months later. Serum samples were stored in 500 μl aliquots at −20 °C. They were used without heat-inactivation.

Lymphocyte culturing for LIF production. 1×10^7 mononuclear cells per serum sample were incubated in 1.5 ml of medium, containing 500 μl of the serum tested, at 37 °C for one hour, washed once, resuspended in 1.5 ml of serumfree medium and divided again into two portions. Half of the samples were completed with 15 μg/ml of PPD (freeze dried, neutralized, purified protein derivate of human tuberculin, Ministry of Agriculture, Fisheries and Food, England), the others served as controls. After 24 hours incubation at 37 °C, the controls were reconstituted with the same amount of the antigen and the cell-free supernatants tested for LIF activity on PMN cells in the indirect agarose microdroplet LMI assay.

Direct investigations of the patients' immune competence

Patients. 20 patients with primary or secondary lung cancer (Stage III) were tested for skin reactions, *in vivo* circulating LIF activity in their sera, and in leukocyte migration inhibition to PPD *in vitro*. Twelve of the patients were retested once, seven twice, five three times and one patient 10 times, both at 72 hours and 14–20 days following sequential combined chemotherapy (cyclophosphamide, adriamycin, vinblastine, prednisolone).

Serum samples. Altogether 31 healthy individuals (laboratory personnel and blood bank donors), 22 patients with chronic bronchitis and 27 patients with disseminated lung cancer served as donors. Human sera as well as fetal bovine serum (FBS) (SERVA, FRG) were used after heat-inactivation.

Direct and indirect agarose microdroplet LMI assay. In the direct investigation of LIF production *in vitro*, the patients' buffy coat leukocytes, sedimented at 1 *g* in the presence of 3.3% sodium citrate and 1% dextran, were divided into four parts, resuspended in medium containing either 5% autologous serum, 5% autologous serum plus 20 μg/ml PPD, 5% FBS, or 5% FBS plus 20 μg/ml PPD, respectively. Following incubation at 37 °C for one hour, the cells were centrifuged and adjusted with 0.2% agarose solution in medium to give suspensions of about $2–4 \times 10^7$ cells/100 μl. 1 μl droplets of these were placed into wells of migrations plates (Sterilin, England), three or four into each. The wells were filled with medium containing the respective serum and hermetically sealed with coverslips. At least three replicate wells, i.e. nine droplets, were prepared. After incubation at 37 °C for 20 hours the migration areas were projected, drawn on paper, cut out and weighed. A migration index (MI) was calculated from the mean weight of the test patterns divided by the mean weight of the control patterns.

For the indirect detection of serum LIF *in vitro* or *in vivo*, normal PMN cells as indicators were handled as above and the wells were filled with either the supernatants or the medium containing the sera tested. In one experiment the leukocytes were preincubated with the serum samples at 37 °C for one hour, washed once and cultured in medium only.

Skin testing. Patients were tested for delayed type hypersensitivity to PPD with intracutaneous injection of 5 U of tuberculin (Humán, Hungary) before and 14–20 days after treatment. Two perpendicular diameters of indurations were measured at 72 hours and the area was calculated. The cutaneous reactivity to PHA was studied after an intradermal injection of 2 μg of Leucoagglutinin (Pharmacia, Sweden) before as well as 48 hours and 14–20 days after chemotherapy. The 24 hour reaction was estimated as above.

Results

Serum testing for CMI blocking activity

The migration indices obtained in the indirect LMI assay with the supernatants from lymphocytes preincubated with different sera and cultured with PPD are shown in Figure 1. A decreased migration inhibition (increased migra-

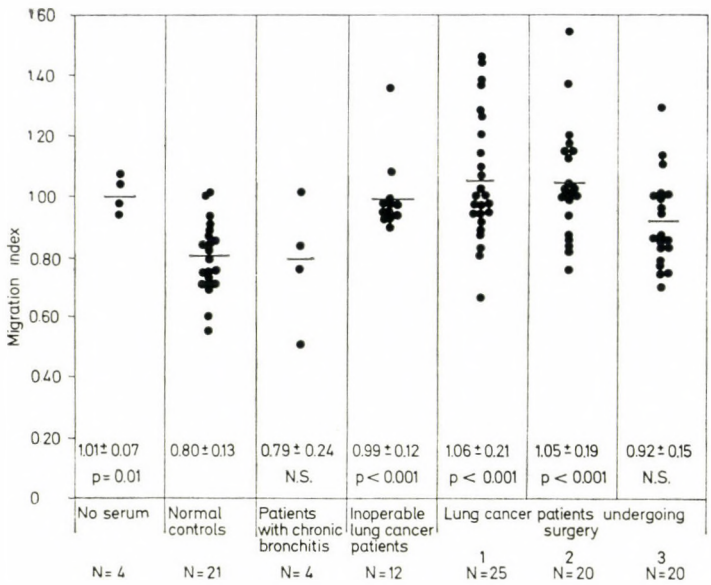

Fig. 1. Effect of preincubation with different sera on the LIF production of normal lymphocytes to PPD. Serum samples of surgically treated patients were collected before (1), two weeks after (2) and six months after (3) the operation. Each point represents an individual MI, while mean values with standard deviation for the groups are indicated. Student's *t* test was used to evaluate the differences

tion indices) characteristic of cancer patients was observed, while the chronic bronchitis group did not differ from the normal controls. Patients treated by surgery could further be divided into two subgroups (Fig. 1a). In 13 cases migration inhibition approached the normal level at six months after the operation while in the 7 other cases it remained depressed.

Direct studies of immune competence

Twenty patients with inoperable tumour were examined for their immune status by estimating five parameters using two tests (Fig. 2) and comparing the results in five various combinations (Figs 2a–e). Significant correlations were

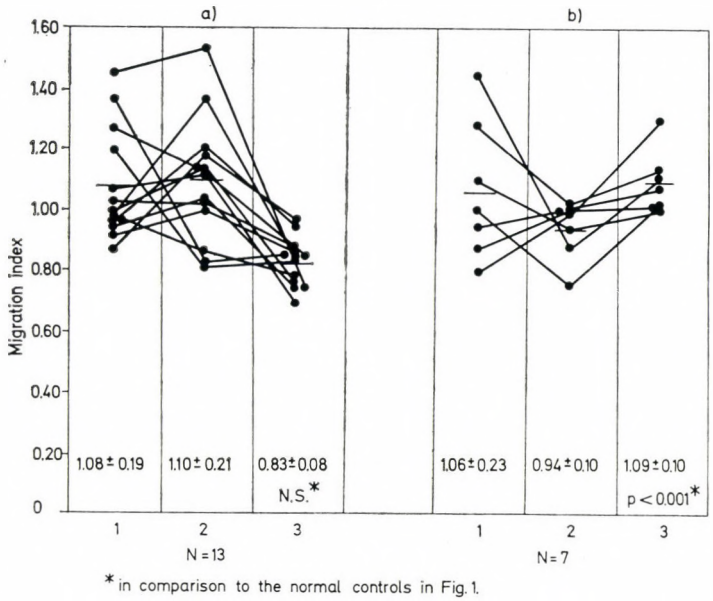

Fig. 1a. LIF production of normal lymphocytes to PPD following preincubation with sera of surgically treated lung cancer patients. Patients are divided into two subgroups: in 13 cases a) migration inhibition approached the normal level by the time when the third serum samples were collected, while in the other 7 cases b) it remained depressed

found between the migration inhibition results obtained against PPD in the presence of autologous serum and the intensity of the Mantoux reaction, as well as between the migration indices expressing the relative effect of human sera on migration to FBS and the skin reactions to PHA.

As an additional study to the putative *in vivo* serum LIF investigations, a comparison among normal controls, cancer patients and patients with chronic bronchitis was made using normal allogeneic PMN leukocytes as indicators. No significant difference among the three groups could be detected at two of the serum concentrations tested (Fig. 3, B and C), while in the other two cases (A and D) pathological sera enhanced cell migration.

Twelve of the above patients, given chemotherapy, were monitored for changes in their immunoreactivity during the treatment. Figures 4 and 5 show the sequential results obtained with skin testing and LMI assay. The reactions measured under drug effect were significantly weaker, except for the migration inhibition in the presence of FBS where the primary results had been negative but reactivity practically returned to the initial level within two to three weeks.

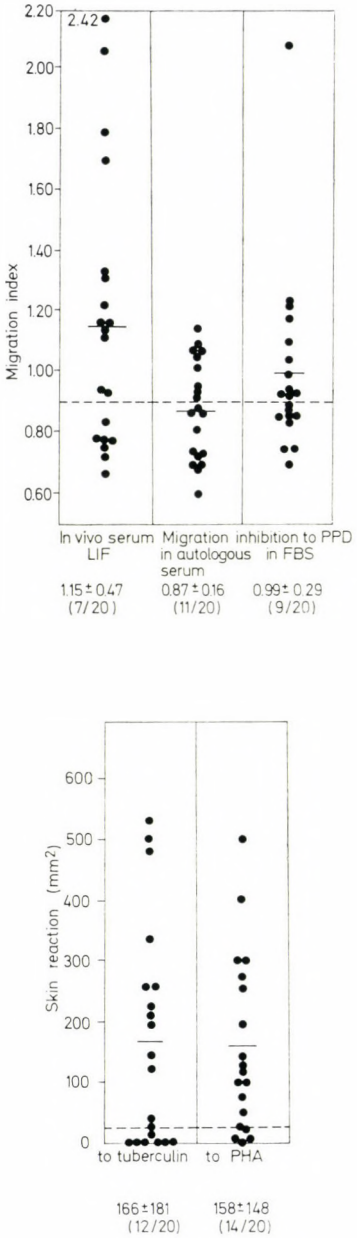

Fig. 2. Migration inhibition and skin reactions of 20 lung cancer patients with inoperable tumour. The points represent the individual results. Mean values with standard deviation are shown. An MI of less than 0.90 was considered to reflect positive LIF activity. Skin reactions were considered positive when the induration exceeded 25 mm²

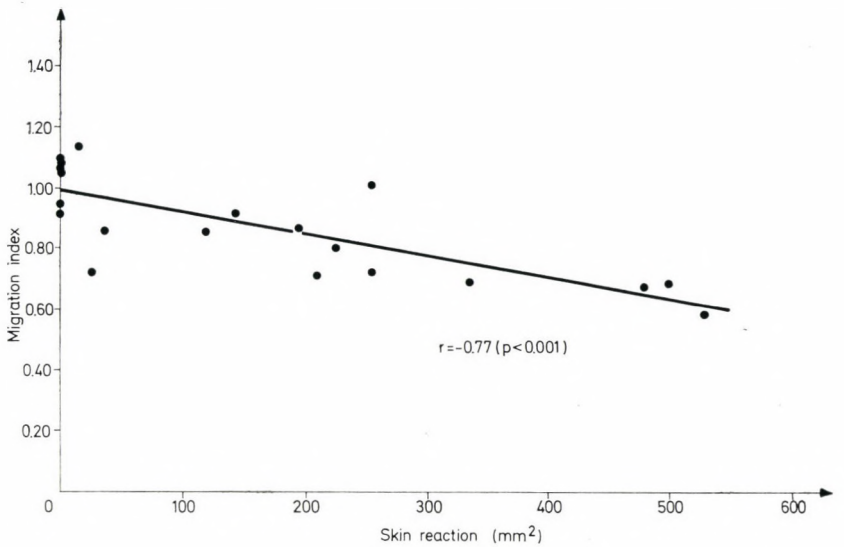

Fig. 2a. Migration inhibition to PPD in the presence of autologous serum, and tuberculin skin reactivity. Linear regression analysis yielded a correlation coefficient: $r = -0.77$ ($p < 0.001$) and a regression line: $y = 0.99 - 0.00074x$

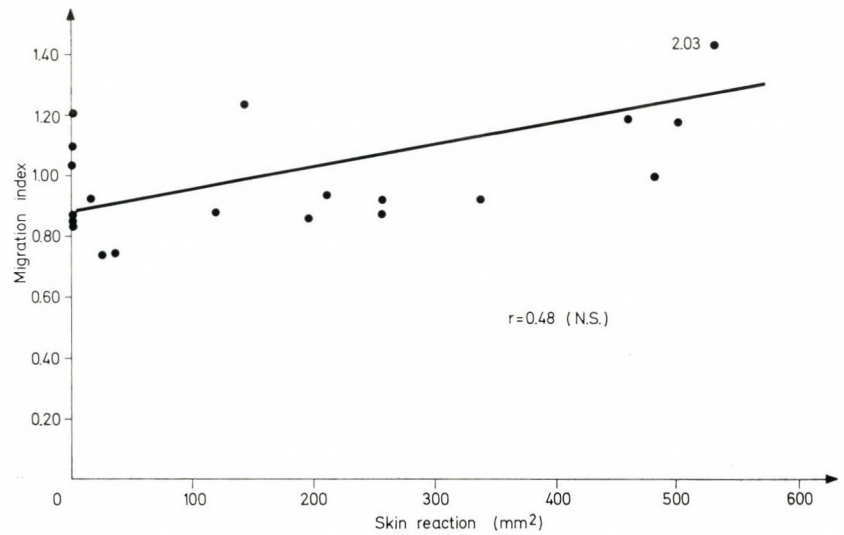

Fig. 2b. Migration inhibition to PPD in the presence of FBS, and tuberculin skin reactivity. No significant correlation was found between the two parameters

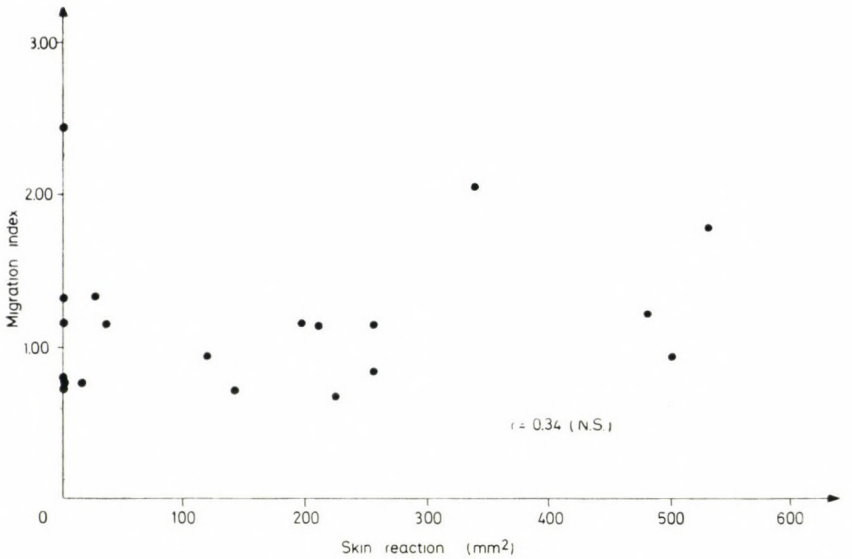

Fig. 2c. Serum migration inhibition activity *in vivo* and tuberculin skin reactivity. No correlation was found between the two parameters

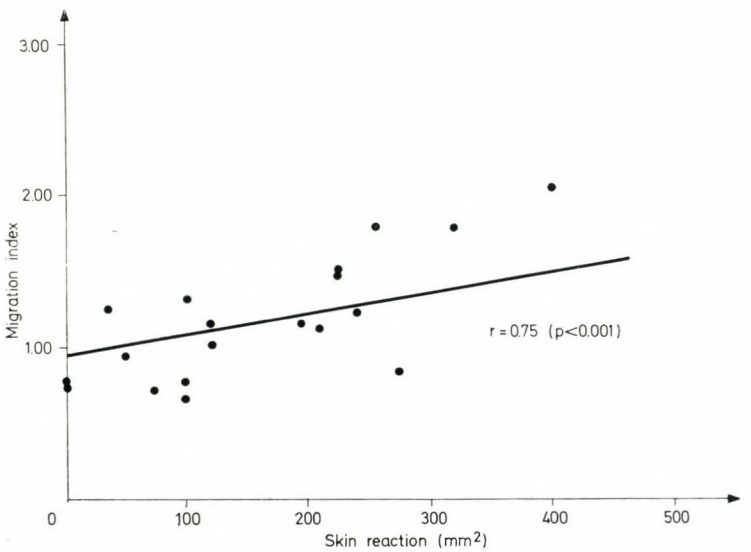

Fig. 2d. Serum migration inhibition activity *in vivo* and skin reactivity to PHA. Linear regression analysis yielded a correlation coefficient: $r = 0.75$ ($p < 0.001$) and a regression line: $y = 0.95 + 0.0014x$

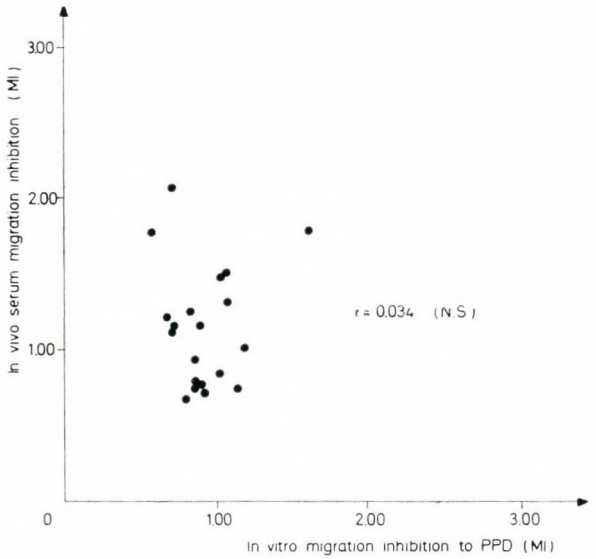

Fig. 2e. Serum migration inhibition activity *in vivo* and migration inhibition to PPD *in vitro* in the presence of autologous serum. No correlation was found between the two parameters

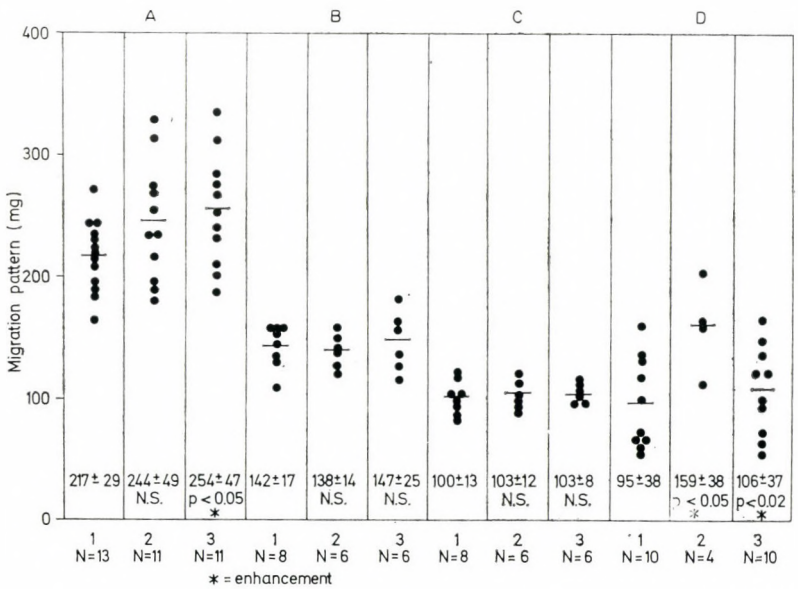

Fig. 3. Effect of different human sera on the migration of normal PMN leukocytes. 1: normal controls; 2: patients with chronic bronchitis; 3: lung cancer patients; A: following preincubation with the sera at a concentration of 50%, B: 10%, C: 20%, D: 50% serum in the migration medium. Each point represents an individual result, while the mean values with standard deviation for the groups are shown. Student's *t* test was used to evaluate the differences

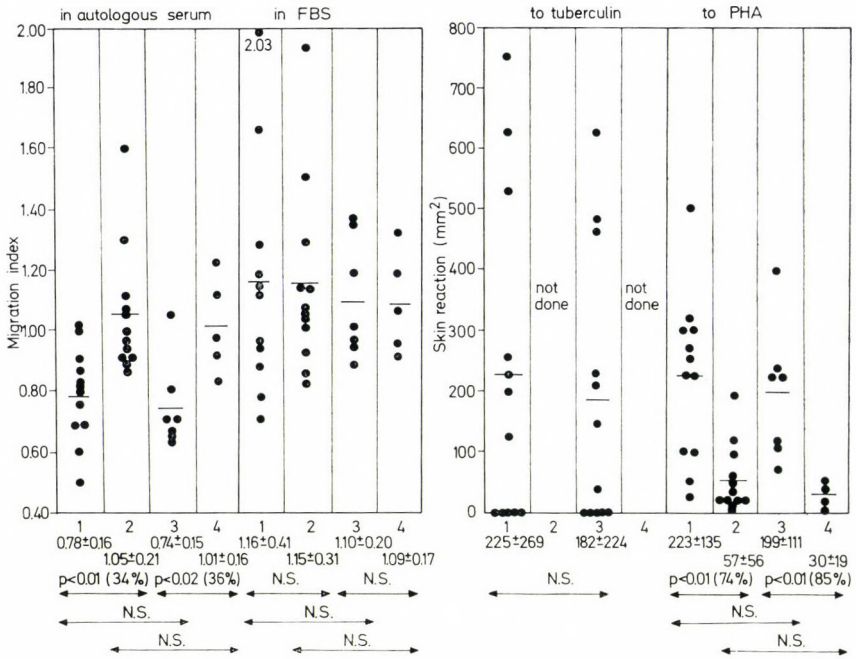

Fig. 4. Migration inhibition to PPD and skin reaction to tuberculin and PHA of patients given sequential combined chemotherapy. 1: before first treatment, 2: 48–72 hours after first treatment, 3: 14–20 days after first treatment, 4: 48–72 hours after second treatment. Each point represents an individual result, while mean values with standard deviation as well as percentile changes are indicated. Student's *t* test was used to evaluate the differences

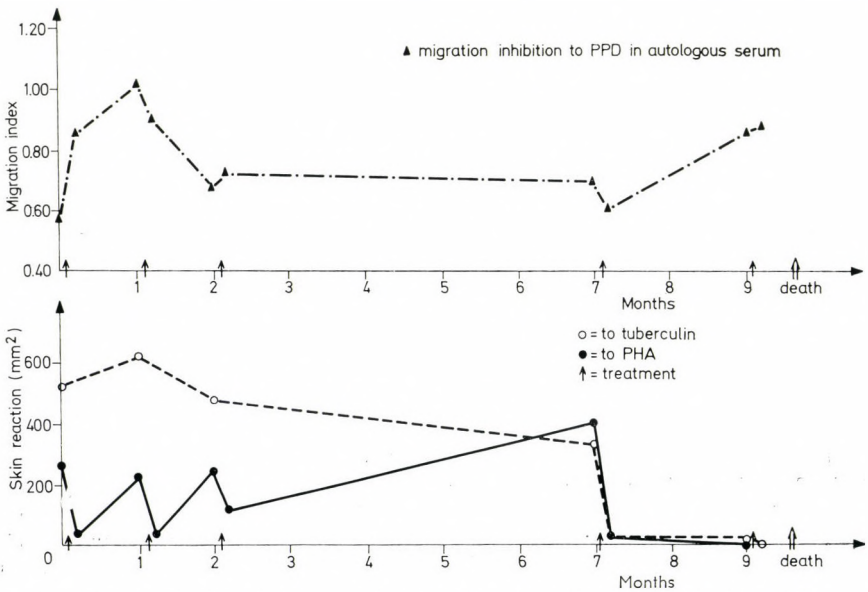

Fig. 5. Immunological follow up of patient N. M., under sequential chemotherapy, until his death

Discussion

A possible explanation of immunodepression often occurring in patients with a wide variety of tumours is the presence of a factor or factors in the plasma that blocks the immune responses. In almost every *in vitro* test measuring specific and non-specific functions of lymphocytes, the serum of cancer patients will often block the effect measured in the presence of control human serum or FBS. The blocking activity has been observed in mixed lymphocyte culture [13], the antibody-dependent and other cell-mediated cytotoxicity reactions [14, 15], the leukocyte adherence inhibition assay [16, 17], in lymphocyte blastogenesis [18-20] and also in the LMI technique [21–24].

Using the LMI assay, we found a CMI blocking activity in the serum of operable and inoperable lung cancer patients, but only in the indirect migration inhibition system. In a pilot experiment, the direct test, where LIF producing lymphocytes and indicator PMN cells are cocultured with the antigen, failed to detect any difference in the effect of the sera tested. The factor does not appear to be specific for CMI against tumour antigens as it inhibited the response of normal lymphocytes to PPD. Moreover, a longitudinal study of surgically treated patients suggests that in the majority of the cases the factor disappears or diminishes by about six months after surgery. For the time being, a prognostic value of monitoring the CMJ blocking activity in serum could not be established, as all the patients tested were in a good condition and none of them showed metastases at the time when the third blood samples were taken.

As already mentioned, assessment of the patients' immune status is a useful and important immunological approach to cancer. *In vivo* testing with recall antigens, dinitrochlorbenzol or PHA is well established and the most frequently used evaluation of immune competence [1–3, 25–28].

We have compared our results obtained with the direct LMI assay with those from the simultaneous skin reaction measurements to evaluate the *in vitro* test. Unfortunately, we could not study the migration inhibition response to PHA as in a pilot experiment the mitogen had been found to inhibit cell migration directly. One interesting observation was that migration inhibition to PPD is better expressed in the presence of autologous serum than in FBS. Moreover, a significant correlation has been found between migration indices to PPD and the skin reaction to tuberculin, but only when autologous serum was present in the migration medium. This finding might serve to explain the lack of a correlation between Mantoux reaction and migration inhibition to PPD, reported in several papers [29–31).

There seems to be a contradiction between the need for autologous serum for an *in vitro* response to PPD and the blocking effect of cancer sera on the LIF production of normal lymphocytes. The migration inhibition reaction to PPD, and perhaps to other antigens seems to be more marked and a better approach when measured in the presence of autologous serum rather than in any other or no serum. This needs to be checked in healthy individuals with several anti-

gens. In our direct LMI investigations, serum and antigen were present together in the system and the blocking factor almost certainly diminished the reactivity, competing probably with PPD for the lymphocytes. So far we have not made a similar study on a normal population for comparison. The reason why the CMI blocking effect seems to be more obvious in the indirect LMI system may be that during preincubation with serum the factor binds strongly to the cells, preventing the PPD to react with them.

It has been reported that MIF, LIF or substances with lymphokine-like activity can be found in the sera of patients with certain diseases associated with anergy [32, 33]. Others have demonstrated *in vivo* migration inhibition activity in patients with various tumours including bronchial carcinoma [24, 34–36], moreover in correlation with delayed type hypersensitivity reactions and a good prognosis [37, 38].

In our lung cancer patients only 35% of the sera were found to inhibit the migration of autologous cells in comparison to FBS. This serum migration inhibitory effect showed a negative correlation with the results of the PHA skin test but not with the Mantoux reaction or the *in vitro* migration inhibition to PPD, indicating that it may be associated more with anergy than with reactivity. A direct comparison of the sera of normal individuals, patients with chronic bronchitis and patients with lung cancer has proved that the agarose microdroplet LMI assay is as sensitive as it can discriminate individual sera, either by their presence or following a preincubation. It has not, however, been confirmed that *in vivo* LIF, characteristic of cancer, existed in the blood circulation.

Some studies of patients receiving cytotoxic drugs indicate that depression of cell mediated immunity occurs during therapy. Reactivity seems to return to or exceed pre-operative levels within a few days after the cessation of therapy [39].

Our results concerning patients on combined chemotherapy indicate that both the direct LMI assay and PHA skin testing are able to detect changes in immune reactivity. Nevertheless, alterations are more marked with the skin test. The importance of autologous serum in the *in vitro* test has to be emphasized. The drugs seem to act mainly on lymphocytes as no decreased migration of the antigen-free control samples was seen. We cannot decide whether or not a combined chemotherapy fundamentally altered the immune status after prolonged application. No significant difference was found between the results obtained before and 14–20 days after the beginning of drug administration, or between those obtained within 72 hours after the first and second dose. Only one patient was monitored for a longer period in which he was given treatment five times until his death. In his case the Mantoux reaction appeared accurately to follow his condition while the temporary improvement of the PHA skin reaction was deceptive. Changes in the migration inhibition results were the least marked.

The indirect agarose microdroplet LMI assay has proved suitable to detect and monitor a probably cell-mediated immunity blocking factor in the serum of lung cancer patients. The direct technique has been found to register altera-

tions in cell-mediated immunity. As a general consequence, we think that any *in vitro* investigation on cell-mediated immunity which does not take account of the role of serum or serum factors, is certainly incomplete and probably inadequate.

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Annotations on the Hyperbilirubinaemia of ABO Incompatible Infants*

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In ABO incompatible infants, a strong association between Coombs test positivity and hyperbilirubinaemia in the first few days of life has been recorded in all samples studied. A remarkable variability between ethnic groups and between different series of infants from the same group has been also observed.

A discriminant analysis carried out on several maternal and neonatal variables in two samples of ABO incompatible infants has shown that the Coombs test is the most important predictor of jaundice. Gestational age and birth weight in white and birth weight in black infants gave also important and independent contributions as discriminating variables.

Among ABO incompatible black infants the incidence of hyperbilirubinaemia was higher than among ABO incompatible white infants. Discriminant analysis suggests that genetic and environmental factors which predispose to jaundice the ABO incompatible black infants may act mainly through immunological and developmental mechanisms accounted for by Coombs test, gestational length and birth weight.

*

ABO feto-maternal incompatibility shows a high prevalence both in Caucasian and Negro populations. Although severe ABO haemolytic disease is rare, milder forms are relatively frequent; in these cases jaundice may not be detected soon after birth and early discharge of the newborn may have serious consequences. All recent contributions have stressed the great practical importance of a possible early identification of newborns at risk of hyperbilirubinaemia [1–3].

In this paper we wish to review these recent studies and our observations in relation to two aspects of the problem: (i) the predictive value of direct Coombs test positivity, and (ii) the racial difference in frequency of manifestations.

Analyses. Three hundred and six white newborn infants of European descent and 76 black infants from the population of New Haven, Connecticut, were considered. All infants were incompatible with their mother in the ABO system and compatible in the Rh (D) system. Previous studies of the relations between haemolytic disease (Coombs test positivity and/or clinical jaundice) and the phenotype of placental alkaline phosphatase and between clinical jaundice and

* This work was supported by the Italian National Research Council.

Coombs test positivity, have already been reported [4, 5]. Sampling and laboratory methods have been described in one of these papers [4]. In the present study the relations between serum bilirubin level and a number of variables (birth weight, gestational age, maternal age, number of previous spontaneous abortions, gestational order, sex, mother and infant ABO blood group) not included in previous studies, were analyzed. Infants were subdivided in two categories according to the maximum serum bilirubin level (< 10 mg/dl or ≥ 10 mg/dl) recorded during the first five days of life. Discriminant analysis was carried out according to Klecka [6].

The predictive value of the direct Coombs test

It has been known for some time that direct Coombs positivity is frequent in ABO incompatible newborns. In 1973, our group reported that the incidence of neonatal jaundice in these infants is positively correlated with direct Coombs test positivity in cord blood [5]. All subsequent studies have confirmed this observation [1–3]. Nevertheless, while Kirkman [1] considers the Coombs test of considerable importance in the routine care of ABO incompatible newborn infants, Risemberg et al. [2] do not regard it reliable for predicting the severity of hyperbilirubinaemia and give preference to the cord blood bilirubin level as a predictor of severe hyperbilirubinaemia in the first days of life. More recently, however, Haque [3] was unable to confirm the association between these two variables and concluded that the cord blood bilirubin level is unreliable for predicting hyperbilirubinaemia due to ABO incompatibility.

Table 1 shows the incidence of Coombs test positivity in ABO incompatible infants in these three studies and in our series. A strong association between Coombs test positivity and hyperbilirubinaemia was present in all samples. A remarkable variability between ethnic groups and among infants from the same group was also evident.

Tables 2 and 3 show the distribution of gestational age and birth weight and a discriminant analysis performed on Coombs test positivity, gestational age and birth weight in newborn infants. In both white and black infants the most

Table 1

Per cent incidence of Coombs test positivity in ABO incompatible infants

	Authors		Kirkman [1]		Risemberg et al. [2] (*)	Haque [3] (*)
	Whites	Blacks	Whites	Blacks		
Serum bilirubin in the first days of life						
<10 mg/dl	23.6	30.4	20.9	35.0	15.9	0.0
≥ 10 mg/dl	56.3	57.9	39.5	79.4	75.0	39.5

* Ethnic group not indicated.

Table 2

Distribution of gestational age (weeks) and birth weight (grams) in relation to serum bilirubin level (Data of Authors)

		Whites			Blacks		
		All ABO incompatible	Serum bilirubin		All ABO incompatible	Serum bilirubin	
			<10 mg/dl	≥10 mg/dl		<10 mg/dl	≥10 mg/dl
Gestational age	mean	39.5	39.7	38.2	38.8	38.9	38.4
	S. D.	1.9	1.5	3.4	2.1	1.9	2.6
Birth weight	mean	3280	3319	2978	3002	3034	2847
	S. D.	543	494	766	490	475	543

Table 3

Stepwise discriminant analysis by Wilks' method on Coombs test, gestational age and birth weight in white and black infants separately. Rao's V is a generalized distance measure. Groups: (A) infants with serum bilirubin <10 mg/dl; (B) infants with serum bilirubin ≥10 mg/dl (Data of Authors)

	Whites				Blacks			
Step no.	Variable entered	Rao's V	Change in Rao'sV	Signif. of change	Step no.	Variable entered	Rao's V	Change in Rao's V	Signif. of change
1	Coombs	24.61	24.61	<0.001	1	Coombs	6.79	6.79	<0.01
2	Gestat. age	40.07	15.46	<0.001	2	Birth weight	12.12	5.33	0.02
3	Birth weight	43.59	3.52	0.060	3	Gestat. age	12.45	0.33	0.57

Standardized discriminant functions coefficients

Coombs test	0.7374	0.8676
Gestational age	−0.4220	−0.1816
Birth weight	−0.3090	−0.5372
Canonical correlation	0.353	0.375
Wilks' lambda	0.8753	0.8593
χ^2	40.555	11.301
D. F.	3	3
Significance	<0.001	0.010

important discriminating variable was the Coombs test. This was followed by gestational age and birth weight in whites and by birth weight in the blacks. All other variables included in the analysis failed to give any significant independent contribution to the separation between infants with a serum bilirubin level higher than 10 mg/dl and those with serum bilirubin levels lower than 10 mg/dl.

Although the discrimination on point of 10 mg/dl may be questionable, we think that as an approach to the problem at the population level, the subdivision is justified on the basis of the general indications for phototherapy.

The importance of other signs such as reticulocytosis, increased number of nucleated red cells and microspherocytosis is well known in the clinical diagnosis of neonatal haemolytic disease due to ABO incompatibility. The main emphasis has, however, been laid on the aim to identify as early as possible ABO incompatible infants carrying a high risk of hyperbilirubinaemia. Such identification should be based on simple methods suitable as a screening procedure.

From this point of view, the analysis of the reported data indicate that the best predictor of hyperbilirubinaemia in ABO feto-maternal incompatibility is the Coombs test. Technical improvement coupled with a better standardization among different laboratories might sensibly improve its predictive value.

It is also well known that low birth weight and decreased gestational age predispose newborn infants to high bilirubin levels. Inclusion of these variables into a discriminant function could increase the predictive value of the Coombs test in order to distinguish at birth the infants which have a low risk of hyperbilirubinaemia and may be discharged early. In fact, as shown by the change in Rao's V (Table 3), in white infants, the independent contribution of gestational age to discrimination is comparable to that of the Coombs test (15.5 vs. 24.6); the contribution of birth weight is comparable to that of the Coombs test in black infants (5.3 vs. 6.8) and appreciable also in whites (3.5 vs. 24.6).

Racial differences in the frequency of hyperbilirubinaemia

We have previously reported that in ABO incompatible black infants some signs of haemolytic disease such as Coombs test positivity and clinical jaundice are more frequent than in Caucasian infants [4, 5]. Subsequent studies have confirmed these observations [1, 7]. An association between these signs and the phenotype of both ABO and placental alkaline phosphatase polymorphism was observed; the differences in the pattern of genetic factors between these two ethnic groups might in part be responsible for the higher incidence of haemolytic disease among black infants [4, 8]. Recent studies have shown that anti-A and anti-B haemolysin and agglutinin titres are higher in black than in white patients. White persons have lower anti-B than anti-A, whereas in the black population anti-B levels are similar to anti-A values [9]. These studies also indicate that genetic factors contribute significantly to the variation in isoantibody levels. Environmental factors, as for example helminthic infection of the mother, might also explain the difference in the incidence of ABO haemolytic disease between ethnic groups [10].

Table 4 shows the incidence of a high serum bilirubin level during the first few days of life in ABO compatible and ABO incompatible infants studied by us and others. Among ABO compatible infants the incidence of hyperbilirubinaemia was higher in whites than in black patients; among ABO incompatible infants

the pattern was reversed. Therefore, while in ABO incompatible white infants there was only a slight increase in the incidence of hyperbilirubinaemia as compared to compatible infants, in black infants this increase was significant.

Table 4

Per cent of a significant serum bilirubin level during the first few days of life in ABO compatible and ABO incompatible infants

	ABO compatible		ABO incompatible	
	* Friedman et al. [11]	** Kirkman [1]	** Kirkman [1]	** Authors
Whites	13.3	12.5	19.4	11.8
Blacks	7.0	4.4	19.5	17.1

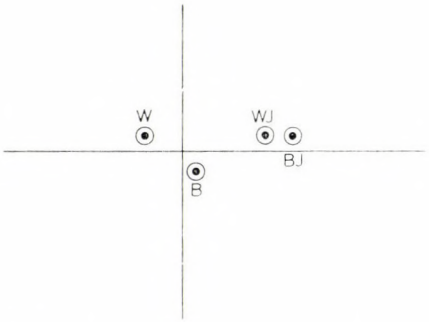
* Serum bilirubin ≥ 12 mg/dl.
** Serum bilirubin ≥ 10 mg/dl.

Racial differences in bilirubin excretion are well known and standards defining physiologic jaundice in Caucasian populations may not be applicable to other races. A lower incidence of hyperbilirubinaemia in ABO compatible black infants as compared to white infants was recorded in two large and independent series reviewed in the present paper and the phenomenon deserves further investigations.

Table 5 shows a discriminant analysis on Coombs test, gestational age, birth weight and race. It appears that "race" contributes nothing as an independent variable to the prediction of hyperbilirubinaemia in ABO incompatible infants. Figure 1 shows the differences between white and black infants. The general configuration is unipolar, suggesting an underlying unidimensional continuum. Whites without hyperbilirubinaemia (W) are clearly separated from all other groups. Black and white infants with a serum bilirubin level of 10 mg/dl or higher show practically the same position. Black infants without hyperbilirubinaemia occupy an intermediate position between W and C infants (WJ and BJ) with hyperbilirubinaemia.

Gestational age and birth weight are somewhat similar in white infants with hyperbilirubinaemia and in both classes of black infants (see Table 2). On the contrary, the values observed in whites without hyperbilirubinaemia are very different from the values observed in the other classes of infants.

The data clearly show that black infants have a higher incidence of Coombs test positivity, a lower mean gestational age and a lower mean birth weight as compared to white infants. It is likely that all these factors contribute to make ABO incompatible black infants highly susceptible to hyperbilirubinaemia. Since "race" does not contribute to the prediction of hyperbilirubin-



Significance of the distance between groups

	W	WJ	B
WJ	≪0.01		
B	< 0.02	< 0.05	
BJ	≪0.01	N.S.	<0.05

Fig. 1. Graphical display of differences between white and black infants. Four-group discriminant analysis on Coombs test, gestational age and birth weight. Groups: (W) white, bilirubin <10 mg/dl; (WJ) white, bilirubin ≥ 10 mg/dl; (B) black, bilirubin <10 mg/dl; (BJ) black, bilirubin ≥ 10 mg/dl. The first function, corresponding to the horizontal axis, showed highly significant discriminant power. The remaining two (vertical axis, and the axis perpendicular to the plain) did not show any appreciable discriminant power. The figure shows the relative position of the four group centroids in the plane defined by the first two functions. (Data of Authors)

Table 5

Stepwise discriminant analysis by Wilks' method on Coombs test, gestational age, birth weight and race. Groups: (A) infants with serum bilirubin <10 mg/dl; (B) infants with serum bilirubin ≥ 10 mg/dl (Data of Authors)

Step number	Variable entered	Rao's V	Change in Rao's V	Significance of change
1	Coombs test	34.11	34.11	<0.001
2	Gestational length	53.24	19.13	<0.001
3	Birth weight	59.63	6.39	0.012
4	Race	60.33	0.71	0.400

Standardized discriminant function coefficients

Coombs test	0.7461
Gestational age	−0.3511
Birth weight	−0.3423
Race	0.1034
Canonical correlation	0.368
Wilks' lambda	0.8642
χ^2	55.746
D. F.	4
Significance	<0.001

aemia, genetic and environmental factors which predispose ABO incompatible black infants to neonatal jaundice might act through immunological and developmental mechanisms largely accounted for by the three variables considered.

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Adherence to Human Monocytes of Red Cells from Autoimmune Haemolytic Anaemia and Red Cells Sensitized with Alloantibodies

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Red cells sensitized with autoantibodies are able to adhere *in vitro* to autologous monocytes and monocytes from healthy individuals. A direct relationship between the degree of sensitization and the percentage of rosettes was not observed, while such a correlation was found if red cells sensitized with anti-Rh alloantibodies were used. Sometimes the adherence of red cell from AIHA was observed although the sensitization was weaker than that of the control erythrocytes sensitized with anti-CD serum which did not adhere to monocytes. In patients with AIHA some relation was found between the adherence assay, haemolysis *in vivo* and treatment with prednisone.

Introduction

It is known that haemolysis in patients with autoimmune haemolytic anaemia (AIHA) is not always parallel to the degree of sensitization of red cells with autoantibodies [8], although there appears to be a direct relationship between the amount of IgG on the erythrocytes and red cell destruction as far as alloantibodies are concerned [8, 15, 16]. The presence of Fc receptors and receptors for certain complement components on human mononuclear phagocytes has been established [3, 6], and there are some data indicating that the adherence of sensitized red cells to monocytes *in vitro* might somehow be related to the process of haemolysis in AIHA [10, 14].

The aim of the present study was to observe the relationship between the degree of sensitization of the red cells with auto- and alloantibodies and the adherence of such red cells to the monocytes obtained from healthy donors and patients with AIHA. The sensitization of red cells in AIHA and rosette formation was compared to the rate of haemolysis *in vivo*.

Material and Methods

Venous blood was collected from 50 healthy donors and 23 patients with autoimmune haemolytic anaemia. The exacerbation of haemolysis and remission of AIHA were recognized according to accepted criteria.

Red cells

(i) Cells sensitized with alloantibodies. One volume of washed, packed red cells O ccDee or O ccDEE from healthy volunteers was incubated at 37 °C (for 1 hour) with 5 volumes of anti-D or anti-CD serum from hyperimmunized donors (one of these sera was Ripley-like [Stas]) [12]. After incubation the red cells were washed 4 times and a 1% suspension in buffered saline was prepared.

Complement fixation was performed by two-step incubation:

1. with the Ripley-like serum as mentioned above, and afterwards
2. with fresh AB serum at 37 °C during 1 hour.

(ii) Cells sensitized with autoantibodies were obtained from patients with autoimmune haemolytic anaemia. The suspension of red cells was prepared as mentioned above.

The sensitization of red cells with antibodies was estimated in the direct antiglobulin test with polyspecific,* anti-IgG and anti-C3 sera. In some cases subclasses of autoantibodies were determined by anti-IgG_1 (KH 161-51-A1), anti-IgG_2 (KH 162-A3), anti-IgG_3 (KH 163-45-A1), anti-IgG_4 (KH 164-46-A1) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service in Amsterdam).

The degree of sensitization of red cells was determined in AutoAnalyzer Technicon II according to Górska et al. with some modifications [4]. A 20% suspension of erythrocytes sensitized with anti-CD serum or with autoantibodies was added to the AutoAnalyzer system. The degree of sensitization was estimated as the difference in peak height (optical density) ΔOD between red cells with saline and red cells with bromeline and methylcellulose (BMC) (Fig. 1). If highly sensitized red cells were investigated a sixfold diluted BMC had to be used in order to visualize differences in sensitization. The degree of sensitization of patient red cells was always compared to that of control erythrocytes. As a control, red cells sensitized with anti-CD serum diluted 1 : 256 were chosen. Such red cells were used because they did not adhere to monocytes although they were strongly sensitized. If the ΔOD of the patient erythrocytes was higher than the ΔOD of control red cells, the sensitization with autoantibodies was regarded as strong, while if the ΔOD of red cells from AIHA was lower, it was accepted as weak.

Monocytes

EDTA blood from healthy volunteers or from patients with AIHA was collected and centrifuged at 400 *g* for 7 minutes in order to remove the platelets. The remaining cells were diluted in three volumes of buffered saline and centrifuged at 400 *g* over Ficoll-Uropoline (density 1.077 g/ml) for 40 min. The inter-

* The polyspecific serum was produced at the Institute of Haematology, Warsaw, and specificity and activity were tested by the methods used by the Working Party on the Standardization of Antiglobulin Reagents, ICSH/ISBT.

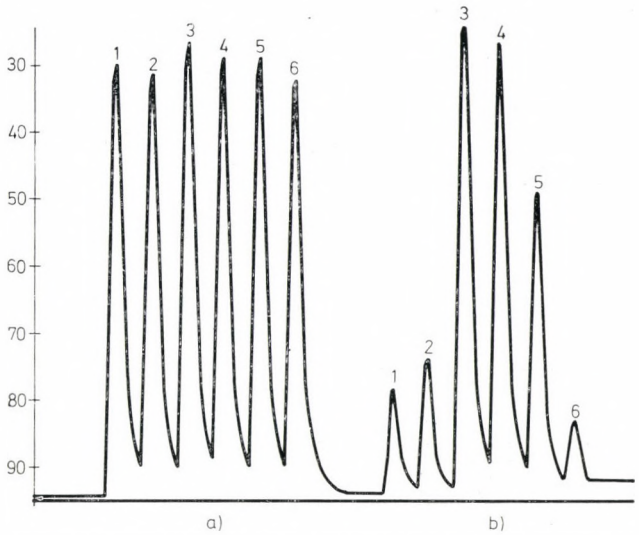

Fig. 1. Example of the results obtained with different red cells by the automated BMC method. a) peak heights of red cells run against saline; b) peak heights of red cells run against sixfold diluted BMC. 1. red cells sensitized with anti-CD serum diluted 1 : 64; 2. red cells sensitized with anti-CD serum diluted 1 : 256; 3. nonsensitized red cells from blood donor; 4. red cells from patient with AIHA with negative Coombs test; 5. red cells from patient Tom (weak sensitization); 6. red cells from patient Mich (strong sensitization)

face layer contained the mononuclear cells washed in Hank's solution (Wytwórnia Surowic i Szeczepionek, Lublin) with 20% fetal calf serum (FCS, Flow Laboratories), adjusted to 1×10^7 cells/ml, layered onto glass Petri dishes and incubated at 37 °C for 1.5 h. Non-adhering cells were removed by threefold washing. Hank's solution with 20% FCS and 15% (12 mM) Xylocaine (Astra) was added to the adhering cells. After half an hour the monolayer was harvested with a 1 cm diameter pipette with a piece of silicone rubber. The cells were washed with Hank's solution and adjusted to a concentration of 1×10^6 cells/ml.

The obtained suspension contained 90% of monocytes as judged by morphological standards (May–Grünwald–Giemsa, and staining for esterase and peroxidase). About 90% of the cells were viable estimated with a 5% solution of eosin yellow (Gurr).

Adherence assay

In preliminary experiments we have shown that the percentage of rosettes depends on the monocyte to erythrocyte ratio. A statistically significant difference was observed with monocyte to erythrocyte ratios between 1 : 25 and 1 : 200, but there were no statistical differences in ratios between 1 : 50 and 1 : 200. For

flurther experiments a 1 % (5×10^7) suspension of erythrocytes and one of 1×10^6 ml of monocytes were chosen.

Equal volumes of erythrocyte and monocyte suspensions were mixed in small tubes (0.5 cm × 4.5 cm) and centrifuged at 200 *g* for 10 min. After resuspension the number of rosettes was determined after adding 1 drop of 5 % eosin to exclude counting of dead cells. Monocytes which bound three or more erythrocytes were counted as rosettes. All tests were performed in duplicate.

Results

Adherence assay with red cells sensitized with alloantibodies

The percentage of rosettes of monocytes from healthy volunteers with red cells sensitized with anti-Rh alloantibodies depends on the degree of this sensitization (Figs. 2, 3, Table 1). Using ccDee or ccDEE red cells sensitized with the same sample of anti-D or anti-CD serum, it was observed that the percentage of rosettes was higher with D homozygous erythrocytes (Fig. 2). Table 1 shows that the percentage of rosettes depends on red cell sensitization obtained with sera with different antibody titres. Among strong sera (Woj, Staś) no difference in the percentage of rosettes was observed. This was confirmed by adherence assay in other cases (Fig. 3). Figure 3 also shows that the percentage of rosettes was the same whether anti-D or anti-CD serum was used.

Figure 4 again indicates the correlation between the sensitization of red cells and the adherence to monocytes – using different dilutions of a particular serum (Staś or Woj). The highest percentage of rosettes (66.8 ± 1.6 %) was obtained when undiluted serum was used. The percentage decreased parallel with the dilution and, finally, with the 1 : 256 dilution the adherence was between 0 and 5 %, although the sensitization of red cells was still strong (score = 20).

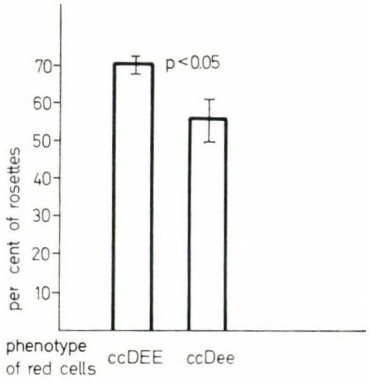

Fig. 2. Percentage of rosettes with ccDEE and ccDee erythrocytes sensitized with the same sample of serum

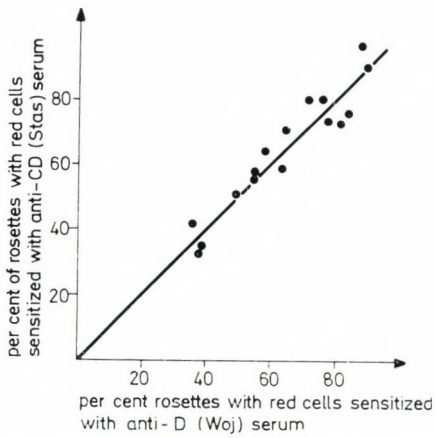

Fig. 3. The comparison of rosette formation of monocytes from individual persons with erythrocytes sensitized with anti-CD (Stas) anti-D (Woj) sera

Table 1

Adherence assay with red cells sensitized with different anti-Rh sera

Anti-Rh sera		Titre of serum in manual technique	Degree of sensitization in AutoAnalyzer (ΔOD) of erythrocytes sensitized with serum diluted 1 : 256	% of rosettes (erythrocytes sensitized with undiluted serum)
Sample	Specificity			
A	D	128	0.00	5
B	D	256	0.02	24
Woj	D	12 800	0.12	50
Staś	CD	25 000	0.20	51

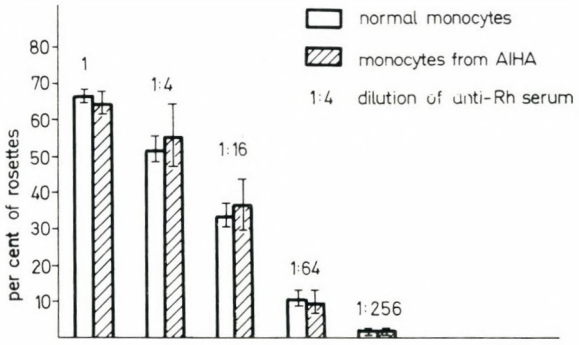

Fig. 4. Rosette formation of monocytes from healthy persons and patients with AIHA with erythrocytes sensitized with different dilutions of anti-CD serum

Figure 4 also shows that this applies to monocytes from patients with AIHA and there is no difference in the adherence to normal monocytes and monocytes from patients if red cells of the same degree of sensitization are used.

The additional presence of complement on the red cells sensitized with IgG did not enhance the ability of adherence. The percentage of rosettes with red cells sensitized with Ripley-like serum was 64, while it was 61 with the Ripley-like serum and complement.

Adherence assay with red cells sensitized with autoantibodies

The ability of rosette formation of monocytes from healthy individuals and monocytes from patients with AIHA with red cells sensitized with autoantibodies, was not significantly different (Fig. 5).

Tables 2 and 3 show the results of adherence assays in patients with AIHA in comparison to haemolysis and to treatment. Among patients with active haemolysis, rosettes were found in untreated patients or those treated with small doses of prednisone (< 30 mg/day). In those who received high doses rosettes were not found except in one patient. Among 8 patients in remission, in 6 cases rosettes were not observed but only one of them received 40 mg of prednisone. In 3 patients in remission the percentage of rosettes was high.

The subclasses of IgG were determined in 10 cases. In all of them IgG_1 was present and in three cases IgG_2 or IgG_3 were also found. No correlation was observed between the subclasses of IgG and the percentage of rosettes.

There was no direct relationship between the sensitization of red cells and the percentage of rosettes. In Fig. 6 the results in individual patients are divided according to the degree of sensitization of red cells with autoantibodies *in vivo*, and according to the presence of complement on the erythrocytes. When the

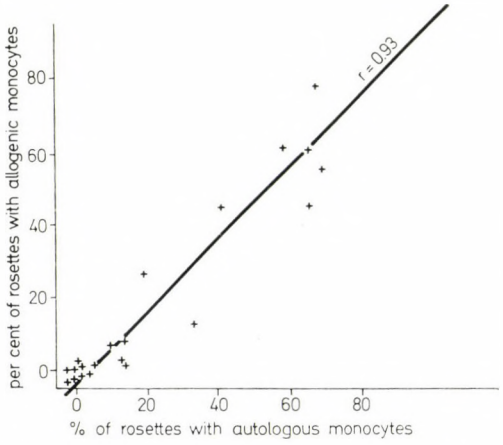

Fig. 5. Rosette formation of monocytes from healthy persons and patients with AIHA with red cells sensitized with autoantibodies

amount of autoantibodies was higher than the amount of alloantibodies on the red cells sensitized with anti-CD serum diluted 1 : 256 (such red cells gave less than 5% rosettes – see Fig. 4), sensitization was considered to be strong.

Table 2

Adherence assay in patients with AIHA

Initials	Treatment		Sensitization of erythrocytes			% of rosettes with monocytes from healthy donors
	prednisone	cyclophosphamide	Degree of sensitization ΔOD in AutoAnalyzer*	Subclasses of IgG	Complement	
AIHA with acute haemolysis						
Pak	60	–	0.01	n. d.	–	0
	0	–	0.01	n. d.	–	35
Gryb	20	–	0.28	IgG_1 IgG_3	–	28
Wiec	80	–	0.02	IgG_1	–	0
Grz	0	–	0.40	n. d.	–	19
	60	–	0.41	n. d.	–	0
Koz	50	–	0.01	IgG_1	–	2
Paj	50	–	0.01	IgG_1	–	0
	0	–	0.03	IgG_1	–	50
Om	30	100	0.06	IgG_1 IgG_2	–	45
	15	–	0.61	IgG_1 IgG_2	–	55
Pec	50	–	0.00	n. d.	++	1
	60	–	0.00	n. d.	–	0
Mich	0	–	0.41	IgG_1	+	45
Sied	10	–	0.41	n. d.	+	41
Zamb	50	–	0.02	n. d.	++	4
AIHA in remission						
Waś	10	–	0.40	IgG_1	–	4
Mit	10	–	0.51	n. d.	–	8
Dom	0	–	0.02	n. d.	–	0
Koł	5	–	0.01	n. d.	–	0
Pach	–	50	0.22	n. d.	–	60
Tom	20	–	0.23	IgG_1	–	27
	40	100	0.22	IgG_1	–	0
Zag	0	–	0.40	n. d.	+	2
Kras	10	–	0.01	n. d.	+++	78
Control eryhtrocytes O Rh+ sensitized with 1 : 256 dilution of anti-CD serum			0.35 ± 0.05	IgG_1** IgG_3	–	0 to 5

* ΔOD > 0.35 – strong sensitization with autoantibodies,
ΔOD < 0.35 – weak sensitization with autoantibodies.

** The subclasses of IgG were determined on the red cells sensitized with undiluted anti-CD serum,

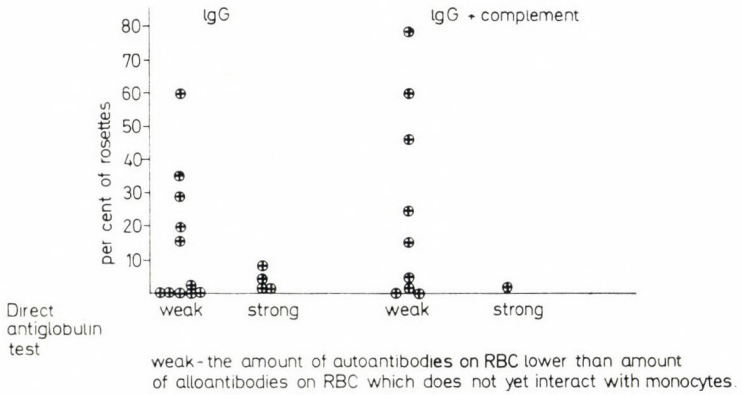

Fig. 6. The comparison of percentage of rosettes with erythrocytes from patients with AIHA with different degree of sensitization with autoantibodies (IgG or IgG and complement)

Table 3

Relation between haemolysis *in vivo* in patients with AIHA and adherence of their red cells to monocytes *in vitro*

Patients	Adherence	Number of patients	
		with active haemolysis	in remission
Non-treated or treated with	present	8	2
<30 mg prednisone/day	absent	0	5
Treated with prednisone	present	1	1
>30 mg/day	absent	7	1

Figure 4 shows that rosettes were often observed when the sensitization with autoantibodies was even weaker than the sensitization of red cells with alloantibodies which caused no adherence. In contrast, in some cases even strongly sensitized red cells in AIHA failed to adhere to the monocytes. These observations concerned patients with IgG on their red cells as well as those with IgG and complement.

Discussion

Our results show that there is a difference between the adherence to monocytes of red cells sensitized with autoantibodies and those sensitized with alloantibodies. The adherence of red cells sensitized with autoantibodies did not depend on the degree of sensitization, while there was a close correlation between the sensitization with alloanti-Rh antibodies and the percentage of rosettes with monocytes. Red cells from some patients with AIHA caused adherence although

the sensitization was weaker than that of control erythrocytes sensitized with anti-CD serum which did not adhere to monocytes.

It is difficult to understand why there is no direct relationship between the adherence and the degree of sensitization with autoantibodies of the red cells, while such a correlation does exist as far as alloantibodies are concerned. The following explanations might be considered. The first one is the difference in subclasses of IgG of autoantibodies. It was found by several authors [1, 7] that macrophages have receptors for IgG_1 and IgG_3 but not for IgG_2 and IgG_4, thus red cells sensitized with such immunoglobulins would not adhere to macrophages and probably neither to monocytes. The observations of Dutch authors [10, 14] on the relationship between the subclass specificity detected by direct antiglobulin test and the presence of haemolysis, confirm this finding. In our material the subclass specificity was checked in 10 patients only, therefore it is difficult to draw any conclusion, but there were some cases in which IgG_1 was detected but no or only few rosettes were observed. Another explanation may be a different distribution of autoantibodies on the red cells. In some patients with AIHA, autoantibodies may occur in clusters on the red cells whereas in others they may be evenly distributed over the membrane. It might be supposed that red cells with clusters of autoantibodies may adhere more readily even if the amount of immunoglobins is low.

The third possibility could be an inadequacy of the methods applied. Van der Meulen et al. [13] using continuous-flow cytofluorometry with fluorescein isothiocyanate-labelled antiglobulin reagents suggested a correlation between the number of IgG_1 antibodies per red cell and the degree of damage as a result of adherence to monocytes *in vitro*.

The additional presence of complement on the red cells seems to have no influence on the adherence irrespective of the red cells being sensitized with alloantibodies or autoantibodies. This is not surprising because the presence of receptors for C3d on the monocytes has been questioned [2, 5, 17, 19]. Although in our material the presence of complement on the red cells was demonstrated only with anti-C3 but not with specific anti-C3d serum, the presence of C3d but not C3b on the red cells was most probable because of the action of C3b inactivator *in vivo* as well as *in vitro* during prolonged incubation of red cells in plasma [18]. Van der Meulen et al. who found C3d in 45% of their patients with AIHA did not even consider the presence of complement in the interpretation of adherence [14].

Because the degree of sensitization with autoantibodies and the presence of complement on the red cells does not correspond to the ability of rosette formation with monocytes, the question arises whether the adherence might be in any correlation with the rate of haemolysis *in vivo* and the treatment. Our observations have made it probable that such a correlation exists, because in most patients with active haemolysis who were not treated or treated with small doses of prednisone, rosettes were present. On the other hand, in most patients in remission no rosettes were found. A close correlation between the presence of active haemol-

ysis and rosette formation in non-treated patients and in most patients treated with prednisone was reported by van der Meulen et al. [14]. In most of our patients treated with high doses of prednisone, no adherence was observed but signs of haemolysis were still present. The same was found by van der Meulen et al. but only in three out of 19 patients. In such cases, although the corticosteroids interfere with adherence, signs of haemolysis might still be present, due to already destroyed red cells. Follow-up of such patients might explain this discrepancy.

MacKenzie [11] found that monocytes from patients with AIHA showed stronger ingestion of IgG-sensitized red cells compared with monocytes from healthy donors and patients with non-haemolytic anaemias. Kay and Douglas [9] showed in most patients with AIHA enhanced rosetting and phagocytosis with autologous sensitized red cells as compared with normal monocytes exposed to the same red cells. Our observations do not confirm these results because the ability of rosette formation with anti-Rh sensitized red cells or red cells from AIHA by autologous monocytes and monocytes from healthy donors was not significantly different. The discrepancy between our findings and the results published by Kay and Douglas cannot be explained.

Although not everything is fully clear with the adherence assay with red cells sensitized with autoantibodies, our data support the assumption that the adherence to mononuclear phagocytes might be an important mechanism in the *in vivo* destruction of red cells in AIHA.

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Book Reviews

Eisenstoffwechsel. Physiologie, Pathophysiologie, Klinik, Therapie. By I. Bernát. Akadémiai Kiadó, Budapest and Gustav Fischer Verlag, Stuttgart, New York 1981. 482 pages, 42 tables, 230 figures.

In this recently issued second, enlarged edition of the monograph a comprehensive review is given of the present knowledge on iron metabolism, based partly on the author's own clinical experience and research, partly on the confirmed and accepted findings of others published in the last 3–4 decades. The enlargement of the second edition finely reflects the development of our knowledge in the treated area since the publication of the first edition in 1973.

The content is divided into 27 chapters. The physiological and pathophysiological chapters comprise the sources (5), absorption (6), transport (7), storage (8), loss and demand of iron (9), erythropoiesis (10), haemoglobin synthesis (11), red cell and haemoglobin destruction (12, 13). Further chapters are devoted to the basic problems of ferrokinetics (14), erythrokinetics (15), cytochemistry (16) and electron microscopy (17). In the physiological and pathophysiological chapters (iron storage, iron absorption) the techniques of diagnostic importance are described and evaluated in subchapters.

Throughout the whole volume the main emphasis laid on the clinical aspects. The various pathological conditions of iron metabolism are described in detail in chapters 18–27. Iron deficiency (importance, incidence, clinical picture, laboratory investigations, diagnostics, differential diagnostics, aetiology, pathogenesis and therapy) and the various forms of iron overload (haemochromatosis, siderosis developed on the basis of liver cirrhosis, dietary siderosis, congenital atransferrinaemia, hypersiderosis developing in the course of various anaemias, transfusion siderosis, etc.) are most extensively dealt with. Diseases related to disturbed iron metabolism, e.g. infections and tumours, burn injury, pernicious anaemia, sideroachrestic anaemias, acute radiation sickness, polycythaemia etc. are also discussed.

The documentation is abundant and demonstrative. The list of references makes up 76 pages.

Especially haematologists, physicians, paediatricians, gynaecologists and obstetricians, as well as physiologists and pathophysiologists will greatly benefit by studying this up-to-date and comprehensive monograph with a clear practical outlook.

Ilma Szász

Immunostimulation. Ed. by L. Chedid, P. A. Miescher, H. J. Müller-Eberhard. Springer Verlag, Berlin, Heidelberg, New York 1980. 236 pages, 39 tables, 44 figures.

The book comprises the No. 1 and No. 2 issues of Volume 2 of the international journal *Springer Seminars in Immunopathology.* It contains 15 chapters on the different possibilities of stimulating the immune system. The chapters follow each other in a more logical order than in the original publication.

In the preface written in September, 1980 L. Chedid says that some results and conclu, sions described in the book have been con-

firmed recently and several new immunostimulating molecules have appeared on the scene since the first publication. After a short introductory chapter (L. Chedid) summarizing the topics of the book, B. H. Waksman discusses the natural regulation of the immune responses and in concise tables and text analyzes targets for different adjuvants in the body and at the level of the cell. According to him it should soon become possible to design a simple adjuvant to accomplish almost any desired effect on the immune system. In the third introductory chapter J. W. Halden stresses the necessity to develop an effective immunopharmacology which should remove the current empiricism from clinical immunotherapy. The next chapter (A. Capron, D. Camus) deals with the possibilities of immunoregulation by parasite extracts. The various immunostimulating effects of bacterial lipopolysaccharides as well as the potential dangers of their application (triggering of an autoimmune response) are summarized by J. A. Louis and P. H. Lambert. Immunostimulation by different bacterial organisms (BCG, *C. parvum*), their possible targets (macrophages, natural killer cells), and the results obtained with these organisms in the immunotherapy of cancer are reviewed in the next chapter (R. W. Baldwin, V. S. Byers). They conclude to the superiority of localized or regional immunotherapy over general immunostimulation. The next two chapters are devoted to the relatively simple immunostimulating compounds produced by or extracted from Mycobacteria, to cord factor and related synthetic trehalose diesters (E. Lederer) and to muramyl dipeptide, MDP (M. Parant). T. L. K. Low and A. L. Goldstein summarize recent findings about purification, chemical characterization and biological activities of various thymic hormones and the mode of preparation and effect of their synthetic analogues. The next four chapters describe simple compounds with significant immunostimulating or immunomodulating effect: tuftsin (E. Tzehoval et al.), synthetic polynucleotides (A. G. Johnson), lysophosphatidylcholine (P. G. Munder et al.) and levamisole (J. Symoens et al.). M. Sela and E. Mozes review the possibilities of combined use (by administering together or by linking covalently) of synthetic antigens and synthetic adjuvants. In the last chapter, L. Chedid and P. A. Miescher, after summarizing the contents of the book discuss some additional avenues (role of I and V genes in the immunogeneicity of antigens, helper/suppressor balance of T lymphocytes) which will probably lead to new developments in clinical medicine in the very near future.

This excellent book offers insight into the current thoughts of a newly developed area of research with more future than past. It gives solid information and includes the latest knowledge on the subject. The volume is recommended to both clinicians and researchers working in the field of immunology, pharmacology and biochemistry.

G. Füst

Abstracts

Action of certain anaesthetic agents on erythrocyte calcium permeability. P. Dube, P. Sagar (Division of Biophysics, Central Drug Research Institute, Lucknow, India). *Indian J. exptl. Biol. 19*, 143 (1981).

Action of a number of anaesthetic agents, viz. chlorpromazine, tetracaine, procaine, xylocaine, centbucridine, dibucaine and phenobarbitone on the calcium permeability of human eryhtrocytes was investigated, using a recently developed procedure [*J. Membrane Biol. 35*, 75 (1977)], which eliminates adsorption artifacts. Only dibucaine amongst these significantly enhanced Ca^{2+} influx and its effect increased sharply with concentration in the range 0.1 to 1.0 mM. Pretreatment of the cells with chlorpromazine or centbucridine and also replacement of external Na^+ by K^+ reduced this action of dibucaine, however, increased calcium influx in its presence was not associated with significant leakage of intracellular K^+, suggesting that unlike the action of propranolol, calcium permeability enhancement by dibucaine is not linked to membrane hyperpolarization. Further, although 1.0 mM dibucaine did partially inhibit Ca^{2+} extrusion from the ionophore (A23187) loaded cells, this did not seem to be related to observed increase in Ca^{2+} influx by the drug since similar inhibition of extrusion was shown also by centbucridine which did not induce any significant calcium uptake.

G. Gárdos

Changes in 2,3-diphosphoglycerate (2,3-DPG) after exercise. H. D. Meen, P. H. Holter, and H. E. Refsum (Institute for Respiratory Physiology, Ullevaal Hospital, Oslo, Norway). *Eur. J. appl. Physiol. 46*, 177 (1981).

Previous studies of the influence of physical exercise on erythrocyte 2,3-DPG have shown conflicting results, including that exercise induces increase, decrease or no changes in 2,3-DPG. Assuming that the interplay between the factors governing 2,3-DPG metabolism may change considerably during the early phase of recovery after exercise, the level of erythrocyte 2,3-DPG was examined over a prolonged period of time after heavy exercise in five healthy men, on 3 separate days, the duration of exercise being 6 min, 6 min + 6 min with 1 h of rest between, and 60 min, respectively. The short exercise periods were accompanied by a substantial lactate acidosis, whereas the 60 min work was essentially aerobic. With the 6 min work, the erythrocyte 2,3-DPG was unchanged or slightly reduced immediately after and 15 min after the end of exercise, then rose to a new level, 8% above the initial level, 30 min after the exercise ($P < 0.001$). With the 6 + 6 min work, the pattern of change followed both bouts of exercise, resulting in a two-step increase of 2,3-DPG, to a new level 12% above its initial value ($P < 0.001$). With the 60 min work, 2,3-DPG was increased after 30 min of exercise, and rose continuously during the early recovery phase, to

a new level, 10% above initial value, 45 min after the exercise ($P < 0.001$). With all three types of exercise 2,3-DPG remained unchanged during the following 4 h. Thus, heavy exercise is followed by a definite, slowly developing increase in erythrocyte 2,3-DPG, reaching a new level 30–45 min after exercise.

G. Gárdos

The insulin receptor is an age-dependent integral component of the human erythrocyte membrane. R. F. Dons, L. M. Corash and P. Gorden (Diabetes Branch, National Institute of Arthritis, Metabolism and Digestive Diseases and the Hematology Service, Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Maryland, USA). *J. biol. Chem. 256*, 2982 (1981).

Human erythrocytes were separated according to age to determine whether there is heterogeneity in the specific binding of ^{125}I-insulin to red cells. The mean cell age of erythrocyte isolates was determined from the cumulative distribution frequency of the cells in an isotonic (290 milliosmolar) density gradient and confirmed by assay for pyruvate kinase, an age-dependent red cell enzyme. An IBM 2997 centrifugal cell separator was used to obtain larger quantities of younger erythrocytes from normal subjects. ^{125}I-insulin binding to red cells including reticulocytes was found to decrease exponentially as a function of their mean cell age in 8 normal subjects. A change in receptor number rather than affinity appeared to account for the observed change in ^{125}I-insulin binding. An exponential, age-dependent change in binding of a hormone to its cell membrane bound receptor has not previously been observed. Consistent with these results is the possibility that regulation of the red cell insulin receptor concentration takes place only in the younger red cells.

G. Gárdos

The rate of transmembrane movement of cholesterol in the human erythrocyte. Y. Lange, J. Dolde and T. L. Steck (Biophysics Section, Boston University School of Medicine, Boston, Massachusetts, and Department of Biochemistry, University of Chicago, Chicago, Ill., USA). *J. biol. Chem. 256*, 5321 (1981).

Cholesterol appears to be abundant on both sides of the human erythrocyte membrane, but its precise distribution and rate of transmembrane movement are undetermined. Although the cholesterol in the intact cell normally was resistant to cholesterol oxidase, enrichment of cells with exogenous cholesterol or preincubation at very low ionic strength rendered the entire cholesterol pool susceptible to attack. Under these experimental conditions, all of the membrane cholesterol was oxidized in a strictly first order fashion with a half-time as short as 10 s at 37 °C. Since the enzyme had access only to the outer membrane surface, these data suggest that the transmembrane movement (flip-flop) of cholesterol is extremely rapid. From an error analysis, we estimate an upper bound on the half-time of the transmembrane movement of cholesterol of 3 s at 37 °C. A physiological function for rapid sterol flip-flop is suggested.

G. Gárdos

Effect of arachidonic acid and the chemotactic factor F-Met-Leu-Phe on cation transport in rabbit neutrophils. R. I. Sha'afi, P. H. Naccache, T. Alobaidi, T. F. P. Molski, and M. Volpi (Departments of Physiology and Pathology, University of Connecticut Health Center, Farmington, Conn., USA and Department of Physiology, College of Medicine, Al-Mustansirah University, Baghdad, Iraq). *J. cell. Physiol. 106*, 215 (1981).

A detailed examination of the effects of exogenous arachidonate on cation metabolism in rabbit neutrophils was undertaken. Arachidonic acid stimulates the movement of ^{45}Ca into and out of the neutrophils with a net result, in the presence of extracellular calcium, of increasing the steady-state level of ^{45}Ca. Arachidonate also increases the uptake of ^{22}Na. These effects of arachidonate are specific to these cations, concentration-dependent, and sensitive to lipoxygenase inhibitors. At the concentrations used in this study arachidonate does not influence the permeability of human erythrocytes to ^{45}Ca.

Furthermore, both arachidonic acid and F-Met-Leu-Phe release calcium from a previously unexchangeable intracellular pool and the effect of the two stimuli are not additive. Arachidonic acid-dependent, but not F-Met-Leu-Phe-dependent, calcium release is sensitive to lipoxygenase inhibitors. These two stimuli thus appear to release calcium from the same pool(s) by separate mechanisms. The results summarized above are consistent with the hypothesis that one or more arachidonate metabolites are involved in the mechanism underlying the chemotactic factor induced permeability changes in rabbit neutrophils.

Ilma Szász

Separate mechanisms of deformability loss in ATP-depleted and Ca-loaded erythrocytes. M. R. Clark, N. Mohandas, C. Feo, M. S. Jacobs and S. B. Shohet (Cancer Research Institute and Departments of Medicine and Laboratory Medicine, University of California, San Francisco, CA., USA; Institut de Pathologie Cellulaire, Hôpital Bicêtre, Kremlin Bicêtre, France). *J. clin. Invest. 67*, 531 (1981).

Membrane rigidity has been widely accepted as the dominant cause of reduced deformability both of ATP-depleted erythrocytes and erythrocytes containing excess calcium (Ca). However, recent studies have shown normal membrane deformability in ATP-depleted erythrocytes. In addition, Ca accumulation causes massive ion and water loss, and it has been shown that extensive dehydration causes an increase in intracellular viscosity with attendant loss of whole cell deformability. To obtain a detailed understanding of the processes accompanying ATP depletion and/or Ca accumulation that limit cell deformability, we have used a viscodiffractometric method to identify the cellular factors contributing to reduced whole cell deformability.

Analysis of the influence of the suspending medium osmolality of deformability showed the presence of two independent processes. One was a Ca-independent reduction in cell surface area/volume ratio, resulting from the spheroechinocyte formation that follows total ATP consumption. The other was a Ca-dependent increase in intracellular viscosity resulting from a Ca-induced loss of intracellular potassium and water. This deformability loss due to increased intracellular viscosity was found for cells depleted of ATP in the presence of Ca and in cells treated with Ca and A23187 without prior depletion. Ionophore-treated cells at high Ca concentration ($> 500\,\mu M$) formed spheroechinocytes with reduced surface area and a further loss of whole cell deformability. The rate of deformability loss associated with Ca-induced spheroechinocytosis was much more rapid than that associated with ATP-depletion-induced spheroechinocytosis, suggesting different mechanisms for the morphologic changes. No major effects of altered membrane elasticity on the reduced deformability of either ATP-depleted or Ca-loaded cells were observed.

Ilma Szász

Erythrocyte membrane sidedness in lectin control of the Ca^{2+}-A23187-mediated diskocyte $\rightleftarrows$ echinocyte conversion. R. A. Anderson, R. E. Lovrien (Biochemistry Department, Gortner Laboratory, University of Minnesota, St. Paul, Min., USA). *Nature (Lond.) 292*, 158 (1981).

Increasing the cytoplasmic calcium concentration of human erythrocytes with ionophore A23187 drives the transformation from diskocyte to echinocyte morphology. This transformation is closely linked to the intracellular ATP level, which drops when calcium is introduced across the membrane. The echinocyte morphology reverts to the diskocyte after restoration of normal ATP and calcium levels. ATP control of cell morphology is thought to depend on the action of a protein kinase and phosphatase which reversibly modify the membrane cytoskeleton. The cytoskeleton in turn is in contact with the outside membrane surface *via* transmembrane proteins. It is shown here that binding of the lectin, wheat-germ agglutinin (WGA), to the erythrocyte membrane surface blocks the morphological conversions diskocyte $\rightleftarrows$ echinocyte in both directions. WGA seems to work *via* a mechanism involving the transmembrane glycoprotein, glycophorin.

Ilma Szász

Activation and deactivation kinetics of Ca transport in inside-out erythrocyte membrane vesicles. J. D. MacIntyre and R. B. Gunn (Department of Pharmacological and Physiological Sciences, University of Chicago, Ill., USA). *Biochim. biophys. Acta (Amst.) 644*, 351 (1981).

The kinetics of active Ca^{2+} transport in inside-out vesicles of human erythrocyte membranes has been studied. Hemolysate or purified calmodulin increased the apparent affinity of the Ca^{2+} transport system for Ca^{2+} and increased the apparent maximum velocity of Ca^{2+} transport. However, as Ca^{2+} concentration was increased above 90 μM in the presence of hemolysate or calmodulin, the extent of activation of Ca^{2+} fluxes decreased. This deactivation was less prominent if the Mg^{2+} concentration was increased. These results may be explained by postulating that calmodulin has a site which binds either Mg^{2+} or Ca^{2+} and if Mg^{2+} occupies this site calmodulin activates Ca^{2+} fluxes, while if Ca^{2+} occupies this site, calmodulin is unable to activate the transport system.

B. Sarkadi

Secretion from rat basophilic leukaemia cells induced by calcium ionophores. Effect of pH and metabolic inhibition. C. Fewtrell, D. Lagunoff, and H. Metzger (Section of Chemical Immunology, Arthritis and Rheumatism Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland and Department of Pathology, St. Louis University, St. Louis, Mo., USA). *Biochim. biophys. Acta (Amst.) 644*, 363 (1981).

Previous experiments on the functional properties of rat basophilic leukaemia cells showed a major anomaly when compared to normal mast cells: though IgE-mediated secretion was dependent on external Ca^{2+} with both types of cells, substantial non-cytotoxic release with ionophore A23187 could be demonstrated with the normal cells but not with the tumour cells. It is shown that when the pH of the incubation medium is increased to 8 it is possible to obtain excellent Ca-dependent, non-cytotoxic secretion from tumour basophils with the ionophores A23187 and ionomycin. These results provide further evidence that secretion from the tumour cells occurs *via* a mechanism similar to that used by normal mast cells and basophils. Experiments with metabolically inhibited tumour cells suggest that their unusual sensitivity to the cytotoxic effects of Ca^{2+} ionophores may be related to their ability to sequester intracellular calcium. Changes in the conditions of cell culture appeared to produce substantial and at least partially reversible changes in responsiveness to IgE-mediated triggering and ionophores.

B. Sarkadi

A spectrin-dependent ATPase of the human erythrocyte membrane. G. S. Baskin and R. G. Langdon (Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, VA., USA). *J. biol. Chem. 256*, 5428 (1981).

Removal of spectrin from erythrocyte membranes results in the simultaneous loss a calcium-stimulated, magnesium-dependent ATPase with an apparent K_D for Ca^{2+} of 1 μM. This ATPase activity with high Ca^{2+} affinity is specifically reconstituted by addition of purified spectrin to spectrin-depleted membranes, and the reconstituted activity is directly proportional to the amount of spectrin that is reassociated with the membranes. Spectrin binding and activation of the high Ca^{2+} affinity Mg^{2+}-ATPase are proportionally inhibited by thermal denaturation, trypsin digestion, or treatment of the membranes with thiol-reactive reagents.

Binding of calmodulin to the Ca^{2+} pump ATPase requires that calmodulin contains bound Ca^{2+}. By contrast, spectrin binding to the erythrocyte membrane is Ca^{2+}-independent. Direct assay of calmodulin in purified spectrin and absence of chlorpromazine inhibition of reconstitution demonstrate that activation of the high Ca^{2+} affinity ATPase resulting from spectrin binding is not a result of contamination of spectrin by calmodulin. Additional evidence that the spectrin-activated ATPase is an entity separate and distinct from the Ca^{2+} pump is provided by other characteristics of the activation phenomenon.

It is suggested that spectrin constitutes part of an ATPase which may function as a component of the "cytoskeleton" controlling erythrocyte shape and membrane flexibility.

B. Sarkadi

INSTRUCTIONS TO CONTRIBUTORS

HAEMATOLOGIA is designed for the publication of original papers, preliminary reports, and reviews which contribute to the advancement in all fields related to haematology and blood transfusion. Manuscripts should be submitted in correct English and are accepted on the condition that they have not been published or accepted for publication elsewhere. Case reports, technical notes, and Letters to the Editor are also welcomed and will be published if appropriate.

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Non-A, Non-B Hepatitis: A Contemporary Assessment

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The current status of non-A, non-B (NANB) hepatitis is reviewed with respect to: (i) epidemiology and incidence of posttransfusion and sporadic NANB hepatitis; (ii) experimental transmission of NANB hepatitis; (iii) serologic tests and the evidence for virus-like NANB agents; (iv) clinical features of NANB hepatitis as compared to hepatitis B; (v) indication and impact of therapeutic and preventive measures against NANB hepatitis. Without specific markers for NANB infection, non-A, non-B hepatitis continues to be a diagnosis by exclusion of infection with hepatitis A, hepatitis B or other viruses. Recent observations suggest that NANB hepatitis may in fact be caused by a group of viruses immunologically different from but genetically similar to the hepatitis B virus.

Key words: chronic and acute hepatitis, commercial and volunteer donor blood, non-A, non-B hepatitis, posttransfusion hepatitis, prevention of non-A, non-B hepatitis

Introduction

Diseases of the liver and particularly jaundice have been described since ancient times, e. g. in the Babylonian Talmud (5th century B.C.) and in the writings of Hippocrates (late 5th century B.C.), and numerous epidemics of jaundice were recorded, especially during times of war [1]. The misconception that epidemic jaundice was caused by a swelling of the ostium of the common bile duct ('catarrhal jaundice') [2, 3] prevailed until the beginning of this century, when it was discarded in favor of a proposed viral etiology of hepatitis [4, 5]. It was not until the second World War, however, that the viral etiology of hepatitis was finally established by experimental transmission to human volunteers [6–10]. Based on the work of MacCallum et al. [8, 9], Havens et al. [10], and Krugman et al. [11], the concept of two types of viral hepatitis emerged:

(i) 'Infectious' hepatitis or hepatitis A with a brief incubation period of 15–45 days and transmission predominantly by the fecal-oral route, and

(ii) 'Serum' hepatitis or hepatitis B with a longer incubation period of 45 to 150 days and a predominantly parenteral mode of transmission through contaminated blood or instruments.

* Recipient of a Heisenberg Award from the Deutsche Forschungsgemeinschaft

Studies by Havens [12], Neefe, Gellis and Stokes [13] and Krugman et al. [11] demonstrated that infection with each agent conferred homologous immunity only, without evidence of cross immunity between the two types of hepatitis.

Despite the accumulated data on clinical, biochemical, histological and epidemiological characteristics, there were difficulties in categorizing individual cases as hepatitis A or B and in distinguishing them from toxic or drug-induced hepatocellular injury. These difficulties are reflected by a multitude of synonyms for viral hepatitis, e.g. infectious hepatitis, epidemic hepatitis, short-incubation hepatitis, 'jaunisse des camps', 'Soldaten Gelbsucht' for hepatitis A, and serum hepatitis, inoculation hepatitis, long-incubation hepatitis, 'hippy' hepatitis, 'tattoo' hepatitis for hepatitis B [14].

The discovery of the Australia antigen [15], its association with hepatitis B [16] and its identification as hepatitis B surface antigen (HBsAg) made it possible to specifically diagnose hepatitis B virus (HBV) infection. The detection of the hepatitis A virus (HAV) by immune electron microscopy [17] provided the basis for serologic identification of HAV infection. Thus, laboratory diagnosis led to specific recognition of two types of hepatitis caused by HAV or HBV. Hepatocellular injury, irrespective of the etiology, is generally defined by elevation of alanine aminotransferase (ALT, previously designated as serum glutamic pyruvic transaminase or SGPT) exceeding twice the upper limit of the normal range.

Non-A, Non-B Hepatitis: A Newly Emerged Clinical Entity

The classical subdivision of clinically indistinguishable viral hepatitis into two serologically and epidemiologically distinct entities, i.e. hepatitis A and B, failed, however, to account for a number of observations: (i) some patients had multiple attacks of acute hepatitis, suggesting the existence of more than two hepatitis viruses [18–20]; (ii) it was noted that some cases of hepatitis had an incubation period of 45 to 55 days, intermediate between that of type A and type B hepatitis, further suggesting the possibility of a third human hepatitis virus [21–23]; (iii) the antibody to HBsAg (anti-HBs), known to confer immunity against HBV infection, failed to prevent posttransfusion hepatitis [24], indicating that a non-B agent(s) serologically distinct from HBV was involved in transfusion associated hepatitis; (iv) most cases of posttransfusion hepatitis were epidemiologically and serologically unrelated to either HAV or HBV [25–33].

Apart from the hepatotropic HAV, HBV and the agent(s) of non-A, non-B hepatitis, which cause a primary infection of the liver, there are at least 24 other viruses associated with hepatitis as part of a more generalized systemic illness, e.g. Epstein–Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus, rubella virus, varicella-zoster virus, Coxsackie virus, yellow fever virus, Marburg virus, Lassa virus, Rift Valley fever virus, etc. [34]. The use of specific tests to identify these viruses has revealed that almost all cases of human hepatitis can be attributed to HAV, HBV, or to another agent(s) distinctly different from the afore-mentioned

viruses. From the evaluation of these findings the concept of non-A, non-B hepatitis has evolved. Hepatitis cases that are serologically unrelated to HAV and HBV or to other known viruses, and for which other causes of hepatocellular damage are clinically excluded, are currently referred to as non-A, non-B (NANB) hepatitis. A specific nomenclature must await further etiologic elucidation of NANB hepatitis, including definition of serologic markers and demonstration of the presumed causative virus(es).

Non-A, Non-B Hepatitis: A Dominant Cause of Posttransfusion Hepatitis

The existence of NANB hepatitis as a disease entity distinct from hepatitis A or B was mostly derived from studies of posttransfusion cases [21, 22, 25–33, 35]. To evaluate the incidence of posttransfusion hepatitis (PTH) and the factors influencing its occurrence, a large number of prospective studies have been carried out. These studies clearly defined three groups of blood donors who are associated with an increased likelihood of transmitting PTH: (i) commercial blood donors, (ii) donors positive for HBV markers (HBsAg or anti-HBc), and (iii) donors with an elevated serum alanine aminotransferase.

(i) *Commercial blood donors.* Blood from commercial donors carries a higher risk of transmitting hepatitis than blood from volunteer donors as first recognized by Allen et al. [36], and by Kunin [37]. The development of assays for HBsAg and their application provided the first direct evidence that commercial blood donors had a 3.5 times higher carrier rate for this HBV marker than volunteer donors [38]. Similarly, commercial donors were found to have an antibody prevalence (anti-HBs) three times as high as volunteer blood donors [39]. With increasingly sensitive assays for the markers of HBV infection it became evident that PTH was not caused by HBV alone and thus the existence of NANB hepatitis was strongly suggested. The relative risk of contracting NANB hepatitis following transfusion of commercial blood was found to be 3.3 times greater than the risk of developing hepatitis from volunteer blood, a figure virtually identical with the relative risk of contracting hepatitis B [26, 27, 40–42]. Based on an analysis of single-donor transfusions, a minimum carrier rate for NANB hepatitis of 1.6 per cent among volunteeer donors and of 5.4 per cent among commercial donors was calculated, indicating again that the risk of acquiring NANB hepatitis from commercial blood is 3.3 times greater than from volunteer blood [27].

(ii) *Blood donors positive for HBsAg or anti-HBc.* The risk of transmitting hepatitis from HBsAg positive blood is more than 4 times that from blood which is HBsAg negative [41, 43–48]. While transfusion of blood containing anti-HBs or anti-HBs and anti-HBc did not transmit hepatitis more often than blood which lacked both antibodies [26, 47–50, 53], blood containing anti-HBc alone carries a greater risk of leading to PTH than blood negative for anti-HBc [51–53]. Even though the identification of anti-HBc positive, anti-HBs negative donors could

further reduce HBV-related posttransfusion hepatitis, but not NANB hepatitis, the cost-effectiveness of this measure appears doubtful.

(iii) *Donors with an elevated serum alanine aminotransferase.* Recently, two prospective studies demonstrated that the incidence of PTH was directly related to the elevated ALT level in blood donors [32, 33], indicating that screening of donor blood for ALT level could reduce the incidence of NANB posttransfusion hepatitis.

As a result of excluding commercial donor blood and HBsAg positive donors, the incidence of PTH decreased after 1970 from 30–35 per cent to about 10 per cent, affecting all forms of PTH (icteric/anicteric) to virtually the same extent [25, 54]. Since commercial donors and HBsAg positive blood were excluded at the same time, it was impossible to assess the relative impact of each. The most significant determinant, however, in the observed reduction of PTH in the USA by about 70 per cent was the adoption of an all-volunteer donor system. HBsAg testing, on the other hand, reduced only the incidence of hepatitis B which accounted even prior to 1970 for only about 25 per cent of PTH [25]. ALT screening of donors has been estimated to prevent 20–30 per cent of the current incidence of PTH with a loss of 1.5 to 3 per cent of the donor population [32, 33].

The proportion of PTH cases classified as NANB in fourteen prospective studies is shown in Table 1 [27, 28, 30–33, 35, 42, 55–60]. While the overall incidence of hepatitis ranged from 4 to 13 per cent (for recipients of HBsAg negative blood from volunteer donors), there is little variation in the percentage classified as

Table 1

Incidence of posttransfusion hepatitis (PTH) in prospective studies

No. of subjects[a]	Donor blood[c]	Incidence of PTH, %	Type of PTH, % of total hepatitis			Reference
			A	B	NANB	
94	C, V	20		11	89	Gocke [55]
139	C, V	11		27	73	Hollinger et al. [56]
204	C, V	25		29	71[d]	Prince et al. [35]
105	V	9	0	11	89[d]	Alter et al. [28]
94[b]	C	14	0	8	92[d]	Knodell et al. [57]
2204	C, V	11	0	22	78	Seef et al. [27]
388	V	8		10	90[d]	Alter et al. [58]
969	C, V	13	0	3	97	Seef et al. [59]
595	C, V	13	0	13	87	Aach et al. [42]
1082	V	12	0	7	93	Tateda et al. [30]
380	V	4	0	0	100[d]	Katchaki et al. [31]
1528	V	11	0	9	91	Aach et al. [32]
283	V	13	0	3	97[d]	Alter et al. [33]
728	V	9	0	6	94[d]	Alter et al. [60]

[a] Recipients of HBsAg negative blood considered only
[b] Placebo subgroup considered only
[c] C = commercial donor; V = volunteer donor
[d] Hepatitis cases with rise in EBV or CMV antibody titer considered as NANB

NANB, ranging from 89 to 100 per cent. No case of posttransfusion hepatitis A was observed and only one case of transfusion transmitted EBV infection was reported [31]. An exceptional case of posttransfusion hepatitis A in two babies transfused with blood from a single donor, retrospectively proven to have IgM anti-HAV, was recently reported to the California State Department of Public Health (personal communication by Dr. S. Hoag). The role of CMV in posttransfusion hepatitis, however, is more difficult to evaluate. Even though serologic evidence of CMV infection was reported in a number of prospective studies [28, 31, 33, 57, 58, 60] and CMV seroconversion was associated with the development of PTH, an etiologic role of this virus in the pathogenesis of PTH could not be established. Except for neonates and patients with acquired or hereditary immune deficiency, transfusion related CMV infection with its mild and transient clinical course does not appear to be a clinically relevant entity [60].

Sporadic Non-A, Non-B Hepatitis

There is now increasing evidence that NANB hepatitis also occurs without prior blood transfusion indicating other modes of transmission of the disease. NANB hepatitis occurring in the absence of a well-defined epidemiologic setting accounts for 15 to 20 per cent of sporadic cases of hepatitis (Table 2) with, except for one study [120], little variation in the percentage of cases classified as NANB [61–69].

Table 2

Type of hepatitis in patients with symptomatic disease

No. of subjects	Type of hepatitis, % of total hepatitis			Reference
	A	B	NANB	
103			12	Villarejos et al. [61]
175		55		Hadziyannis [63]
845		55		Mueller [62]
417	23	54	23	Dienstag et al. [64]
329	17	64	19	Mueller et al. [67]
480	23	62	15	Norkrans [65]
148	27	57	16	Norkrans et al. [66]
73	37	49	14	Kryger et al. [68]
368	58	27	13	Farrow et al. [69]
1284			2	Villarejos and Visona [120]

Mode of Transmission of Non-A, Non-B Hepatitis

Although discussion of the mode of transmission of NANB hepatitis must be considered tentative until specific serologic markers are identified, it is probable that its transmission pattern will be similar to hepatitis B. Certainly, NANB hepa-

titis is prevalent following transfusion of blood or blood products, or other covert or overt percutaneous exposure such as in drug addicts [20, 70, 71], in hemodialysis patients [72–74], in renal transplant recipients [75–77], and in patients undergoing plasmapheresis [78, 79]. In non-transfused, hospitalized patients the overall incidence of nosocomial hepatitis was found to be 2.2 per cent: NANB hepatitis was contracted by 94 per cent of the patients while 6 per cent acquired hepatitis B [42]. This is in marked contrast to sporadic hepatitis cases (Table 2) where the incidence of hepatitis B is considerably higher than NANB hepatitis.

A person-to-person transmission of NANB hepatitis is suggested by secondary cases of NANB hepatitis among family contacts [61, 79] and the increasing frequency of NANB hepatitis in homosexual men [80]. Also maternal-fetal [81] and mother-infant transmission [82] of NANB hepatitis in women who had acute NANB hepatitis during the third trimester of pregnancy have been recorded. Finally, there is growing evidence for the existence of epidemics of NANB hepatitis, resembling HAV infection, i.e. transmission *via* contaminated water, especially in certain parts of India [83, 84].

Even though it is reasonable to conclude that the mode of transmission of NANB hepatitis resembles hepatitis B more closely than hepatitis A, an accurate estimate of the frequency of infection with these agents and their mode of spread will have to await the development of specific serologic tests for markers of NANB hepatitis.

Experimental Transmission of Non-A, Non-B Hepatitis

Although to date there are no confirmed serologic tests for NANB hepatitis [60], and no confirmed observations of a virus particle, the existence of NANB hepatitis as an infectious, transmissible disease seems well established. The data on NANB hepatitis following blood transfusion cited above have been complemented by conclusive experimental evidence of serial transmission of infection to volunteers and to chimpanzees.

In studies conducted in the early 1950's, sera from several asymptomatic blood donors, who were suspected of having transmitted hepatitis to transfusion recipients, were shown to be infectious when inoculated into human volunteers [85–87]. A reanalysis of serum samples from these studies identified three donors whose HBsAg negative serum induced NANB hepatitis in nine volunteers [88]. Transmission of NANB hepatitis was also shown in another volunteer study of malaria transmission by blood inoculation [89], demonstrating in addition serial passage of the illness.

Utilizing serum or plasma derived from patients with presumed non-A, non-B hepatitis, several groups of investigators have transmitted NANB hepatitis to chimpanzees [90–96]. The findings in these studies are very similar and can be summarized as follows:

(i) Acute and chronic phase sera from patients with acute NANB hepatitis were infectious.

(ii) Sera from patients with chronic NANB hepatitis were infectious.

(iii) The incubation period in chimpanzees was similar to that in humans, both short (2 to 6 weeks) and long (10 to 20 weeks) incubation periods being observed.

(iv) The illness was relatively mild, almost invariably anicteric and asymptomatic, and self-limited in most cases.

(v) The histologic changes on liver biopsy were typical of viral hepatitis, resembling those seen in chimpanzee hepatitis B. The degree of histologic change paralleled the extent of transaminase abnormality.

(vi) The transmissible agent could be filtered through a 220 nm filter consistent with its presumed viral nature [93]. Furthermore, it could be inactivated by formalin [97].

(vii) Chronicity and carrier state of NANB hepatitis were demonstrated by transmitting hepatitis to chimpanzees from asymptomatic patients and donors implicated in transmitting NANB hepatitis to human recipients as long as 1 to 5 years ago [88, 90, 91]. The frequency of this carrier state may be in the range of 1 to 2 per cent [54].

The transmission studies mentioned above document two important points in the epidemiology of NANB hepatitis: (i) NANB hepatitis is caused by a transmissible agent and blood-borne transmission is possible from human to human, from human to chimpanzee and from chimpanzee to chimpanzee. (ii) As demonstrated for hepatitis B, there is a chronic carrier state for NANB agent(s) with no demonstrable abnormalities of liver function as well as clinical evidence of chronic NANB induced liver disease.

Cross-challenge studies with different infectious human sera showed that after one NANB inoculum chimpanzees were protected from infection with two unrelated inocula, suggesting that a single agent was involved in NANB hepatitis [98]. However, there is now evidence from several sources pointing toward the existence of more than one NANB agent: (i) NANB hepatitis presents itself clinically with both long and short incubation periods. The most marked difference is found between PTH cases with a mean incubation period of 7 to 8 weeks [27, 35, 58, 99] and NANB hepatitis with an incubation period of 1 to 4 weeks after infusion of factor VIII concentrates or cryoprecipitates [100] and in a nosocomial outbreak [101]. Based on different incubation periods and clinical/biochemical presentation of the disease two [102] or even three types [30] of NANB hepatitis are suggested. (ii) Multiple attacks of NANB hepatitis have been reported in drug addicts [20, 70, 71], in hemophiliacs [19, 100] and in renal transplant recipients [75–77]. (iii) The most convincing evidence for the existence of two different NANB agents, however, is derived from cross-challenge studies in chimpanzees, demonstrating two types of NANB agents inducing two different types of NANB hepatitis [103–105]. (iv) Plasma from a patient with acute NANB hepatitis and from a patient with chronic NANB hepatitis, when inoculated into chimpanzees lead to different ultrastructural changes in liver biopsies [106], further suggesting the existence of two distinct etiological agents.

Non-A, Non-B Serologic Tests

A number of different serologic tests for NANB hepatitis have been reported [93, 102, 107–110]. All of these have, however, suffered from any or all of the following: lack of reproducibility, inability to be confirmed in other laboratories, inability to identify coded sera correctly, failure to demonstrate specificity for the infectious agent [111, 112]. A recently developed solid-phase radioimmunoassay employed in the testing of a NANB panel, however, holds out great hope that a specific test for NANB hepatitis will be available soon, even though the panel has not proved the specificity or established the efficacy of this test system [60]. Autoantibodies against polymerized human albumin (PHALB) have been described as a nonspecific marker of liver disease [113]. Using a solid-phase radioimmunoassay [114], anti-PHALB was detected in 40 per cent of patients with acute NANB hepatitis and in 55 per cent of patients with chronic NANB hepatitis (unpublished observations), similar to recent data obtained using a hemagglutination assay [115]. After the specific serologic exclusion of HAV or HBV infection, anti-PHALB may thus provide an interim nonspecific marker, useful in the differential diagnosis of NANB hepatitis. The establishment of a specific test for NANB hepatitis, however, remains a matter of highest priority.

Virus-like Particles in Serum and Ultrastructural Characteristics in Liver Biopsies

The identification of virus particles distinct from HAV and HBV and specific for NANB hepatitis is hampered by the failure to detect an antibody suitable for immune electron microscopy, i.e. an antibody proven by other methods to be specific for the NANB agent(s). Virus-like particles associated with NANB hepatitis of humans and chimpanzees were demonstrated by (immune) electron microscopy in plasma/serum, in hepatocyte nuclei and in hepatocyte cytoplasm as shown in Table 3 [94, 105, 106, 116–126]. Even though there is strikingly little variation in the morphology of these virus-like particles associated with NANB hepatitis, compatible with the assumption that they indeed represent the NANB agent(s), the interpretation of these findings must await the specific identification of components of the presumed virus(es).

Liver biopsies obtained during acute or chronic NANB hepatitis in humans and chimpanzees display a number of ultrastructural cytoplasmic changes [94, 105, 106, 117, 118, 121, 123, 126–128]. These ultrastructural changes consist of cytoplasmic tubular abnormalities which mainly involve the smooth endoplasmic reticulum, even though the rough endoplasmic reticulum is also affected. Somewhat similar endoplasmic reticulum membrane alterations occur in early differentiating fetal lung cells [129] and have been observed in vascular endothelial cells of kidney and skin and in circulating lymphocytes of patients with systemic lupus erythematosus and systemic sclerosis and also in muscle cells in polymyositis [124]. Obvi-

Table 3

Virus-like particles in non-A, non-B hepatitis

Localization	Diameter nm	Buoyant density g/cm³	Reference
Plasma/serum	60 (40 nm inner core)		Coursaget et al. [116]
	27	1.31	Bradley et al. [94]
	32	1.30	Mori et al. [117]
	27	1.28	Yoshizawa et al. [118]
	15–25 (spheres and filaments)		Hantz et al. [119]
	35–40 (double-shelled particles)		Hantz et al. [119]
	27/25 (NANB 1/NANB 2)		Yoshizawa et al. [105]
	38–40 (double-shelled particles)		Villarejos and Visona [120]
Hepatocyte nucleus	20–27		Shimizu et al. [106]
	15–27		Tsiquaye et al. [121]
	25–30		Hantz et al. [119]
	27		Gmelin et al. [122]
	20		Burk et al. [123]
	15–20		Tsiquaye et al. [124]
	15–27		Busachi et al. [125]
	20–27		De Wolf-Peeters et al. [126]
Hepatocyte cytoplasm	37		Burk et al. [123]

ously, the virus specificity of such cytoplasmic changes remains to be determined and more information is needed on the immunologic and biochemical properties of these membranous structures, as compared to normal endoplasmic reticulum. In addition, immunohistochemical confirmation is essential to lend meaning to these histologic observations.

Clinical Characteristics of Non-A, Non-B Hepatitis

Even though NANB hepatitis is a relatively recently recognized entity, its clinical pattern displays several remarkable features.

(i) *Incubation period.* The average incubation period of NANB hepatitis following blood transfusion is about 8 weeks (5 to 10 weeks) as compared to about 11 weeks for hepatitis B (Table 4) [27, 28, 35, 42, 55, 58–60, 99]. In about 10 per cent of patients, however, the incubation period may be as short as 1 to 2 weeks, especially after administration of factor VIII to hemophiliacs [100, 130], and after percutaneous inoculation with contaminated blood products [88, 89], or as long as 26 weeks. In general, however, the incubation period of NANB hepatitis describes a sharp unimodal curve with a peak at about 8 weeks after exposure.

Table 4

Incubation period of posttransfusion hepatitis (PTH)

Hepatitis B		NANB hepatitis		References
No. of subjects	Mean incubation period, weeks	No. of subjects	Mean incubation period, weeks	
21	9.0	17	6.4	Gocke [55]
15	10.4	36	8.0	Prince et al. [35]
4	14.5	8	9.4	Alter et al. [28]
18	14.4	22	7.3	Purcell et al. [99]
52	9.4	189	7.2	Seef et al. [27]
3	16.6	26	8.2	Alter et al. [58]
4	11.8	119	8.4	Seef et al. [59]
10	10.3	65	6.3	Aach et al. [42]
4	17.5	45	10.5	Alter et al. [60]
Weighted mean incubation period[a]	10.8		7.8	

[a] Sum of number of subjects × mean incubation period, divided by the sum of subjects

(ii) *Biochemical characteristics of acute NANB hepatitis.* The biochemical characteristics of PTH (Table 5) indicate that acute NANB hepatitis is in general less severe than hepatitis B with lower peak ALT activities, lower peak bilirubin levels and a higher proportion of asymptomatic/anicteric cases [27, 28, 35, 42, 55, 58–60, 99]. Although NANB hepatitis is usually a mild subclinical illness, 40–50 per cent of fulminant viral hepatitis cases are attributed to this agent(s) (Table 6)

Table 5

Biochemical characteristics of acute posttransfusion hepatitis (PTH)

Hepatitis B				NANB hepatitis				Reference
No. of subjects	Average max. ALT level U/l	Average max. bilirubin mg/100 ml	Icteric patients, %	No. of subjects	Average max. ALT level, U/l	Average max. bilirubin, mg/100 ml	Icteric patients, %	
21	1159	9.0	80	17	414	1.7	12	Gocke [55]
15	318		47	36	259		37	Prince et al. [35]
4	857	7.8	75	8	470	1.3	12	Alter et al. [28]
18	768		63	22	395		27	Purcell et al. [99]
52	448		28	189	311		19	Seef et al. [27]
3	1168	7.3	100	26	744	2.8	31	Alter et al. [58]
4	395		0	119	286		12	Seef et al. [59]
10	625		20	65	647		25	Aach et al. [42]
4	1050		75	45	655		24	Alter et al. [60]

Table 6

Etiology of fulminant viral hepatitis

No. of subjects[a]	Type of hepatitis, % of total hepatitis			Reference
	A	B	NANB	
71	8	41	51	Rakela et al. [131]
173	2	61	37	Acute Hepatic Failure Study Group [132]
21	19	43	38	Mathiesen et al. [133]
63	30	27	43	Wyke and Williams [134]
Mortality, %	66–75	66	87–100	Acute Hepatic Failure Study Group [132] Mathiesen et al. [133]

[a] Patients with evidence for both hepatitis A and B excluded

[131–134] with a mortality rate of 87–100 per cent, exceeding that of fulminant hepatitis A or B [132, 133].

(iii) *Fluctuation of enzyme levels.* Another clinical characteristic of NANB hepatitis is the tendency for ALT/AST levels to fluctuate markedly over relatively short time intervals and to remain abnormal for prolonged periods of time. These recurring enzyme elevations over six months and longer are typical of NANB hepatitis and are rarely, if ever, seen in other types of viral hepatitis. Due to these fluctuating enzyme patterns, the biochemical resolution of the disease is difficult to assess. Arbitrarily, a minimal criterion for resolution should be normal ALT/AST levels in at least six consecutive monthly serum samples. It should be noted, however, that even normal ALT levels do not preclude persistence of NANB hepatitis [135].

(iv) *Extrahepatic manifestations.* Unlike in hepatitis B, extrahepatic manifestations such as urticaria, purpura, arthritis or arthralgia are rarely, if ever, seen in acute NANB hepatitis despite the frequency of immune complexes in this disease [136]. Also antimitochondrial, antinuclear or antibodies against smooth muscle are usually not detectable.

(v) *Chronic sequelae.* Chronic viral hepatitis, defined as continuous or recurrent inflammation of the liver over at least 3 to 6 months [137, 138] without lasting clinical or biochemical improvement, has been observed in 6 to 82 per cent of patients with NANB hepatitis [26, 27, 42, 58, 60, 61, 65, 67, 72–74, 77, 89, 99, 139–143]. Two important factors predictive of a high frequency of chronicity emerge from these studies: (a) The incidence of chronicity is significantly higher following blood transfusions (40–60 per cent) [141–143], among drug addicts (58 per cent) [142] or among renal transplant recipients (82 per cent) [77] than in cases of sporadic NANB hepatitis (9–20 per cent) [61, 65, 142]. (b) Patients with peak ALT levels during the acute phase of NANB hepatitis of less than 300 U/l were less likely to develop chronic hepatitis (11–27 per cent) as compared to patients with peak ALT

levels greater than 300 U/l (70–88 per cent) [60, 140]. The presence of icterus during acute illness, on the other hand, does not consistently appear to be of predictive value [60, 141–144].

The histologic features of chronic NANB hepatitis following blood transfusions are chronic active hepatitis (CAH) in 61 per cent and chronic persistent hepatitis (CPH) in 17 per cent of the patients. Evidence of cirrhosis has been observed in 0 to 36 per cent of patients with CAH in the transfusion setting [60, 139, 141–144]. Again, the histologic alterations tend to be more severe in chronic NANB hepatitis following transfusion as compared to chronic NANB hepatitis in non-transfused patients with a 72 per cent incidence of CPH [71, 72, 142].

While NANB hepatitis is usually asymptomatic, biochemical and histologic evidence of chronic hepatitis do occur in 6 to 82 per cent of patients depending on the epidemiological background (see above). It is probable, however, that the majority of cases of non-A, non-B CAH is mild and relatively benign with ultimate complete resolution. However, CAH has been observed to progress to cirrhosis. If it does so, it represents the most severe outcome of this disease, in general evolving over many years. Recent findings, however, suggest that cirrhosis of the liver can develop in the posttransfusion setting in a relatively short period of time (mean period 2.6 years, range from 6 months to 11 years), particularly in an elderly population [144].

Treatment and Prevention of Non-A, Non-B Hepatitis

Treatment of acute non-A, non-B hepatitis should follow the guiding principles applied to the management of acute viral hepatitis A or B. Hospitalization is indicated during the early icteric phase, when symptoms are severe (e.g. vomiting) and when there is evidence of serious illness, particularly fulminant hepatitis. There is some evidence for the value of bed rest during the acute phase of viral hepatitis and a daily intake of at least 2000 calories should be maintained. Corticosteroids, however, should not be given [145]. Since most patients with acute NANB hepatitis are asymptomatic or only mildly symptomatic, there is in general no need for hospital management. If fulminant hepatitis evolves, intensive care with monitoring and support of vital functions is required. A variety of measures designed to support the patient in acute liver failure have been applied (e. g. exchange transfusions, plasmapheresis, charcoal hemoperfusion, polyacrylonitrile-membrane hemodialysis, cross-perfusion with a human volunteer or primate, extracorporeal perfusion through a mammalian liver). Unfortunately, except for very few cases, these measures and combinations thereof, could not significantly reduce the very high mortality of fulminant NANB hepatitis [132–134].

Considering the asymptomatic clinical course of chronic NANB hepatitis in most patients, i. e. CPH and asymptomatic CAH, there is in general no need for drug therapy. However, in symptomatic patients with severe CAH (i.e. histologic evidence of severe disease, AST level 5 to 10 fold elevated, gamma globulin level

more than 2 fold elevated over at least three months) corticosteroid therapy alone or in combination with azathioprine was shown to improve certain biochemical abnormalities (serum bilirubin, globulin and albumin levels), to control complications from portal hypertension and to increase immediate life-expectancy [146]. By contrast, more recent studies on HBsAg positive CAH have shown no apparent benefit [147] or even a detrimental effect of steroids [148], especially in HBeAg positive patients [149], raising the possibility that no form of truly chronic viral hepatitis is improved by steroid therapy. The testing of this hypothesis, however, must await the identification of the NANB agent(s) and the results of further controlled trials. While chronic hepatitis B seems to improve upon antiviral therapy (interferon and/or adenine arabinoside), with a concomitant loss of infectivity [150], similar therapeutic trials in NANB hepatitis must await the specific serologic detection of markers of NANB virus(es).

A number of measures to prevent posttransfusion hepatitis, such as pre- or posttransfusional administration of globulin preparations, transfusion of packed red cells, frozen red cells or frozen deglycerolized red cells are not convincingly effective in reducing the incidence of PTH [54]. Similarly, studies designed to determine the preventive effect of lysozyme [151] and of vitamin C [152] on PTH showed no benefit from these two compounds.

As mentioned above, the measures conclusively established to reduce the risk of transmitting viral hepatitis following blood transfusions are: (i) exclusion of commercial blood donors, (ii) exclusion of HBsAg (and anti-HBc) positive donors, and (iii) exclusion of donors with persistently elevated serum ALT levels. The development of a specific and practical test for the detection of the NANB agent(s), however, is of highest priority to effectively screen for NANB virus carriers.

Conclusions and Perspectives

(i) NANB hepatitis is a newly emerged clinical entity accounting for more than 90 per cent of cases of posttransfusion hepatitis and up to 20 per cent of cases of sporadic hepatitis. NANB hepatitis displays distinct clinical features (incubation period, biochemical characteristics) and is in general only mildly symptomatic or asymptomatic. The ALT/AST levels, however, tend to fluctuate markedly and to remain abnormal for prolonged periods of time. Depending on the epidemiologic background, there is a high incidence of transition from acute hepatitis to chronic forms of this disease, including the development of liver cirrhosis.

(ii) NANB hepatitis is predominantly transmitted by overt or covert percutaneous exposure (transfusion of blood or blood components, drug addicts, hemodialysis patients, renal transplant recipients, patients undergoing plasmapheresis). However, a person-to-person transmission including maternal-fetal and mother-infant is also suggested.

(iii) NANB hepatitis is caused by one or more virus-like agents based on experimental transmission and serial passage in human volunteers and chimpanzees.

(iv) Effective measures to reduce the incidence of PTH are exclusion of commercial blood donors, HBsAg positive persons and individuals with persistently elevated ALT levels.

(v) In view of the epidemiologic and clinical similarities between NANB hepatitis and hepatitis B and based on recent findings that liver biopsy specimens of patients with NANB hepatitis do contain HBV DNA [153], one might hypothesize that the NANB agent(s) are similar to HBV and have indeed arisen from point mutations in the genes coding for characteristic biologic properties of HBV [154].

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Administrative Aspects of the Preparation and Issue of Blood and its Components

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For administrative and medico-legal reasons it is essential to maintain accurate records of blood donors, blood donations and the preparation of components of a blood donation. In smaller blood banks this can be done by keeping simple ledgers and in larger blood banks by punched cards. In very large centres computers are essential and data capture is best achieved by the use of laser or light pens to transmit information to computers. International standardization is desirable so that international transfers of blood or its components would not present any problem to the receiving blood bank. In the USA and Europe the CODABAR method is the most popular with some support for OCR-B. The fundamentals of the application of a bar-coded data capture system are stated and discussed.

Key words: allocation, bar-coding, blood bank record, check digit, labelling

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In the days when only whole blood and plasma were supplied to hospitals by transfusion centres the keeping of records of issues was a very simple matter. In the present era of blood cell components and plasma fractions, the maintenance of records is much more complicated.

It is important to keep accurate records, not only for the day to day control of issues of blood or its products, and for the preparation of statistical returns (e.g. monthly, quarterly and annual figures), but it is also essential, in certain situations, that a transfusion centre should be quickly able to trace the distribution of blood components. For example, if a recently bled donor reports that he has developed hepatitis, it is obviously necessary, for administrative and medico-legal reasons, quickly to trace the various destinations of his blood products so that all concerned are promptly informed; and the components, if not already used, returned to the transfusion centre for destruction. If they have been used, the patient is entitled to immediate treatment with immunoglobulin, preferably specific anti-HBs immunoglobulin, particularly if the donor, who should be immediately retested by a specialist laboratory, is positive for HBs.

Above all, the accuracy of blood bank records is fundamental to the avoidance of transfusing a patient with incompatible blood.

The procedures to be adopted will of course depend on the size of the transfusion service and the availability of funds. Inexpensive methods such as the main-

tenance of several ledgers with suitable cross-references are now out-moded because of the greatly increased work-load in most transfusion centres. Similarly, systems using punched cards are currently inadequate in very large centres, in which the use of computers has become inescapable.

I shall briefly describe how computers are applied to this problem in Britain, and, in particular, in the Birmingham Centre. The various relevant sections are:

1. Blood donor organisation.
2. Labelling of blood and its products.
3. Automated blood grouping.
4. Control of issues.

In Birmingham there are also schemes for the Antenatal Serology Service and for Organ Transplantation. It is possible to link and co-ordinate all our computer schemes.

The Blood Donor Organisation

In Birmingham all donors are on the computer. Calling letters are prepared by the computer which also prepares a session slip for each donor. Medical notes and other relevant observations are made on the slip at the session. The session slip itself is used also to record the results of laboratory tests. A complete and comprehensive administrative and laboratory record of every donor is held on computer file and regularly updated. The computer also prepares lists of donors who are suspended for various reasons, donors due for awards or retirement, and provides statistical information for administrative reports.

Labelling of Blood and its Products

Preferably there should be an internationally agreed system for labelling blood and its products. The achievement of uniformity is being actively sought in North America and in several European countries.

The shape, size, colour and layout of the various labels is a matter for agreement. I do not intend to discuss these aspects in great detail, but I shall devote some time to the important matter of donor identification, donation identification, and blood product identification, with particular reference to bar-coded systems of labelling.

Bar-Coding

In Britain and the USA the CODABAR system has found favour, whereas in Finland and Sweden the OCR-B system is preferred. My comments are confined to CODABAR.

The donation identification number is of fundamental importance. In some

countries it is the individual's national number. It is the link between the blood donor, the donation, the products obtained from the donation and the recipient or recipients. In the CODABAR system the donation number is printed in both bar-coded and eye-readable forms. In the OCR-B system the same number is readable both by eye and by automated equipment.

The introduction of bar-coding, for example, requires a fast method of capturing information. Bar-codes are read by automatic blood grouping machines by means of a laser scanner, and from blood or blood product labels by means of a light pen. The information is transmitted to the computer.

Bar-coding is not new. It has been used by the larger supermarkets for some years. The bar-code consists of different lines and spaces (both thick and thin) arranged in different ways to represent different characters. The American Blood Commission symbol is recommended. This is a linear bar-code which consists of four bars and three spaces, the bars and spaces are either wide or narrow. Each set of seven wide and narrow bars and spaces represents one character. The wide bar or space represents a binary 1 and the narrow bar a binary 0. A '7-bit' pattern of zeros and ones represents each number.

The ABC character symbol set consists of 20 characters: the figures 0 to 9, special characters, i.e. : (colon), + (plus), $ (dollar), − (minus), . (full stop), and / (slash, stroke, oblique), and start or stop codes, a, b, c, d.

When an item of information is recorded it must be preceded by a start code and ended by a stop code. These codes are not part of the eye-readable information.

There is an extremely remote possibility (1 in 10^6) that a light pen may make an error by the substitution of one number for another. To prevent this from happening a check digit is necessary. The introduction of a check digit will reduce the possibility of substitution to 1 in 10^9.

Check Digit

Calculation of check digit. The International Standards Organisation (ISO) modulus 11-2 check digit is recommended. The method of calculation is as follows:

'Weighting factors' 765432

1. Multiply each digit of donation number by the corresponding weighting numbers.
2. Sum the products from step 1.
3. Divide the sum by 11.
4. Subtract the remainder from 11.

An example is given below:

Donation number = 2 2 8 9 5 7
Weighting factor = 7 6 5 4 3 2
Products = 14 12 40 36 15 14
Sum of products = 131
Divide by 11 = 11 remainder 10

Subtract remainder from 11 = 1

The check digit is therefore 1. If the check digit is 10, it is written as X.

The full donation number for the Birmingham Centre would therefore be: 228957H1. H is the code for the Birmingham Centre, and 1 is the check digit.

Standardization of Labels

Blood pack labels should preferably be standardized in accordance with the recommendations of the American Blood Commission (ABC) Committee for Commonality in Blood Banking Automation (CCBBA). Similarly, blood group labels should be standardized in respect of both format and colour. Colour is a problem, because different countries use different colours. It is recommended that colours should be given up for some years, and, if necessary, be later re-introduced on a uniform international basis.

Besides the donation number, the blood group label of the primary pack should be overstuck with the date of collection, and with the blood groups (ABO and Rhesus positive or negative), and with information on type of pack and the anticoagulant used, as well as information such as 'whole blood' or 'whole blood, platelets removed' or 'whole blood, cryoprecipitate removed' or 'red blood cells' or 'red blood cells, washed', etc. All this information can be presented in both bar-coded and eye-readable forms.

Satellite packs should be labelled with the name of the component e.g. platelet concentrate, cryoprecipitate, the method of preparation e.g. plasmapheresis, on the lines mentioned above. The volume of the donation or component should be stated on the primary or the satellite pack.

Ideally, bar-coded information should also be given on plasma fractionation products derived from pools of plasma e.g. anti-tetanus immunoglobulin, anti-D immunoglobulin, anti-HBs immunoglobulin, etc.

Blood Group Labels

Rh-positive labels are printed in black, and Rh-negative labels in white against a red background underneath which is the statement 'tested with anti-D, anti-C and anti-E'.

Code assignments for blood group labels are as under:

Group	Bar-code
O, Rhesus positive	51
A, Rhesus positive	62
B, Rhesus positive	73
AB, Rhesus positive	84
O, Rhesus negative	95
A, Rhesus negative	06

B, Rhesus negative	17
AB, Rhesus negative	28
O	55
A	66
B	77
AB	88

These bar-codes have d as a start and 0b as a stop code.

Similarly code assignments should be standardized for products, e.g. whole blood, red cells; for components, e.g. cryoprecipitate, platelets; for anticoagulants, e.g. ACD, CPD; for the package, e.g. plastic, double; for the method of preparation, e.g. pooled from many donors; and for volumes.

In order to cope with the large workload and to eliminate clerical and related errors, bar-coded labels (CODABAR) were introduced into the Birmingham Transfusion Service. All labels in current use carry bar-coded and eye-readable information. One or two Visual Display Units (VDU) and light-pens are located in each laboratory.

Our sessional Donor Record consists of a single computer document to which serial numbers are attached and, when the blood group is known, a blood group label. A document known as the Session Count, which shows a breakdown of the number of donors bled for each ABO and Rhesus group, is returned from each session. The session documents are manually sorted into a folder in numerical order. The system is activated by raising a file on the computer on the basis of date bled and the code for a particular session. The serial number and, when known, blood group relevant to this document is then read into the computer. A programme confirms that the session documents have been sorted in correct numerical sequence, and a hard copy Session Analysis is produced and checked against the Session Count to ensure that all documents have been returned to the laboratory. When the ABO and Rhesus groups of new donors have been determined the results are put on file.

Labelling of New Donor Units

The serial number of the unit is read into the computer and if the unit is ready for labelling the appropriate ABO and Rhesus group is displayed, and the unit labelled accordingly.

Production of Components

A file is raised as above and serial numbers are entered. In addition, components produced are read into the computer from bar-coded 'menu' cards (these are produced by attaching selected bar-coded labels to a single firm backing sheet from which they can be read into the computer as required). For pooled products batch numbers are allocated. If, as the result of component production, the red cells are left 'plasma-reduced' this will also be recorded at this stage.

Allocation of Blood

The serial number and blood group labels are read into the computer and checked against information on file. Instructions for allocation of units are then displayed. For example:

1. General issue.
2. Plasma production only.
3. Further investigation required.

Issue to Hospitals

Information related to each unit is read into the computer *via* (i) serial number, (ii) blood group label, (iii) whole blood/plasma reduced/anticoagulant label, (iv) component label. If information on a unit does not agree with that on file an appropriate message is displayed and no further issue can take place until the unit has been withdrawn for investigation.

The computer prepares, as required, a list of issues to a particular hospital. The list is filed at the hospital and replaces the hospital register of receipts. It is hoped that hospitals will adopt the use of light-pens and computer terminals to improve the efficiency of their blood bank administration.

The International Society of Blood Transfusion is understandably very interested in these matters and keeps in close touch with the CCBBA. It has recently produced a special edition of *Vox Sanguinis* on 'Automation and Data Processing' [1]. Because of the possibility of international exchange of rare bloods, country identification numbers are required. In the United Kingdom they are:

England	70
Scotland	71
Northern Ireland	72
Wales	73

Any country wishing to automate on these lines should write to the Secretary of the ISBT for the allocation of a national number, and for the allocation of all the other codes required.

I have attempted briefly to state the principles on which the application of bar-code procedures are based.

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Cryopreservation of Human Platelets. General Protocols, Development of Freezing Techniques and Clinical Applications

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A review is presented of the recent advances in cryopreservation of human platelets with particular reference to the technical protocol reported and its development, using either DMSO, glycerol or HES as additives. The methods for assaying viability of frozen-thawed platelets are described and the results are reported according to the cryoprotector used. The clinical applications of frozen platelets are discussed with reference to the clinical experience with DMSO and glycerol. An organization scheme for a frozen platelet bank is proposed.

Key words: cryoprotector, platelet freezing, platelet transfusion

Introduction

The preservation of platelets by freezing has been one of the main points of applied research in blood transfusion in the past ten years. Numerous publications on this subject bear witness to the desire of the laboratories concerned to be able one day to propose a relatively simple technique which will above all be reproducible from one centre to another [14, 26, 36, 45, 58, 59, 69].

Ever since the original work of Cohen and Gardner [9, 10] using glycerol as the cryoprotector of platelets, we have been faced with a great diversity in the methods proposed. Glycerol and dimethylsulfoxide (DMSO) [60] have been retained among the numerous cryoprotectors tested. But technical variations have been proposed for each one [3, 15, 16, 28, 39, 61, 66, 71]. They are principally concerned with the dilution medium, the concentration of the cryoprotector, the freezing rate, the storage temperature, the thawing temperature and the washing step. Each laboratory appears to be attached to its 'personal recipe' and this is most often difficult to reproduce elsewhere. So as to better understand the meaning of these divergent techniques, the American National Red Cross organized a 'Platelet Cryopreservation Conference' in October, 1978, which was run by H. T. Meryman. The unpublished expectations of this Congress gave weight to the fact that 'the DMSO protocol reviewed by the participants, represents a consensus agreed to by all. If preliminary results are confirmed, platelets frozen with glycerol should be equal to those frozen in DMSO. However, a clinical evaluation of the glycerol method must be done'.

In 1981, these conclusions are still topical. The cryopreservation of platelets with DMSO is well known and considered to be technically feasible for use in blood centres.

The protocol for the use of glycerol is not yet well standardized. The dilution medium and the cryoprotector concentration cause fundamental differences [15, 28].

In reality, DMSO is the cryoprotector used most frequently. It permits storage at $-80\,^{\circ}C$ and so a reduction in the cost of preserving the platelets. The choice of glycerol is most often linked to the fact that it is not useful to wash the platelets between thawing and transfusion. This technical simplification is very important in the emergency service of a blood centre.

Recent work has brought forth some interesting results with hydroxyethyl starch (HES). This cryoprotector, either alone or in association, could perhaps find its place in platelet cryobiology [8, 60].

A laboratory which would like to apply some method of platelet freezing will encounter many problems in the choice and the exact reproduction of a protocol. There exists a mass of sometimes contradictory information and functional explorations of the platelet are not carried out under identical conditions. It is very difficult for laboratories to master identically all the parameters which interfere before freezing, after thawing and in each laboratory test.

And yet we consider it useful to make a synthesis of the main works concerning the freezing of platelets. We shall show how, starting from an initial protocol, ideas can evolve which make the protocol increasingly simple, and reproducibility can decrease its cost. The principal steps will be discussed in detail. For the interpretation of laboratory tests we must not forget that each handled platelet is already activated.

Preservation of Platelets: General Observations

A) *Theory of freezing injury*

The phenomena which occur during the freezing of platelets are not well interpreted yet, due to a lack of basic cryobiological knowledge of factors related to the freeze-thaw survival of platelets. It seems useful to recall the principal theory of freezing injury. Mazur [40, 41] has shown that the optimum cooling rate varies widely from one cell type to the other. At present it is well known that very slow rates of cooling are destructive to living cells. Intracellular ice occurs when insufficient time is available for water to leave the cell to contribute to extracellular ice [17]. This critical cooling rate is related to intracellular viscosity and to the permeability to water of the membrane. At excessive cooling rates only extracellular ice occurs and this concentrates the extracellular solution. This phenomenon has been called 'solution effects' by Mazur [40]. According to Meryman [43], cell damage is associated with the cell dehydration and volume reduction that occurs in response to the increased extracellular osmolarity.

Cell dehydration creates compressive stress and membrane constituents may be lost, thus altering the cell's permeability and integrity. According to this hypothesis, freezing injury would be an essentially physical rather than biochemical event. At slow rates of cooling the cell is dehydrated beyond a tolerable extent. As the cooling rate is increased, less time is available for water to diffuse from the cell and excess dehydration may be avoided. With an increase in cooling rate, however, the cell interior may remain so diluted that intracellular ice is precipitated. Farrant and Morris [19] have suggested that the cell injury that occurs during rewarming is primarily osmotic in nature.

Recently some advances have been made towards a better understanding of the platelet's freezing injury. Rowe et al. [52] have postulated that loss of platelet function after freezing may be due to damage to lysosomes resulting in the release of hydrolytic lysosomal enzymes into the platelet cytoplasm.

Some questions remain: what are the origins and mechanisms of freeze resistance? Is thawing a lethal process rather than freezing? What is the difference between chill and freeze injury?

B) *Cryoprotection* [42]

The cryoprotective agents which have a high solubility in water must be nontoxic at high molar concentration both to the platelets and the recipient. They must penetrate the cell and cause minimal osmotic damage during thawing and washing [51]. Figure 1 shows the osmolarity values of DMSO and glycerol diluted in plasma.

The cryoprotection concept is based on the minimization of intracellular freezing and the reduction of damage to the cell from the environment of concen-

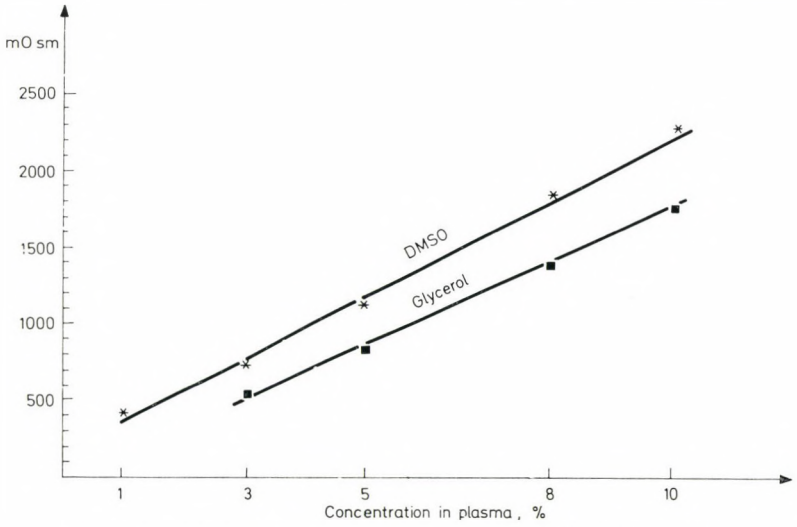

Fig. 1. Osmolarity values of DMSO and glycerol diluted in plasma

trated solutes. Cryoprotective agents may work by allowing the shrinkage required to minimize intracellular ice formation. They avoid exposure to high concentrations of other solutes (e.g. salts).

Glycerol is the best known and the least toxic of the colligative cryoprotectants [67]. The low concentrations of glycerol needed to freeze platelets are very attractive since cryopreserved platelets can be used for transfusion with no post-thaw processing. High concentrations of glycerol cause cell swelling and ultrastructural alteration [2].

DMSO is effective at low concentrations but its platelet toxicity increases with the concentration. Aggregation and prostaglandin synthesis are inhibited even by a low level of DMSO. The inhibition is reversed on washing the platelets [56]. A critical factor for the use of DMSO is its systemic toxicity, not the effect on platelets [75].

Dayian et al. [14] have related the differences in human platelet permeability to glycerol and DMSO. There is a partial barrier to the entry of glycerol into platelets and this may account for the difficulties other investigators have experienced using glycerol as the cryoprotective additive.

Polymers such as HES are non-penetrating and they cannot function colligatively. HES possesses the property of being able to prevent the loss of cell membrane material, to stabilize macromolecules and to reduce the rate of membrane degradation during hyperosmotic stress.

It is necessary to define the procedure for the addition of the cryoprotective agent before freezing and its removal after thawing. The optimum cooling for platelets frozen with DMSO is between 1 and 2 °C/min, whereas with a comparable concentration of glycerol the optimum rate is reported to be 30 °C/min. In the context of Meryman's model for freezing injury, this would suggest that DMSO reduces the permeability to water of the platelets.

C) *Methods for assaying viability of frozen-thawed platelets*

The problem of assaying platelet preservation by freezing depends on the ability to measure the function and viability of frozen-thawed cells. Our objective is to briefly describe the most commonly used methods of assessing platelet viability. The detailed methods for carrying out each of the studies will be found in the referenced literature. Unfortunately, no single, simple test is sufficient to determine the nature of the lesion observed in frozen and thawed platelets.

The *in vitro* tests of platelet viability measure various stages of platelet functions operative in haemostasis. It needs to be emphasized that the platelets prepared for transfusion, being activated by the isolation procedure, are different from the platelets prepared in a haemostasis laboratory. We must dispose of a panel of laboratory tests clearly defined by the laboratory concerned in order to evaluate a freezing method [5, 20]. It is important to choose tests which can allow to foresee the *in vivo* activity after transfusion.

In vitro assay of platelets

Morphology. Nomarsky's phase contrast observation allows only for one analysis. We can propose a classification of these cytomorphological changes (Table 1).

Table 1

Classification of morphological alterations of platelets after freeze-thaw procedure (according to Potron et al., unpublished)

Name	Morphological aspect	Function
Discs	Regular membrane, diffuse granules	Normal
Spheres	Loss of lenticular form	Subnormal
Dendrites	Beginning of activation	Variable
Balloons	Spherical increased diameter	Disturbed
'Capping'	In general ballooned, granulations off centre set at periphery	Impaired
No granules	In general ballooned, devoid of granules	Nought

Electron microscopy [2, 11, 63, 74] cannot be used routinely, but allows an analysis of the internal structure.

Size distribution [20]. It is obtained by use of a Coulter Counter Model ZBI and size distribution analyser model channelyser (Coultronics). The mean cell volume of platelets can be compared before and after the addition of the cryoprotector and the freeze-thaw procedure.

Mepacrine test (number of granules by cell) [38]. The dense granules can be counted easily by their fluorescence after the incorporation of mepacrine.

Adhesion test [5, 24]. A number of tests have been devised to measure platelet adhesion. These include immersion of glass slides and coverslips in platelet-rich plasma; and passage of blood through columns filled with small glass beads, a test that is difficult to standardize.

Aggregation test with ADP, collagen and adrenaline [4, 6, 13, 29, 32, 50]. This classical method needs a rigorous technology and creates numerous problems of quantitative interpretation. At an acid pH, aggregation is inhibited. We must take the generally used ACD into account at the preparation of the platelet concentrate. After thawing, the platelets might not respond to a single stimulant but only to an association of two aggregants such as adrenaline and ADP.

Hypotonic shock [18, 20, 23, 34, 47]. Platelets kept in a hypotonic medium absorb water, then actively release it. This phenomenon seems to be linked to the entirety of the platelet's energetic system. A photometer with a recorder must be available.

This test is greatly influenced by the intracellular presence of a slow penetration and exit cryoprotector such as glycerol. If the platelets have been frozen with glycerol the test is not interpretable.

Enzymatic assays [50]. Beta glucuronidase and lactic dehydrogenase activities released from platelets during freeze-thawing process. They show an adequate parallelism with morphological lesions.

Serotonin uptake [23, 49]. The incorporation of serotonin in the dense granules is an active phenomenon. Using ^{14}C-labelled serotonin we can appreciate the kinetics of incorporation.

All *in vitro* studies must include a calculation of platelet yield.

Accurate counting of platelets is still a difficult task [54]. Automatic methods have improved the accuracy of measurements but microscopic counting is still the reference method used. Important differences in the counts of thawed platelets between Technicon (Hemalog 8) and Coulter Counter ZBI have been shown. This is not the case when the counting concerns fresh platelets before freezing [72].

Choice of a study methodology

To evaluate a freezing method it is useful to have a panel of laboratory tests available which explore the principal platelet functions. Each step of the preparation of the cryopreservation of platelets must be the object of control in order to be aware of modifications induced by each manipulation. A cryobiology laboratory which routinely freezes platelets must be able to control its freeze and thaw procedure regularly (quality control) and the choice must lean towards simple tests which give the best possible reflection of the quality of the platelets.

We propose a panel as follows:

platelet yield
platelet morphology
mepacrine test
enzymatic assay
aggregation test
hypotonic shock (only if DMSO is used).

In vivo assay of platelets

Platelet counts are made prior to transfusion. Per cent recovery is calculated on the basis of estimated body surface area (BSA) using the formula

$$\text{per cent recovery} = \frac{\text{post-transfusion count} - \text{pre-transfusion count}}{\text{platelet transfusion} \times 10^{11}} \times \text{BSA} \times 100$$

Evaluation of platelet survival after transfusion is a logical way to assess the quality of frozen platelets. Evaluation is made using platelets marked before freezing with sodium chromate (^{51}Cr) or ^{111}indium [1, 53, 70, 73]. By this means we can study the survival time and the splenic sequestration.

Another criterion for clinical evaluation of frozen platelets has been the correction of bleeding time abnormalities in thrombocytopenic patients after transfusion [44]. In fact, the best clinical evaluation concerns the haemostatic effectiveness of thawed platelets with a bleeding syndrome [31].

Cryopreservation of Platelets: Review of the Principal Protocols

Numerous methods for platelet freezing have been proposed. We shall describe in detail the main techniques known at present and give for each of them essential parameters which characterize them. It is undeniable that the variations brought to the technical parameters of each protocol may affect the results, such as cryoprotective additive type and concentration, dilution medium, rates of cooling, temperature of storage, thawing and washing procedure.

If the present article is mainly concerned with problems of theoretic and practical cryobiology, it is useful to recall the importance of platelet collection and separation steps before freezing. It seems that several parameters can influence the quality of the platelets prior to freezing, such as

– the method of platelet collection: with a cell separator the procedure depends on the apparatus used and must be well defined. There may be a difference between platelets collected from random units of whole blood and those prepared by cytapheresis

– the nature of collection bags

– the centrifugation speed to obtain the platelet-rich plasma (PRP) and the platelet concentrate (PC)

– the delay between collection and freezing

– the storage temperature in the liquid state prior to cryoprotection

– the pH of the collecting media, in close relationship with the quantity of ACD added.

General protocol for freezing platelets with DMSO (ME_2SO). Review of the methodology

Various procedures have been reported during the past decade [22, 30, 35, 36, 39, 45, 56, 69]. The protocols recommended vary only in details. We shall consider the main variables selected among the protocols proposed:

Origin of the platelets. Either random donor units or single donor preparation by cytapheresis (discontinuous or continuous flow centrifugation).

Anticoagulant used. In most cases it was ACD. Sometimes the random units were collected in CDP.

Optimum volume for a platelet concentrate. It varied from 30 to 100 ml. If platelet concentration is too high, many platelets can be lost during transfer.

After cytapheresis it is also possible to add the cryoprotective additive directly into the PRP but the final volume to be frozen will be large. The advantage of this method is that it eliminates a rapid centrifugation step which is behind the activation of the platelets.

DMSO concentrations. Most of the investigators use 4 to 6 per cent which appears to give the best recovery.

Dilution medium for the DMSO. Tissue culture medium has been used and the addition of dextrose has been investigated. It has, however, been shown that no additive other than plasma should be used; the addition of undiluted DMSO di-

rectly to the platelet concentrate is hazardous. There is enough positive heat effect to kill the cells.

Rates of DMSO addition. To reduce the osmotic stress, most investigators added DMSO slowly, over a 5 to 30 min period. Mixing is essential during addition of the DMSO plasma solution to the platelets.

Container materials for freezing. Polyvinyl chloride, Teflon and polyolefine have been investigated. According to Kim and Baldini, polyolefine appears to be substantially better than the others [33].

Cooling rates. The best results have been obtained within a 2 to 4 °C/min rate to either −80 °C or −120 °C. Valeri and others place the bag directly in a −80 °C freezer which produces a 2 to 3 °C/min rate [71]. A rigidly controlled, preferably automated cooling rate minimizes technical error.

Storage temperature. The platelets can probably be stored for as long as six months at −80 °C. Below −120 °C no storage limit has been reported. The storage time is an important parameter for the preservation of autologous platelets or HLA-matched single donor.

Thawing step. It should be relatively rapid, without agitation, in a water-bath at 37 to 40 °C.

Washing step. It is essential to reduce the quantity of residual DMSO. To minimize the osmotic stress, during dilution, several authors used plasma with 2 per cent of DMSO [39, 71]. Other investigators used plasma alone + 10 per cent ACD. Plasma is essential in the dilution and resuspension solution. Autologous fresh frozen plasma is recommended. After extensive dilution, a centrifugation allows the removal of the supernatant with DMSO.

Storage times before transfusion. Four to six h post-thaw at room temperature have been reported to yield satisfactory results. There should be minimum delay of at least one to two h before the transfusion of thawed platelets.

The influence of these different parameters on the quality of frozen-thawed platelets has been evaluated by many laboratory tests and by clinical explorations *in vivo* [3, 48, 61, 62, 68]. The laboratory test panel varies according to authors but it is nevertheless dominated by the study of platelet yield, morphology, aggregation, serotonin uptake and hypotonic shock response. Even with an optimized protocol, the frozen platelets are different from fresh platelets, but the importance of the alterations varies according to the *in vitro* assay used.

In vivo studies have shown that recovery *in vivo* was reduced but the life span was normal [73]. The frozen platelets were also shown to be capable of correcting aspirin-induced lengthening of the bleeding time [69].

In order to illustrate this review of DMSO protocol, we shall describe in detail a method proposed by Valeri et al. and which can act as a reference.

Method of freezing human platelets using DMSO according to Valeri et al. [71]

Collection and isolation of the platelets

Platelets were prepared from 450 ml of blood collected in 63 ml CPD in a triple blood pack.

The PRP was obtained after blood centrifugation in a Sorvall RC-3 centrifuge at 4500× *g* for 3 min.

To obtain the PC, the PRP was centrifuged at 4500× *g* for 10 min.

The PC was stored undisturbed at 22 °C for one h in 30 ml of plasma to prevent clumping.

Cryopreservation and freezing

The cryoprotective solution contained 26.4 ml of platelet-poor plasma (PPP) + 3.6 ml sterile DMSO = 30 ml of 12 per cent DMSO solution.

The PC was placed on an oscillating shaker and 30 ml of 12 per cent DMSO were added over a 30 min period.

The 60 ml volume of PC with 6 per cent DMSO was transferred to a hemoflex bag (Union Carbide Corp.).

This bag was then placed in a freezing frame for storage in a −80 °C mechanical refrigerator (Harris Refrigeration Co.).

The rate of freezing was not controlled.

Thawing

The frozen platelets were thawed for one min in a 37 °C water-bath without agitation.

Platelets were diluted with 100 ml of 2 per cent DMSO in plasma followed by 16 ml of ACD.

The suspension was centrifuged at 4500× *g* for 10 min and the supernatant was removed.

The platelets were resuspended in 30 ml of autologous plasma. The washing procedure takes about 30 minutes.

The washed platelets were stored at room temperature for about 3 to 4 h before transfusion.

With this method, *in vitro* recovery after washing was about 65 per cent and *in vivo* recovery of the ^{51}Cr-labelled platelets was about 35 per cent. The prolonged bleeding times were usually corrected within two h after transfusion, indicating that the thawed platelets were haemostatically effective.

Evolution of the parameters of the DMSO protocol

Platelet collection. At present the platelets to be frozen are more frequently obtained by thrombocytapheresis from a single donor. This can be done either by a variety of continuous or intermittent flow centrifugal systems, or by a multiple plastic bag system like conventional plasmapheresis (an equivalent of 4 platelet units is obtained from a single donor).

Centrifuging speeds. According to the recommendations of the International Society of Blood Transfusion (1980), the first step to separate PRP from the red cell mass calls for the use of one of these centrifugation protocols:

1000× *g* for 9 min
1740× *g* for 3 min
2240× *g* for 1.5 min

In the second step to separate platelets from plasma, the PRP is centrifuged more heavily; there are three possibilities:

3000 × *g* for 20 min
3400 × *g* for 10 min
5000 × *g* for 5 min

Volume of platelet concentrate. The present tendency is to increase this volume. The minimal volume for a platelet concentrate appears to be about 100 ml. If the platelet concentration is too high, clumping may occur after thawing.

After cytapheresis it is possible to freeze the totality of the PRP to avoid a centrifugation step, even if the plasma volume to be frozen is large. A small volume of highly concentrated DMSO is directly added to the PRP.

DMSO concentration. Most of the authors used 5 per cent in plasma.

Washing step. The addition of 2 per cent of DMSO to the dilution medium is not a main point. Most methods use plasma (preferably autologous) with 10 per cent of ACD. Dilution occurs in a single step followed by rapid centrifugation at 5000 × *g* for 5 min. The volume of resuspension must be at least 100 ml.

General protocol for freezing platelets with glycerol

The main works concerning the freezing of platelets with glycerol have been carried out by Dayian and Rowe [14, 15]. The literature which refers to this cryoprotector is much more limited than that for DMSO. The varying techniques are less numerous, but they relate to fundamental points such as concentration and the type of dilution medium for the cryoprotective agent [28].

Review of methodology

Origin of the platelets and anticoagulant used. There was no difference between glycerol and DMSO.

Freezing solution. There are two possibilities:

1. it was composed of 10 per cent glycerol and 8% glucose diluted in saline and 30 per cent plasma with a final concentration of glycerol to 5 per cent and of glucose to 4 per cent;

2. it was composed of 6 per cent glycerol alone without glucose, diluted in autologous plasma (the cryoprotective properties of human plasma complete those of glycerol). The final concentration of glycerol was 3 per cent.

Optimum volume for a platelet concentrate. The platelets were either highly concentrated in a final volume of 10 to 20 ml or largely diluted in a final volume of 360 ml.

In the original technique published by Rowe the concentrated platelets were resuspended in a minimum quantity of residual plasma (10 ml) and 300 ml of the freezing solution was added. The suspension of glycerolized platelets was centrifuged, the supernatant removed and the PC resuspended in some residual freezing solution (7 ml).

The rate of addition of the freezing solution. It was relatively rapid (over a 5 min period) with gentle mixing during addition of the glycerol.

The freezing bag. It was used either in polyolefin (Union Carbide Corp.) or in Teflon Kapton (Gambro). Tests of *in vitro* function did not show platelet alteration after incubation in these bags, the inner wall of which is of Teflon [28].

Cooling rate. The rate was not greater than 30 °C/min until the heat of fusion, then a similar rate of 30 °C/min until the temperature reached a point between −80 °C and −120 °C. Then the freezing bag was transferred into liquid nitrogen.

This cooling rate was achieved by using an electronic freezer with a programmer. A recorder indicated the temperature obtained in a reference bag containing the cryoprotective solution of identical volume.

Storage temperatures. The frozen platelets are stored either in vapour phase (−120 °C) or directly in liquid nitrogen (−196 °C). No storage limit has been reported at these temperatures.

Thawing step. Submersion was done in a 42 °C water-bath for 30 s to 1.5 min according to the previously frozen volume. Fifty to 100 ml of autologous plasma was added to reduce the glycerol concentration.

Storage time before the transfusion. The thawed platelets can directly be infused after an incubation at room temperature for one to two h.

Functional preservation of the frozen-thawed platelets was studied by measuring the serotonin uptake, the mepacrine test, the aggregation response to ADP, and the ability of the platelets to cause clot retraction. Morphology was judged by interferential phase contrast (Nomarsky) and ultrastructural lesions were evaluated with an electronic microscope.

All these results have been published before [13, 14, 28, 52, 67].

The autologous frozen platelets labelled with ^{51}Cr, had a life-span of four to six days.

To illustrate this method we shall describe our own method in detail [28].

Own method

Collection and isolation of platelets [25, 26]

The platelets destined for freezing are taken from a single donor. Thrombopheresis is achieved by discontinuous flow centrifugation (Haemonetics Model 30) or continuous flow centrifugation (IBM 2997). The platelets are collected in a plastic bag and 10 per cent ACD is added to the PRP to avoid platelet clumping.

The platelet suspension is centrifuged at $260 \times g$ for 8 min (centrifuge Sorvall RC3) in order to remove the contaminating erythrocytes and leucocytes.

The minimum quantity of platelets in the PRP destined for freezing must be at least 400×10^9 cells.

The average volume of PRP is 340 ml. No supplementary centrifugation is carried out in order to concentrate the platelets.

Cryopreservation and freezing

The sterile glycerol solution is highly concentrated (60 per cent). It is provided by the Regional Blood Centre of Strasbourg, France.

After adjustment of the volume of PRP, it is separated into 3 Teflon-Kapton bags with 114 ml in each bag. The air is removed and 6 ml of 60 per cent glycerol is added in each bag with a syringe through a sampling site coupler over a 5-min period. The bag is gently shaken during addition of the glycerol.

The bags are heat-sealed and placed between two metallic grates adapted to ensure a homogeneous distribution of the liquid to be frozen.

A controlled rate of cooling is achieved by using an electronic freezer with a programmer ('Nicool' Air Liquide, France). The cooling rate is 10 °C/min from 25 °C down to −3 °C followed by 35 °C/min down to −120 °C.

After freezing, the bags are placed without their holders in the vapour phase of liquid nitrogen (−150 °C).

Platelet thawing

For a platelet transfusion, three bags are thawed one after the other by submerging them in a 42 °C water-bath without agitation for 1.5 min.

The platelet suspension is slowly diluted with 60 ml of autologous fresh frozen plasma.

No washing is done.

The three bags are pooled and stored at room temperature for about 1 to 2 h before transfusion.

Glycerol protocol

Dayian and Pert have worked out a simplified method for freezing platelets in glycerol-glucose [16]. This simple and inexpensive method allows to obtain a reproducible cooling rate which replaces the mechanically controlled cooling rate apparatuses. The device is called 'Freezing Cassette'. The freezing bag containing 10 ml of platelet suspension is placed between 2 sheets of corrugated cardboard. The cardboard and freezing bag are then placed between thick hinged aluminum plates. The entire assembly is placed inside a plastic bag and then immersed for 15 min in liquid nitrogen. Depending on the thermocouple location, the maximum cooling rates vary from 22 to 38 °C/min averaging 33.6 °C at the centre of the bag. The cardboard and platelet bag are removed as a unit for storage in the vapour phase of liquid nitrogen.

It has been shown that for post-thaw platelet clumping mostly damaged red cells and leukocytes are responsible. These contamination cells must be removed before freezing by slow centrifugation.

The Teflon-Kapton freezing bags are very suitable for use in cryobiology. They are highly resistant and this quality is indispensable for the preservation of platelets stored in liquid nitrogen.

Cryopreservation of platelets using hydroxyethyl starch (HES)

Choudhury et al. have determined the value of HES as a cryoprotective additive for freezing platelets [8].

Platelet-rich plasma is obtained from a whole blood unit collected in 75 ml ACD. After the second centrifugation, the PC is resuspended in 30 ml of residual plasma and left at room temperature under gentle agitation for 40 min.

Two volumes of 6 per cent HES in normal saline (plasma-steril Mw 450 000, Fresenius, FRG) are added to one volume of PC to obtain a final concentration of 4 per cent HES; this concentration is critical. The platelet suspension is transferred into a polyolefin bag and frozen at a controlled rate of 1 °C/min and stored in vapour phase liquid nitrogen.

Thawing is done in a 37 °C water-bath immediately before transfusion without post-thaw manipulation.

As with glycerol, a major advantage of HES is that it is not toxic at the given concentration and can be infused without demonstrable side effect. It has been shown that the HES concentration used to freeze the platelets did not affect coagulation *in vivo* [64].

The indices studied were platelet recovery, platelet morphology, platelet Factor 3 availability and the hypotonic stress response. Platelets frozen by HES gave better results *in vitro* than did platelets stored at 4 °C for 48 h.

A preliminary clinical test of HES-preserved platelets has confirmed the haemostatic effectiveness *in vivo*. The results of this preliminary report were encouraging and merit confirmation by other laboratories.

Clinical Applications of Frozen Platelets

Platelets frozen with DMSO

The clinical experience with cryopreserved platelets has been the object of numerous reports and their effectiveness needs no further demonstration [7, 12, 31, 57, 70, 76]. In most cases the platelets used were collected by plateletpheresis of normal random donors. Before freezing or after thawing (depending on the investigators) the multiple units of the same ABO type were pooled and on the average 6 to 8 units corresponding to 4×10^{11} platelets were given to each patient. Sometimes HLA-matched units obtained by cytapheresis of a single donor were injected. All transfusions were tolerated well and there were no side effects attributable to the small amounts of DMSO infused.

In the report of Schiffer et al. [55] the recipients were patients with leukaemia or other malignancies who were profoundly thrombocytopenic, the platelet count was usually below 20×10^9/l.

The platelets were given as 'prophylactic' transfusions under the most favourable clinical conditions.

In vivo recovery was approximately two-thirds of that seen with fresh platelets and the corrected increment at one h was $12.8 \times 10^9/l \pm 1.4$.

Frozen autologous platelets have also been transfused to patients with leukaemia [57]. In this group of 25 patients, 13 were alloimmunized and refractory to andom-donor platelet transfusion.

Plateletpheresis was performed shortly after the patients had entered remission. No patient was receiving chemotherapy at the time of platelet donation.

Four to five units, 3.0 to 3.5×10^{11} platelets for each patient, were pooled before freezing; this represented the dose of platelets needed to provide good clinical results.

In most cases the transfusions were given to the patients on a prophylactic basis when they were profoundly thrombocytopenic. The count increment of 13 700 per ml represented 67 per cent of the increment with fresh platelets and these results were identical with those obtained with frozen allogeneic platelets.

Similar post-transfusion recoveries and *in vivo* haemostatic effectiveness have been described by Valeri and Zaroulis [70, 76]. The mean count increment at 1 h was 46 and 32 per cent at 18 to 24 h. However, in the cases of splenectomized patients, recovery at 24 h could be the same as that at one h post-transfusion.

Platelets frozen with glycerol

Pertinent clinical studies are scarce [26, 27, 65]. We have previously reported our clinical results with thawed platelets. The platelet HLA phenotypes were obtained by thrombopheresis. For each transfusion, about 430×10^9 (range 270 to 610) platelets were infused from the Haemonetics and 502×10^9 (range 263 to 740) from the IBM.

We have assessed the clinical efficiency after transfusion of 183 frozen platelet units into 150 patients during the period 1977–1981. Thawed platelets were transfused to patients with acute leukaemia (105 cases), aplasia (19 cases), lymphosarcoma (12 cases) and solid tumour (14 cases). All the patients had a platelet count between 20×10^9/l. We transfused 120 bleeding patients to judge the haemostatic effectiveness of thawed platelets. Bleeding was mucocutaneous (74 per cent), visceral (15 per cent) or retinal (11 per cent). The platelets were effective in 81 per cent of the cases, bleeding stopped in about 4 to 8 h, 67 per cent remained free of bleeding for 72 h. *In vivo* recovery 2 h post-transfusion averaged 18 per cent (range 0 to 30.5 per cent). In 30 patients the frozen platelets were given prophylactically with a recovery *in vivo* of 28 per cent (range 11 to 50.5 per cent).

Thirty-seven patients were immunized and refractory to histoincompatible platelets. They were transfused with HLA-matched frozen platelets which were well-tolerated (25 patients) or autologous platelets (12 patients).

In the following, a clinical observation is reported to demonstrate the privileged place of frozen platelets in haematological reanimation.

Report of a case

H.A.S., a one-year-old female patient, presented a bone marrow aplasia in the course of a preleukaemic syndrome. The child was profoundly thrombocytopenic, platelet counts were below 15×10^9/l and was alloimmunized by previous fresh platelet transfusions. In three months this patient received 19 transfusions of frozen HLA-matched platelets which were prepared by cytapheresis from a single donor. For each transfusion a third of one unit was thawed and infused. The same donor allowed three platelet transfusions. On the average, 1.48×10^{11} platelets (range 0.8 to 1.8×10^{11}) were administered with each transfusion. The platelets were given twelve times as prophylactic transfusion and seven times in the course

of mucocutaneous bleedings. In all the cases the transfusions were tolerated well and effective in preventing the bleeding syndrome or to stop haemorrhage.

Supportive therapy with only frozen platelets is feasible even over a long time.

Organization of a frozen platelet bank (Fig. 2)

Such a bank must be integrated in the cryobiology centre so as to dispose of all the cryogenic equipment necessary. In practice the PRP meant for freezing must contain at least 4×10^{11} platelets. Nevertheless, if the donor possesses a phenotype which is compatible or even identical with that of the immunized recipient, the threshold limit becomes less strict.

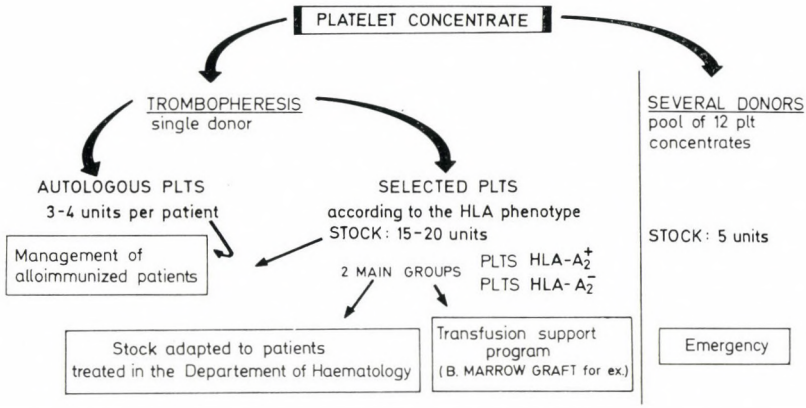

Fig. 2. Frozen platelets storage organization in a Regional Blood Centre

Selection of donors

The donors are chosen in terms of their phenotype HLA and classified in 3 categories:

1. The first one allows to set up of a reserve of HLA-A_2^+ and HLA-A_2^- platelets. The great frequency of anti-HLA-A_2 immunization justifies this distinction. It is necessary to avoid the appearance of antibodies with respect to common antigens when the aim is to prevent alloimmunization.

2. The second one concerns the HLA phenotypes corresponding to those of sick people who must be protected from immunization because they belong to a complex transfusional programme.

3. The third one allows to conserve platelets devoid of antigens specific to the antibodies present in a recipient.

Autologous platelets come from hyperimmunized patients or those who must receive multiple platelet transfusion, as for example in the case of a bone marrow graft.

Collaboration with different departments

– with the cytapheresis department;

– with the histocompatibility laboratory to have access to the immunized receiver file and to deliver the PC after a test of cross-compatibility;

– with the control laboratory: a freezing method imposes systematic verification and random selection (functional quality);

– with the clinical haematology department: this relationship is essential because it facilitates access to the file of hospitalized patients. Such collaboration is an element indispensable for making an objective judgement of the quality of the product.

Discussion

Due to its advantageous intracellular diffusion, DMSO remains the most widely used cryoprotector for platelet freezing. DMSO permits cryopreservation of platelets at −80 °C in a mechanical freezer and consequently does not need liquid nitrogen equipment. Although there has been no evidence of serious side effects of DMSO infusion, the compound's smell is a disadvantage and the washing step increases the time of manipulation. At present there are potential difficulties in licensing a procedure using DMSO because of the questions regarding DMSO toxicity. The ideal cryopreservation procedure would involve a clinically acceptable cryoprotectant such as glycerol or perhaps HES, with little or no post-thaw processing.

'The present understanding of freezing injury and cryoprotection is not yet sufficient to suggest novel approaches to platelet preservation' (conclusions of the Platelet Cryopreservation Conference, Washington, October, 1978). A simple practical procedure for the cryopreservation of platelets accepted by all is yet to be defined.

There remains a fundamental question: what is the goal of platelet freezing? At the beginning it meant being able to dispose of a reserve supply of effective platelets in an emergency situation. Preservation of platelets in a liquid phase at room temperature during 72 h has lowered the risk of unavailability in case of emergency for a well-organized blood centre [21, 37, 46].

The establishment of a security reserve is not in itself justifiable for platelet freezing, all the more because new polyolefin bags will allow to conserve platelets at room temperature for five days.

The concern with cryopreserved platelets is immunological in nature. Their use should be limited to HLA-matched platelets from single donors or autologous transfusions.

Alloimmunization of thrombocytopenic patients remains the essential problem: more than 50 per cent of multiple transfusion patients are sensitized.

The Haemonetics Model 30 and the IBM celltrifuge are two available systems for obtaining large numbers of platelets from a single donor by plateletpheresis;

other systems too are being developed, such as the CS 3000 cell separator produced by Travenol Co.

Elimination of the great majority of leukocytes in the platelet concentrate offers a double advantage, i.e. for sensitized recipients, weakened or even no transfusion reaction, and for the platelets, a reduction of the noxious effect of enzymes liberated by granulocytes lysed during freezing.

It has been demonstrated clinically that the thawed platelets cryopreserved either by DMSO or glycerol are unquestionably effective. There is, however, little clinical information concerning the glycerol method as compared to the DMSO method.

It is necessary to adapt the transfusion dosage of frozen platelets to the number of effective platelets. It is more difficult to evaluate the therapeutic effectiveness of a homologous platelet transfusion in a patient than to study an autologous platelet transfusion in a healthy individual. Preservation injury is more readily recognized after an autologous platelet transfusion to a healthy voluntary donor.

Diseases may effect the circulation and the function of the donor's platelets. Immediate sequestration of transfused platelets occurs in patients with enlarged spleen. If the platelets are infused during a bleeding syndrome, the variability in the yield *in vivo* may be explained by the immediate consumption of part of the platelets.

Several clinical factors can falsify the appreciation of the effect of a frozen platelet transfusion: fever, infectious disease, splenomegaly, alloimmunization, chemotherapeutics such as cytosine arabinoside or daunorubicin. Comparison of transfusion results using fresh and frozen platelets and the same donor-recipient pairs is the ideal way to study such technical variations [55].

In spite of the evolution of the techniques of platelet cryopreservation and the demonstration of their clinical effectiveness, there remains a certain reticence among physicians using them. Frozen-thawed platelets undeniably possess altered functional qualities in relation to those of fresh platelets, but it is useful to remember that platelets conserved at 22 °C for 72 h have lost some of the characteristics which define the fresh platelets.

All has not been done and all has not been said concerning the cryopreservation of platelets. At present there are still too many unknowns on the subject of platelet freezing injury. Further propositions must seek to define a highly technical protocol reproducible by all and which depends on a better understanding of the phenomenon.

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Beneficial Effect of Delayed Washing on the Quality of Frozen-Thawed Red Cell Concentrate

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If the washing off of glycerol is started only 1 h after complete thawing of red cells, the supernatant haemoglobin content will be less than in preparations deglycerolated immediately after thawing. The observation is explained by the time needed for the normalization of red cell volume after freezing and thawing.

Key words: frozen-thawed, red cell concentrate, washing

Introduction

Freeze-preservation of human red cells in liquid nitrogen is a routine blood bank procedure. The different steps of processing are, however, not completely optimalized. Although red cell recovery after the freeze-thaw-wash procedure is over 85 per cent, the free haemoglobin content of the last washing supernatant, a good indicator of the quality of recovered cells, is significantly higher than in the case of simply washed red cell concentrate. Storage of frozen red cell concentrate in a cold room after deglycerolation results in a further substantial increase in free haemoglobin.

Material and Methods

The red cell freezing procedure of Krijnen et al. [2] was used. Freezing takes place in liquid nitrogen using aluminium freezing bottles of Dutch make. The glycerol containing supernatant is discarded before freezing [5], the first solution in the washing process (serial centrifugation) is a hypertonic NaCl solution. Second and third washing is made with physiological saline.

In the experiments, 40 min were allowed for complete glycerolation of red cells at room temperature, then the freezing bottles were immersed into liquid nitrogen.

Free haemoglobin in the third (last) washing supernatant was estimated with spectrophotometry at 415 nm, the data were expressed generally in relative values in per cents of the maximum optical density checked, to avoid individual differences between blood units.

A Knauer (FRG) half-micro osmometer was used to control osmolarity values.

Extracellular K^+ and Na^+ concentration was measured with a type 243 Instrumentation Lab. flame photometer.

The paired *t*-test was used for statistical comparison.

Results

Different aspects of the freeze-thaw-wash procedure were examined, but only the time between complete thawing and the start of the washing procedure was found to have importance (Table 1). If the time between thawing and washing is 0.5–1 h, then the free haemoglobin content of the last washing supernatant is significantly decreased.

Table 1

Effect of the time lag between thawing and washing on the free haemoglobin content of the last washing supernatant (optical density values at 415 nm were expressed in per cent of the highest value in the same experiment)

	Time lag, min				
	0	15	30	45	60
n	27	16	27	19	24
mean	94.96	68.63	59.44	51.74	46.54
±SD	15.85	23.95	19.55	16.96	18.72
	$p < 0.0002$	ns	ns	ns	
	$p < 0.00005$		$p < 0.05$		

Osmolarity of the extracellular medium after thawing showed a slight decrease, from 2820 to 2780 mOsm/kg, especially immediately after thawing. (The significance between osmolarities checked immediately after thawing and 20 min later was $0.1 < p < 0.05$).

Extracellular K^+, Na^+ and free haemoglobin contents are shown in Figure 1.

Discussion

As 40 min were allowed for complete glycerolation of red cells at room temperature, therefore a complete equilibration is supposed [4].

The most significant changes of extracellular K^+, Na^+ and haemoglobin content in the thawed system are observed immediately after thawing, but at the same time the osmolarity is slightly decreased. This can be explained by some facts. First, during freezing and thawing, when the system is in the liquid phase but around 0 °C,

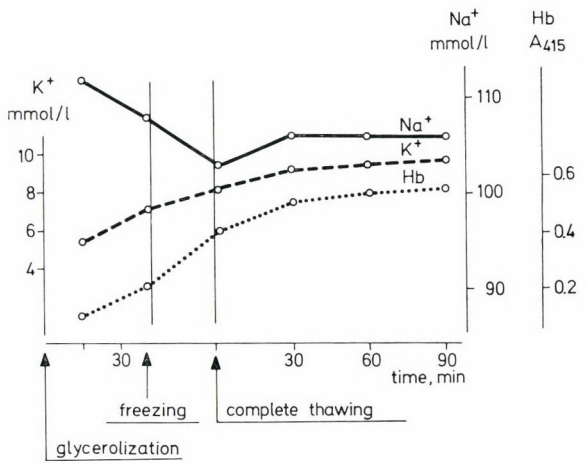

Fig. 1. Change of extracellular K^+, Na^+ and haemoglobin content (circles represent mean of five experiments)

the biochemical mechanisms maintaining the 'normal' intracellular ion-concentrations are practically stopped; thus, an ion-movement towards the balance is initiated. Second, during freezing, extracellular freezing results in a slight increase of glycerol concentration in the unfrozen liquid phase and red cells exposed to this elevated extracellular osmolarity will suffer an osmotic injury [6]. Because of the geometrical characteristics of the freezing bag or freezing containers, the substantial mass and volume of the sample, the extent of these processes are very different at the various sites of the sample.

After thawing the above processes will show a condition of the system different from that before freezing. After complete thawing the membrane transport mechanisms become activated, but formation of the new equilibrium requires some time. Resealing of injured membranes may perhaps also play a role in the phenomenon [1].

A similar phenomenon of post-thaw lysis and osmotic reactivation was displayed by Chinese hamster fibroblasts [3].

If there is no time for the new equilibrium to set completely, i.e. deglycerolation washing is started immediately after thawing, a certain number of red cells will suffer an osmotic injury resulting in haemolysis.

The findings have a practical use: with delayed washing, started 0.5–1 h after complete thawing, the final therapeutical blood unit will be of improved quality, with low free haemoglobin and increased stability of cells.

The phenomenon observed has practically no influence on red cell recovery *in vitro*.

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Banking-Related Decline of Erythrocyte N-Acetyl-Neuraminic Acid and Phospholipids

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The alterations of some membrane components of the red blood cells were investigated on the day of drawing and after storage for 42 days. During banking the erythrocyte membrane looses N-acetyl-neuraminic acid (NANA), phospholipids and double bonds. A sex-related dependence of these changes is assumed. The losses could be related to (i) sequestration of limited membrane area (vesiculation) and (ii) to molecular changes of the membrane influenced by peroxidative events as well as by endogenous and exogenous hydrolases.

Key words: erythrocyte, N-acetyl-neuraminic acid, phospholipids, storage

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Beside the modern processes of cryopreservation, liquid blood banking is very important in transfusion practice. Alterations described as 'preservation damage' influence the life-span of the red cell. In contrast to the investigations of *in vivo* alterations of the erythrocyte membrane, few experiments have been published about membrane alteration under storage conditions [1–3]. This has stimulated us to investigate the phospholipid and NANA contents of the red cell membrane with regard to the degradation mechanisms under conditions of banking. The mentioned membrane components were determined on the day of drawing and after a storage time of 42 days, as has been done by Devenuto et al. [1] and Moore et al. [3]. Furthermore the extent of lipid peroxidation after 6 weeks of banking was investigated.

Material and Methods

From 20 A_1D blood samples banked in ACD-AG or CPD-AG medium, the required blood volumes were taken out sterile on drawing day. After banking at 4 °C for a period of 42 days the procedure was repeated. The applied quantity of erythrocytes was characterized by cell counting (counter type: Laborscale PSL-1, Medicor, Budapest). Evaluation of lipid phosphorus was performed according to Bartlett [4]. For a quantitative isolation of the required cell membranes the erythrocytes were haemolysed in the following scheme:

1. Twenty ml of blood were washed ten times with 10 volumes Tris buffer (10 mmol/l, pH 7.6, 300 mOsm).

2. The sediment of the last washing was diluted to a haematocrit of about 40 per cent with Tris buffer (10 mmol/l, pH 7.6, 300 mOsm) followed by cell counting.

3. From the adjusted cell suspension four samples of 0.2 ml were taken. To each sample 6 ml of the Tris buffer containing 0.015 per cent Alcian blue 8 GS were added. After 10 min the haemagglutinates were centrifuged at 5 to 6000 rpm for 30 min and the supernatants removed by cautious suction.

4. For haemolysis, 8 ml Tris buffer (pH 8, 20 mOsm, 0.015 per cent Alcian blue 8 GS) were added to each sediment. After 10 min the samples were centrifuged at 5 to 6000 rpm for 30 min and thereafter the supernatants removed by cautious suction. This haemolysis procedure was repeated four to five times until the supernatants showed no more haemoglobin.

All operations were carried out at 5 to 10 °C. 'Alcian blue ghosts' prepared in this way were analysed according to Bartlett's procedure for lipid phosphorus.

Oxidation of the unsaturated fatty acids was carried out by means of H_2O_2 according to Stocks and Dormandy [5]. This procedure leads to the formation of malonedialdehyde (MD) from fatty acids, which contain C_n/C_{n+3} positioned double bonds. MD was determined by thiobarbituric acid [5]. The estimated decrease of MD is proportional to the extent of lipid peroxidation during the time of storage. NANA was evaluated according to Warren [6] whereby a glutaraldehyde fixation was performed before hydrolysis of the test material [7].

Results and Discussion

Alcian blue is a strong positively charged dye molecule of copper phthalocyanine. It binds firmly to the negatively charged glycocalyx of erythrocytes, thus leading to agglutination of the cells [8] or their membranes, as well as to an increase of the specific gravity of the Alcian-blue-loaded ghosts. In this way it is possible to precipitate the blue coloured and aggregated ghosts in a quantitative manner by low speed centrifugation (6000 rpm). During the washing steps the metabolic phosphorus can be removed completely without remarkable loss of lipid phosphorus. The procedure does not require the extraction of membrane lipids, therefore the lipid phosphorus estimation is essentially simplified.

The lipid phosphorus and sialic acid (NANA) values showed considerable individual variations (Table 1), which is in accordance with the results of others (for details see [9]). The same applies to MD. When the mean values of lipid phosphorus, NANA and MD after banking for 42 days are compared with those observed on the drawing day, the values of the stored erythrocytes were not significantly decreased (Table 1). On the other hand, it is well documented that erythrocytes lose phospholipids and NANA during storage *via* vesiculation [10, 11]. For this reason the individual losses of lipid phosphorus, NANA and MD after six weeks of banking are given in Table 2.

Table 1

Lipid phosphorus-, sialic acid (NANA)- and malone dialdehyde (MD)-contents of unstored erythrocytes and erythrocytes banked for 42 days

Sample No.	Sex	Lipid P_0	Lipid P_{42}	$NANA_0$	$NANA_{42}$	MD_0	MD_{42}
		($\times 10^{-17}$ mol P/cell)		($\times 10^{-18}$ mol NANA/cell)		($\times 10^{-19}$ mol MD/cell)	
1	M	61.07	54.91	28.52	26.24	16.19	15.18
2	M	75.02	61.87	28.11	26.28	17.39	16.47
4	M	56.28	41.20	15.93	14.54	18.22	14.10
5	M	50.21	44.99	15.76	15.12	16.00	14.64
6	M	55.79	42.92	16.35	15.83	18.13	14.05
8	M	53.22	45.24	14.36	13.55	18.35	17.71
14	M	58.70	54.21	32.15	29.58	17.96	13.68
15	M	64.19	58.08	28.47	25.93	16.59	12.48
	mean	59.31	50.42	22.45	20.88	17.35	14.78
	SD	7.7	7.76	7.45	6.67	0.96	1.65
3	F	57.12	51.41	26.92	24.66	13.60	12.29
7	F	55.58	42.99	13.5	12.12	18.07	14.11
9	F	47.59	37.08	14.84	13.57	19.59	15.08
10	F	57.47	52.48	28.06	27.92	n. d.	n. d.
11	F	52.46	49.67	17.88	17.63	22.09	19.23
12	F	55.89	50.41	18.53	18.35	n. d.	n. d.
13	F	51.78	49.41	14.01	13.76	n. d.	n. d.
16	F	58.80	48.63	16.91	16.60	18.85	17.38
17	F	50.31	38.92	13.51	13.27	19.08	14.81
18	F	47.37	37.87	16.46	16.24	20.92	15.31
19	F	60.70	50.70	27.18	26.93	n. d.	n. d.
20	F	65.46	55.90	n. d.	n. d.	16.66	14.69
	mean	55.04	47.1	18.89	18.27	18.60	15.36
	SD	5.39	6.26	5.71	5.67	2.61	2.1

Table 2 shows that

1. the decreases of lipid phosphorus, NANA and MD differ considerably from sample to sample;

2. the decreases of lipid phosphorus and NANA are not related. A constant quantitative relation between the decrease of phospholipid and NANA should, however, occur if vesiculation was the sole disintegration mechanism;

3. only the erythrocytes of some female donors (samples 10 to 13) gave slight losses of phospholipid and NANA. This finding might be explained on the basis of the results of Devenuto et al. [1]. These authors observed a stabilizing effect of progesterone against storage lesions of the erythrocyte membrane. In the luteal phase the plasma progesterone of the adult female reaches high levels. Therefore, erythrocytes from female donors obtained later than twenty days after the onset of menstruation are expected to be more resistant to banking-related disintegration than erythrocytes of male donors, as well as of female donors in the follicle phase.

Table 2

Decline of membrane components of erythrocytes after banking for 42 days. The differences between male and female samples are not significant (U-test according to Mann and Whitney [17]). The samples 10–13 from female donors are listed separately. They show very low values as compared to the other female samples

Sample No.	Sex	Lipid-P, %	NANA, %	MD %
1	M	10.05	7.99	6.24
2	M	17.53	6.51	5.29
4	M	20.72	8.72	22.63
5	M	10.40	4.06	8.42
6	M	23.07	3.16	22.49
8	M	15.00	5.61	3.54
14	M	7.65	7.99	23.81
15	M	9.52	8.90	24.78
	mean	14.24	6.61	14.65
	SD	5.72	2.16	9.50
3	F	10.00	8.40	9.64
7	F	22.66	10.20	21.91
9	F	22.09	8.52	23.03
16	F	17.30	1.80	7.77
17	F	22.64	1.74	22.41
18	F	20.06	1.31	23.77
19	F	16.48	0.91	n. d.
20	F	14.61	n. d.	11.85
	mean	18.23	4.69	17.19
	SD	4.50	4.1	7.08
10	F	8.69	0.49	
11	F	5.32	1.39	
12	F	9.81	0.97	
13	F	5.10	1.78	
	mean	7.23	1.15	
	SD	2.37	0.55	

The alterations occurring during banking lead to a stiffening of the erythrocyte membrane [12, 13] and a formation of vesicles which could be detected in the spontaneous sediment of blood preserves [10, 11, 14, 15]. Results of parallel isolation of vesicles from banked blood samples together with our knowledge about the composition of these vesicles [15] led us to suppose that vesiculation was responsible for most of the losses. Another part of storage-conditioned descreases of membrane material can be attributed to the possible action of exogenous and endogenous hydrolases. The MD values show that peroxidation processes occur during banking, as has been shown by Tannert et al. [16]. These values demonstrate a strong individual variation.

A trend of homogeneous decline of NANA, phospholipids and unsaturated fatty acids can be expected only if all the disintegration processes develop with constant quantitative relations. This is improbable with regard to the different decomposition mechanisms. Our finding of an inhomogeneous decline of lipid phosphorus, NANA and MD point rather to a multifactoral event.

The different ACD-AG and CPD-AG media did not influence the results. This was confirmed by parallel tests.

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Variants of Congenital Dyserythropoietic Anaemia: An Update

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During the past decade a large number of variant congenital dyserythropoietic anaemias (CDA) have been described. The morphological, genetical, clinical and serological features of the classical types (I, II and III) are not well delineated, so, there are no exact borders between classical and variant forms. In the present review attention will be given to variant serological features, variant globin chain synthesis, variant genetical and morphological features and variant clinical presentations. Finally, it is emphasized that the existence of transitional forms between dysplastic and hypoplastic affections of bone marrow function points to the stem cell origin of these disorders.

Key words: classification, congenital dyserythropoietic anaemia, family disorders

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The congenital dyserythropoietic anaemias (CDA) are rare family disorders characterized by grossly ineffective erythropoiesis, distinct erythroblastic morphological features and frequently by secondary haemochromatosis. Since 1968 three different classical types have been described and widely accepted on the basis of morphological, serological and genetical data [1]. Unique features for these classical forms of CDA do not exist: many so-called specific morphological and ultrastructural findings can not only be found in all three types, but in a variety of acquired disorders, as well. As a consequence it is not surprising that an ever increasing number of aberrant or mixed forms is described.

At the Third Meeting of the European and African Division of the International Society of Haematology, held in London, August '75, a special session on CDA was organized: 19 'variant' patients belonging to 6 different families and representing 6 different variants were presented [2].

The 1981 review of the literature contains reports of at least 49 patients belonging to 29 different families. This represents a fivefold increase in the known variants of the classical CDA types.

When summarizing the 1968 to 1981 CDA literature, a distinct pattern can easily be discerned, the variants becoming roughly subclassified in five major categories: variants in serological features, variants in globin chain production, variants in morphology, variants in inheritance and – the most heterogeneous group – the clinical variants. Some overlap in these categories can logically not be avoided and some variants do not fit any category.

To describe aberrant forms, the presence of clear and precise delineations for the classical forms of CDA is a *conditio sine qua non*. Those who have been involved in this field, however, will immediately recognize that there are no exact borders between classical and variant forms.

Differential Diagnosis of Classical CDA

As a reminder, the more or less typical features of the three congenital types of dyserythropoietic anaemia, are shown in Table 1.

Table 1

Differential diagnosis of classical CDA

	Type I	Type II	Type III
Erythroblasts	Megaloblastoid Chromatin bridges	Binuclearity Gaucher-like cells	Multinuclearity Gigantoblasts
Erythrocytes	Macrocytosis	Normocytosis	Macrocytosis
EM	Nuclear pores (cytoplasmic penetration) Spongy appearance	Peripheral cisternae	Clefts and blebs
Acid serum test	−	+	−
Anti-i agglutination	−	+++	+++
Anti-I agglutination	−	++	++
Inheritance	Recessive	Recessive	Dominant

Many of the 'classical' features, which have been amply described elsewhere [3], are not reviewed in detail and we have only concentrated on the anomalies which may be helpful in the differential diagnosis of the three types.

As a rule, peripheral macrocytosis is more often found in type I and type III, while type II, the HEMPAS type, usually shows a normocytic anaemia.

Bone marrow examination can be helpful: typically one finds chromatin bridges and megaloblastoid features in type I, while binuclearity in 10 to 30 per cent of the erythroblasts, and Gaucher-like cells are more prominent in type II. Furthermore, the typical gigantoblasts, with ten or more nuclei, and the overwhelming multinuclearity in marrow smears of the rare IIIrd type of CDA are well known to most cytopathologists.

The electronmicroscopical appearance of the congenital dyserythropoietic anaemias is well defined:

1. wider and more numerous nuclear pores in CDA type I, giving rise to cytoplasmic penetration into the nucleus or even, in the more severely affected cells, to the spongy appearance of the nuclei.

2. Peripheral cisternae or 'double membranes' are typical of type II.

3. Nuclear clefts and blebs are said to be more prominent in type III.

The serological features of the three types are unequivocal: the positive acidified serum lysis test with 30 per cent of normal sera, is only found in type II, while the higher agglutination titres with anti-I and anti-i are found in both types II and III.

Finally, an autosomal dominant inheritance might be more specific for type III, especially when compared to the recessive mode by which the majority of CDA I and II is inherited.

Serological Variants

Since 1973, 28 patients, representing 13 families, have been described as so-called 'serological' variants (Table 2).

The most prominent aberration in this category is the non-expression of the HEMPAS antigen. Therefore, these variants have also been called the HEMNAS type (Hereditary Erythroid Multinuclearity with Negative Acidified Serum test) [2]

Table 2

Serological variants of classical CDA
Data of 28 patients belonging to 13 different families

Reference	No. of patients (family)	Morphological type	HEMPAS-antigen (number of sera tested)	i-antigen	Other aberrations
Weatherall [5]	6 (1)	II (2–4%EB)	− (10)	+	Inheritance (globin chain synthesis) Hb-inclusions
Hruby [13]	1 (1)	II	− (?)	−	Globin chain synthesis
Weatherly [11]	3 (1)	II	− (10)	−	Inheritance Cytoplasmic vacuolisation
Dvilansky [6]	1 (1)	I−II	− (3)	−	Morphology
Benjamin [14]	1 (1)	II	− (?)	−	No cisternae
Seip [7]	1 (1)	I−II	− (12)	+	Morphology
McBride [4]	2 (1)	II	− (?)	−	Globin chain synthesis
Eldor [8]	3 (1)	II	− (30)	+	Globin chain synthesis
Kenny [12]	1 (1)	I−II	− (17)−	−	Cytoplasmic vacuolisation
Miller [9]	3 (2)	II	− (20)	−	− −
Lowenthal [10]	1 (1)	II	− (38)	−	Clinical
Borst-Eilers [15]	5 (1)	II	±(1–5%)	+	− −

and some authors even designated them as the fourth type of CDA (CDA IV) [4]. However, this classification has never been universally accepted.

While most of the cases logically show type II morphology, the Weatherall variants [5] did show the classical binuclearity in only 2 to 4 per cent of the erythroblasts, and the cases of Dvilansky [6] and Seip [7] represented mixed type I and type II ultrastructural characteristics. A recent re-examination of the Seip case, however, disclosed some serological positivity, and maybe this case represents a true HEMPAS type (personal communication).

About 30 per cent of normal sera contain a naturally occurring IgM antibody that reacts specifically with an antigen on HEMPAS red cells. To make a statistically sound statement about a negative acidified serum test, and to obtain a P value of less than 0.005, about 30 normal sera should be tested each time. This is only the case for the patient of Eldor et al. [8], Miller et al. [9] and Lowenthal et al. [10] (Table 2). While the cases of Weatherall et al. [5], Weatherly et al. [11], and Kenny et al. [12] probably also represent true negative acidified sera tests, nothing sure can be said about the variants of Hruby et al. [13], Benjamin et al. [14] or McBride et al. [4].

The family described by Borst-Eilers et al. [15] showed a typical CDA II morphology, but the acid serum lysis test was only positive with 1 to 5 per cent of the tested sera. This again indicates that the expression of the HEMPAS antigen might be weakened or altered and that a much larger number of sera ought to be tested before one could state with any degree of certainty that any CDA patient lacks the classical HEMPAS antigen.

Four of the serological variants did not show the classical HEMPAS antigen but did show a strong agglutination with anti-i. Considering the altered membrane status of the CDA's, if more sera had been tested, some positivity might have been found for HEMPAS. On the other hand, this might only represent some degree of foetal haematopoiesis.

Finally, many of the serological variants displayed some other aberrations, as well. These are shown in the last column of Table 2. An additional variation in globin chain production was most frequent, while some morphological variations and one clinical variant, are represented.

Variants in Globin Chain Synthesis

Variant globin chain synthesis has been described in 14 CDA patients belonging to 6 different families (Table 3). The patients of the Weatherall family [5] showed an imbalance in globin chain synthesis of bone marrow cells and reticulocytes. Alpha-chains were synthesized in excess of non-alpha-chains, and consequently values of Hb A2, but also of Hb F were increased in all affected family members. The amount of excess alpha production paralleled the excess rate seen in thalassaemics. Haemoglobin-like inclusions could also be noted.

Table 3

Variants in globin chain synthesis. Data of 14 patients, belonging to 6 different families (n. d. = not done)

Reference	Hb F	Hb A2	Imbalance in chain synthesis
Weatherall [5]	increase	increase	excess alfa
Hruby [13]	increase	normal	excess alfa
McBride [4]	?	?	excess beta
Van Dorpe [16]	slight increase	normal	n. d.
Berrebi [17]	normal	increase	n. d.
Eldor [8]	normal	increase	excess alfa (1/3)

Similar aberrations were reported by Hruby et al. [13] and Eldor et al. [8], while the cases reported by Van Dorpe [16] and by Berrebi and Nir [17] displayed increases only in Hb F or Hb A2, without referring to chain synthesis studies. Solitary increase in Hb F simply may point to a regression towards foetal erythropoiesis.

Remarkable in this category are certainly the two sisters described by McBride et al. [4], as they showed an excess in beta-chain production in bone marrow cells.

However, beside these variants, authentic cases of thalassaemia with coexistent CDA have been described. The difference is not always very clear. Recently, Berrebi and Barak have described a possible double heterozygote for the HEMPAS antigen and the thalassaemia trait [18].

Variants in Mode of Inheritance

While most CDA's of type I and type II are inherited in an autosomal recessive way, the cases reported by Weatherall et al. [5], Weatherly et al. [11] and Sansone [19] clearly showed a dominant trait. The cases of Weatherall and Weatherly also showed serological abnormalities, the case of Sansone displayed an aberrant (rapidly deteriorating) clinical course.

Variants in Morphology

Variants in morphology are difficult to define. The number of variations of the so-called 'typical' CDA types is so great, that the description of further variants rather seems hazardous (Table 4).

Most of the variants one can select from the literature are 'mixed' forms of CDA I and CDA II morphology. The case of Van Dorpe [16] showed the typical serological findings of the HEMPAS type, but failed to show the bone marrow

Table 4

Variants in morphology. Data of 16 patients, belonging to 9 different families. Morphological data designate electron microscopical (EM) findings

Reference	No. of patients (family)	Morphology (EM)	Expression of antigens: HEMPAS and i
Weatherall [5]	6 (1)	II (only 2–4% binuclears) Inclusions (Hb)	HEMPAS−/i +
Morgenstern	1 (1)	I–II	HEMPAS+/i +
Weatherly [11]	1 (1)	II (Vacuolization)	HEMPAS−/i −
Rozman	1 (1)	I–II	HEMPAS+/i +
Dvilansky [6]	1 (1)	I–II	HEMPAS−/i −
Seip [7]	1 (1)	I–II	HEMPAS−/i +
Van Dorpe [16]	2 (1)	II	HEMPAS+/i +
Kenny [12]	1 (1)	I–II (Vacuolization)	HEMPAS−/i −
Papayannis [20]	2 (1)	I Inclusions Absence centrioles	HEMPAS−/i −

binuclearity. The electronmicroscopical study, however, disclosed the presence of the characteristic 'double' membranes or peripheral cisternae in the erythroblasts.

The case of Weatherall et al. [5] fitted the HEMPAS type, but only 2 to 4 per cent of the erythroblasts showed binuclearity, where one would expect 10 to 30 per cent binuclears. All family members of the Weatherall patient showed large inclusion bodies in the normoblasts, in the marrow and after splenectomy in the peripheral blood as well.

Recently, Stephanoudaki-Sofianatou et al. [20] also described siblings who showed similar haemoglobin-like inclusions. These cases, however, differed in several aspects: they had a typical type I morphology (instead of II), did not show an inbalanced chain synthesis and they did not follow a dominant inheritance. Careful ultrastructural examination disclosed the absence of centrioles in mitotic erythroblasts. While the presence of inclusion bodies in the presence of excess alpha chain production can easily be understood, the haemoglobin inclusions in the last-mentioned cases are more difficult to explain. They were perhaps the result of 'dys-maturation', the haemoglobinization being out of phase with the enzymatic system. In the absence of e. g. the methaemoglobin reductase system, methaemoglobin inclusion bodies could have easily been formed.

Another type of cytoplasmic inclusion bodies has been reported by Weatherly et al. [11] and Kenny et al. [12]: striking red cell and erythroblast vacuolization, with typical myelin figures on electronmicroscopical examination. These probably represent autophagocytosis of degenerating cytoplasmic material.

A number of other minor morphological variants have been excluded from this category, because we considered the borders of the classical morphology sufficiently loose to finally fit them into one or the other type.

Clinical Variants

Clinical variants are difficult to define. Whereas the classical clinical features of CDA include chronic anaemia, variable jaundice, some degree of hepato- and/or splenomegaly and eventually haemochromatosis in the older patients, a number of rare clinical aberrations have been described, most often in connection with some other variations of the classical CDA phenotype.

Independently, Heimpel et al. [21], Clauvel et al. [22] and Jean et al. [23] reported the occurrence of membraneous syndactylies in some forms of CDA, while Clauvel and Erlinger [24] reported the association of CDA and Dubin-Johnson syndrome, an equally rare type of congenital conjugated hyperbilirubinaemia.

The occasional occurrence of mental retardation is mentioned by a number of authors, but no exact data nor an exact description of the kind of mental lesion are available [25]. The patient described by Lowenthal and colleagues [10] suffered from severe tophaceous gout, which previously had not been reported in CDA.

Of particular interest is the patient described by Homberg et al. [26], who was diagnosed in early infancy as having typical CDA type I, but who also showed skeletal anomalies typical of congenital hypoplastic anaemia of the Fanconi type with involvement of the radial or preaxial ray. Recently, Gasser [27] reported two non-related cases, both CDA I, with skeletal malformations this time restricted to the postaxial ray. The association of CDA and skeletal malformations usually seen in Fanconi or Diamond syndromes, suggests possibly related genetic aberrations between congenital dysplastic and congenital hypoplastic anaemias.

A number of conditions have been described where there seems to be considerable overlap between quantitative and qualitative deficient erythropoiesis [28]. The ultimate variants of the classical CDA seem to belong to this intermediate category.

During the last years we had the opportunity to follow two of these patients. They are unrelated examples of a complex congenital disorder in which qualitatively and quantitatively grossly aberrant haematopoiesis is linked to congenital dyskeratosis and severe peripheral neuropathy, typical of Charcot-Marie-Tooth disease [29]. Morphologically they rather fitted the CDA type II classification, but with negative HEMPAS-serology, while anti-i-agglutinability was definitely increased. These cases will be published in detail elsewhere.

Conclusion

Finally, it should be emphasized that numerous authors have stressed the presence of structural abnormalities in the granulocytic and thrombocytic series of patients with classical CDA [10, 31] and recently the group of Breton-Gorius [30] have presented *in vitro* evidence for the occurrence of the defect at least at the BFU_E level. Moreover, the cases in which a transition between dysplastic and hypoplastic affection of bone marrow function are described, illustrate and support the idea that the primary genetic defect of CDA lies at the stem cell level.

Thorough study and adequate analysis of rare variants may contribute to the ultimate understanding and physiopathological elucidation of the primary disease. Therefore, it seems important to collect more data on the unusual presentations of congenital dyserythropoietic anaemias.

We should, however, realize that the exact limits of the CDA syndrome will probably not lie within presently known morphological, clinical, biochemical, genetical or serological boundaries. The *in vitro* techniques might in the future provide the answers people have been looking for in the past decade.

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Analysis of Criteria in Staging in Multiple Myeloma

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One hundred and thirty patients suffering from multiple myeloma were reviewed for retrospective study of their classification according to Durie and Salmon's staging system. No significant difference was found in survival between patients in stage I, II and III. Since then, each criterion developed by Durie and Salmon was evaluated separately. Only the Hb level showed a significant influence; a Hb level under 8.5 g/dl or corrected calcaemia above 3 mmol/l were never found separately. When survival according to the number of criteria was considered there was no difference. These findings agree with the well-founded classification of Durie and Salmon but the real importance of each criteria is questionable.

Key words: multiple myeloma, prognosis, staging

*

The clinical staging system recently developed by Durie and Salmon [5, 7] for multiple myeloma (MM) achieved wide-spread acceptance [2, 6, 9]. We studied retrospectively 130 charts of MM and failed to find any correlation between prognosis and stages I, II or III. Since then, we evaluated separately each criterion developed by Durie and Salmon for stage III, *viz.* the Hb level, calcaemia corrected for albuminaemia, M component level and extent of bone lesions.

Patients and Methods

Three institutions contributed a total of 130 cases to the study. All the patients fulfilled the usually accepted criteria for MM [3]. The study began in September, 1974, and admission of new patients was terminated in September, 1978. For each patient the records included the Hb level, calcaemia corrected for albuminaemia, skeletal X-rays, M component level, search and quantitation of Bence-Jones proteinuria (BJP), creatininaemia and BUN. All the patients were given orally the same treatment [1]: melphalan 0.25 mg/kg and prednisone 2 mg/kg during four days, every sixth week until death. Of these patients, 45 discontinued their treatment and consequently were regarded as 'non-treated'. Patients were classified using the method of Durie and Salmon [5, 7] and subgrouped according to the presence or absence of renal failure (serum creatinine $\geq$ 170 mmol/l, and/or

BUN $\geq$ 9 mmol/l). They were regarded as 'early death' if they died less than 3 months after staging.

Life tables were drawn using the computer AIDE-PLD developed by J. P. Chantalou (Hôpital La Salpétrière, Paris). The significance of the differences in survival of the sample groups was assessed by the logrank test, and the correlation between the different variables was calculated.

Results

Median survival (MS) of the 130 patients under investigation was 22.0 months.

Table 1 shows the patients' classification according to stage and substage and the distribution of substages within the treated and untreated patients. There was no significant difference (data not shown) in survival of groups I, II and III (MS, 24.8, 32.1 and 31.2 months, respectively). Each criterion of classification was assessed separately (Table 2). Figures 1*a*, 1*b*, 1*c*, 1*d*, 1*e*, show survival to Hb level, X-ray bone lesions, M component level, calcaemia corrected for albuminaemia and Bence-Jones proteinuria. Of the parameters affecting survival, only the Hb level

Table 1

Clinical staging and substaging of 130 multiple myeloma patients

Stage no.	No. of patients	Renal function	+/−	MS, months
I	27	A 23	11/12	A + B : 25
		B 4	3/0	
II	29	A 26	15/11	A + B : 32
		B 3	3/0	
III	74	A 54	42/12	A + B : 17
		B 20	10/10	A : 32
				B : 9

+/− : treated/untreated, MS: median survival

(Fig. 1*a*) showed significant differences between the groups (MS, 32.3 months, 15.2 months and 9.6 months, and a Hb level above 10 g/dl, between 8.5 and 10 g/dl, and under 8.5 g/dl, respectively). There was no difference according to X-ray bone lesions (MS, 24.2 months, 16.3 months and 20.7 months, respectively, for less than one osteolytic bone lesion, 1 to 10 osteolytic bone lesions or more than 10 osteolytic bone lesions; Fig. 1*b*), nor according to the M component level (MS, 26.2 months, 20.1 months and 17.7 months for IgG or IgA levels, respectively, $<$ 30 g/l for IgA or 50 g/l for IgG, between 50 and 70 g/l for IgG or 30 to 50 g/l for IgA and $\geq$ 70 g/l for IgG or $\geq$ 50 g/l for IgA; Fig. 1*b*).

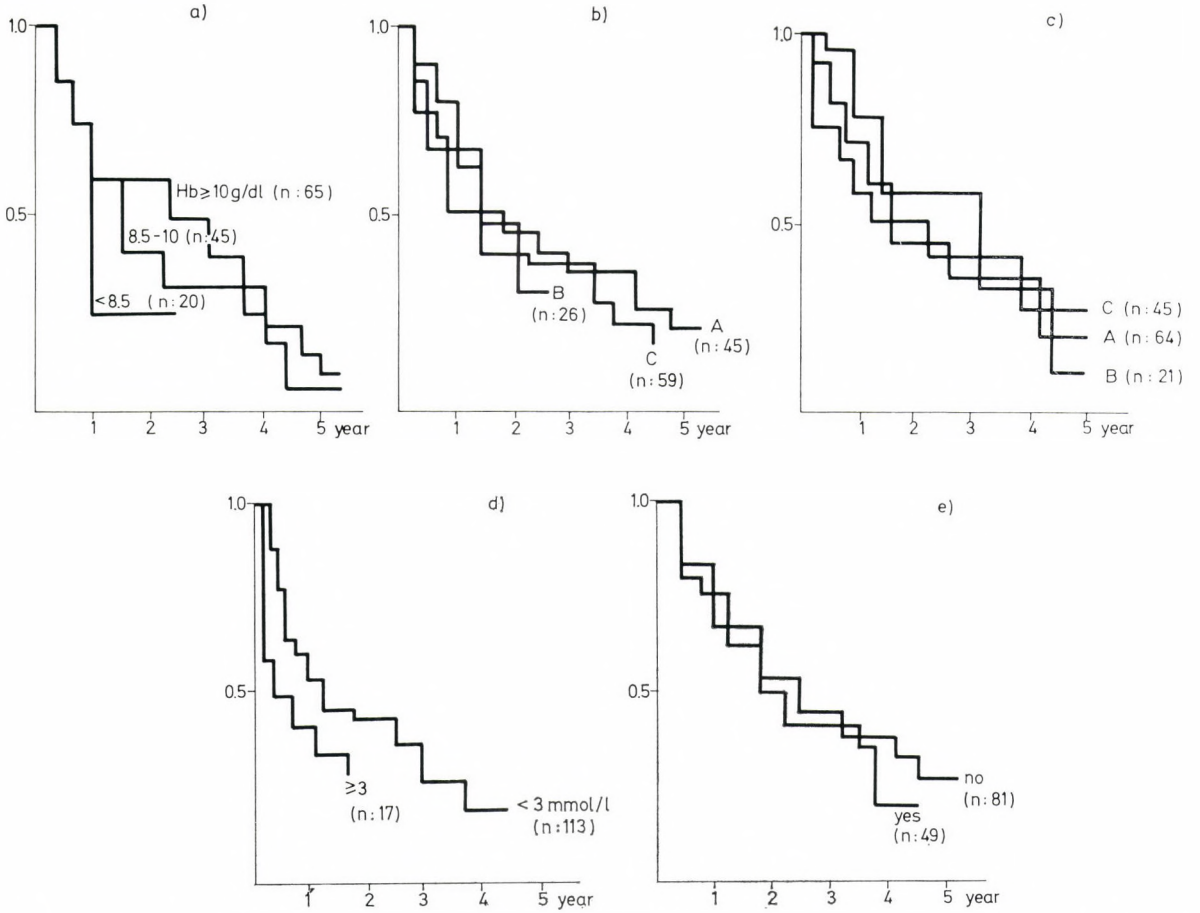

Fig. 1. Life-table analysis according to: *(a)* Hb level: >10 g/dl, median survival (MS) = 31.8 months, VS <8.5 g/dl, MS = 9.1 ($p < 0.04$); *(b)* M component level: A. IgG <50 g/l or IgA <30 g/l, MS = 26.2 months. B. IgG 50–70 g/l or IgA 30–50 g/l, MS = 20.1. C. IgG >70 g/l or IgA >50 g/l, MS = 17.7; *(c)* X-ray bone lesions: A. <1 osteolytic bone lesion MS = 24.2 months. B. 1–10 osteolytic bone lesions, MS = 16.3. C. <10 osteolytic bone lesions, MS = 20.7; *(d)* Corrected calcaemia: >3 mmol/l, MS = 7.0 months. VS <3 mmol/l, MS = 17.1; *(e)* Bence-Jones proteinuria: yes, MS = 15.3 months, VS no, MS = 24.0

Table 2

Survival and observed/expected deaths according to each criterion of Durie and Salmon's classification

Criteria		Stage no.	No. of patients	MS, months	O	E	O/E	Chi square	P
Hb	> 10 g/dl	I	65	32.3	13	8.26	1.57	6.46	0.0396
	8.5–10	II	45	15.2	33	27.52	1.20		
	< 8.5	III	20	9.6	41	51.22	0.80		
Calcaemia	≤ 3 mmol/l	I + II	113	17.1	13	9.54	1.36	1.49	0.2228
	> 3	III	17	7.0	73	76.46	0.95		
X-rays	≤ 1 geode	I	45	24.2	29	30.12	0.96	0.69	0.7083
	1–10	II	21	16.3	9	11.07	0.81		
	> 10	III	64	20.7	42	38.81	1.08		
M-comp	low	I	45	26.2	31	29.74	1.04	1.89	0.5964
	median	II	26	20.1	15	16.94	0.89		
	high	III	59	17.7	37	36.32	1.02		

MS: median survival, O: number of observed deaths, E: number of expected deaths

On the other hand, a difference was noted between the subgroups according to corrected calcaemia MS, 17.1 months and 7.0 months, respectively, for calcaemia under 3 mmol/l and above 3 mmol/l; Fig. 1*d*). BJP was not significantly related to survival (MS, 15.3 months and 24 months, respectively, with and without BJP; Fig. 1*e*). Therefore, we wanted separately to consider the importance of each criterion of the classification. Only stage III was taken into account since a single criterion is sufficient to consider a patient to belong to stage III. Table 3 shows the association between these different criteria. Of the 74 patients belonging to stage III,

Table 3

Association between the different criteria in stage III patients

No. of criteria	No. of patients	
1	21	18 X-ray bone lesions 3 M component
2	30	
3	19	
4	4	
Total	74	

21 were classified then on the basis of one criterion (18 because of X-ray bone lesions, 3 because of high M component level). Thirty patients presented two criteria, 19 with three and only 4 with four. Table 4 shows the relationship between the four criteria. When survival was considered according to the number of criteria, no difference was found (MS, 10.3 months, 15.4 months and 11.1 months for one, two or three associated criteria, respectively; Fig. 2).

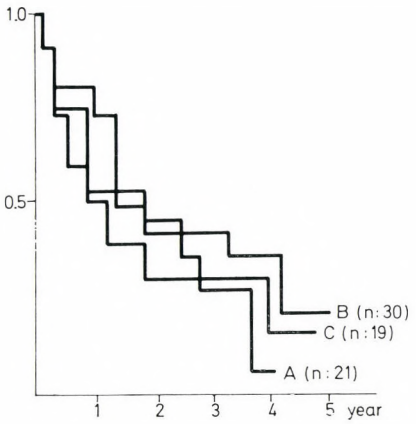

Fig. 2. Life-table analysis according to the number of criteria present in stage III: A.One criterion, MS = 10.3 months. B. Two criteria, MS = 15.4. C. Three criteria, MS = 11.1

Table 4

Relationship between the four criteria

	Hb	X-ray bone lesions	M component	Ca
Hb	0			
X-ray bone lesions	33	18		
M component	20	31	3	
Ca	3	3	2	0

Discussion

Survival in the above series was nearly in agreement with most previous reports [2, 4, 9]. Contrary to other findings, survival was not significantly different in the groups I, II and III. Of the four staging criteria, the Hb level was the only one which by itself proved to be of prognostic significance. Conversely, corrected calcaemia, X-ray bone lesions, M component level or BJP did not characterize survival. This observation failed to correspond to other findings [2, 4–6, 9]. In addition it appears that of the four criteria, a Hb level of < 8.5 g/dl or calcaemia

> 3 mmol/l were never found separately whereas this was not the case with the X-ray bone lesions or the M component level. Moreover, analysis of survival according to the number of criteria also failed to show differences.

These findings on the whole agree with the well-founded classification of Durie and Salmon but the real importance of the single criteria is more than questionable.

With this in view we think that other criteria such as for instance bone marrow plasmocytosis should also be taken into account, its value being related to survival [8].

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A Comparison of Fc-Receptor-Bearing Lymphocytes in Normal Individuals and in Patients with Lymphoproliferative Diseases, Using EA Tests with Rabbit and Human-Sensitizing Antibodies

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A comparison between the percentage of rosettes in EA tests, using rabbit and human-sensitizing antibodies and the depletion experiments of rosette-forming lymphocytes, showed that both systems can be used equally well to detect Fc-receptors on mature peripheral blood lymphocytes. The observations made in B-chronic lymphocytic leukaemia and immunocytoma patients suggested, however, that both EA tests determine lymphocytes that differ in Fc-receptors from normal lymphocytes. The possibility that the differences in Fc-receptors are connected with the stage of maturation of lymphocytes is discussed.

Key words: EA_{human}, EA_{rabbit} tests, Fc-receptor-bearing lymphocytes, lymphoproliferative diseases, Ripley-like serum

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Ripley serum (anti-CD), unique for its ability of complement-binding, was introduced by Natvig and Frøland [17] to the rosette technique with human erythrocytes (EA_{hum} test) in order to detect Fc-receptor-bearing lymphocytes. It has been found that some other anti-Rh sera can also be used in EA_{hum} test, but Ripley serum was regarded to be the best one [12, 17, 23]. Recently, Maślanka and Żupańska [16] have detected that the usefulness of anti-Rh sera in this test does not depend on the quality of antibodies and their ability to bind complement but on the amount of antibodies absorbed onto red cells.

Natvig and Frøland [17] suggested that the EA_{hum} test specified a population of lymphocytes different from that determined in the test with SRBC sensitized with rabbit serum (EA_{ra} test), a 'third population of lymphocytes', which have Fc-receptors but no surface immunoglobulins.

According to Lobo and Horwitz [14] the 'third population' is identical with the L-lymphocytes, because of the membrane-labile Ig which is bound by avid Fc-receptors and is easily released. Arbeit et al. [1] showed that red cells sensitized with Ripley serum bind to cells with avid receptors, whereas SRBC sensitized with rabbit serum bind to both L-lymphocytes and B cells. This would be in agreement with the observations of Pang and Wilson [18] who reported that the EA_{ra} formed consistently more rosettes than the EA_{hum} test.

A new approach has recently been worked out by Dunne et al. [6]. Rosette formation in the EA_{hum} and EA_{ra} test with ox red cells showed that both systems gave a similar number of rosettes. In depletion and inhibition experiments they

indicated that human and rabbit IgG are equivalent in the system and can be used equally well to detect Fc-receptor-bearing lymphocytes.

The aim of the present study was to compare the rosetting of lymphocytes from healthy individuals and from patients with different lymphoproliferative diseases, using human red cells sensitized with human serum and sheep red cells sensitized with rabbit serum (EA_{hum} and EA_{ra} tests).

Material and Methods

Lymphocytes for rosette tests were separated from defibrinated peripheral blood of 45 normal individuals and of 88 patients with lymphoproliferative diseases: 33 patients with Hodgkin disease (HD), 38 with chronic lymphocytic leukaemia (CLL), and 17 patients with non-Hodgkin lymphoma (NHL). In most cases the investigation was performed two to five times.

In all patients the diagnosis was confirmed histologically. The histological classification of NHL was done according to Lennert [13]. Among NHL of low-grade malignancy there were 5 patients with immunocytoma (IM) and one with centrocytoma. Among patients with NHL of high-grade malignancy there were 5 with centroblastic, 2 with lymphoblastic and 4 with immunoblastic lymphoma without a leukaemic picture. CLL in all cases was of B-cell origin diagnosed by the E and SIg tests [21]. Clinical staging of CLL was assessed according to Rai et al. [20]. All patients with HD were investigated during remission, patients with NHL prior to any treatment. Of the CLL patients, 20 were under treatment.

Separation of lymphocytes was performed by Ficoll-Isopaque gradient centrifugation [3]. Phagocytes were removed by carbonyl-iron powder (Fluka, Buchs). The lymphocyte suspensions contained 95 to 100 per cent viable cells, while the content of nucleated cells other than lymphocytes never exceeded 5 per cent.

The EA_{hum} rosette test was performed according to Natvig and Frøland [17]. For sensitization of human red cells (HRBC) undiluted Ripley-like serum was used as described previously [16].

The EA_{ra} rosette test was carried out with sheep erythrocytes (SRBC). SRBC were sensitized with a subagglutination dose of IgG antibodies (previously fractionated on Sephadex G-200 at 37 °C) for 30 min. 0.5 ml of sensitized SRBC were mixed with an equal volume of 10^6 lymphocytes in Hank's medium (Biomed, Lublin, Poland), centrifuged at 200 *g* for 3 min and incubated at 37 °C for 30 min. Two-hundred lymphocytes were examined in the preparations and the percentage of rosetting cells was calculated.

The E and EAC tests were performed with SRBC according to WHO/IARC Workshop [21]. For EAC test a subhaemolysing dose of rabbit serum (Cracow Laboratories of Sera and Vaccines) and mouse complement were used.

Depletion studies were performed by differential cent rifugation of lymphocytes rosetting in the EA_{hum} or EA_{ra} test. Equal volumes of lymphocytes (2×10^6

cells/ml) and 1 per cent suspension of either HRBC or SRBC were rosetting in EA_{hum} or EA_{ra} test, respectively. The rosette suspension was layered onto Ficoll-Isopaque gradient and centrifuged at 1000 *g* at room temperature for 10 min. The interface cells were removed, washed twice in PBS with 1 per cent FCS and re-rosetted in the EA_{hum} or EA_{ra} test.

To establish the relationship between EA_{hum} test and rosette tests with SRBC correlation coefficients were calculated by the method described by Ladosz [15].

Results

Figure 1 shows the percentage of rosettes in the EA_{hum} test and in tests with SRBC in normal individuals. The correlation was observed only between EA_{hum} and EA_{ra} tests. Correlation coefficient, EA_{hum} rosettes *vs.* EA_{ra}, was $r = 0.59$,

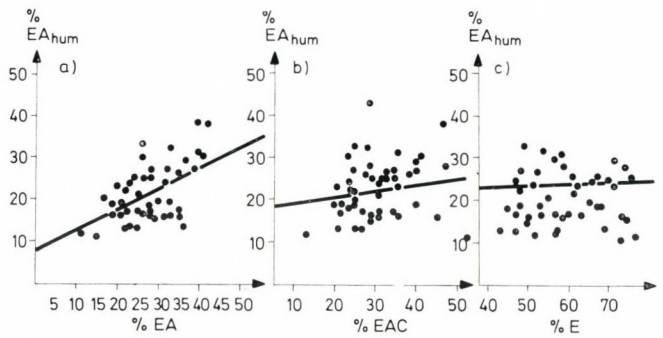

Fig. 1. Percentage of rosettes in the EA_{hum} and rosette tests with SRBC in healthy persons

while *vs.* EAC was $r = 0.16$ and *vs.* E rosettes $r = 0.07$. No significant difference was found between the mean values obtained by both EA tests, the EA_{hum} giving a mean of 24.0 ± 6.7 per cent and the EA_{ra} with 27.5 ± 7.4 per cent (Fig. 2).

Experiments were performed to detect whether depletion of cells rosetting with either EA_{hum} or EA_{ra} would also deplete lymphocytes that bind in the other system. Table 1 shows that depletion of lymphocytes with the EA_{ra} reduced not only the number of lymphocytes rosetting with the EA_{ra} but also, to a similar extent, the number of cells bound in the EA_{hum} test. Similarly, depletion of lymphocytes with EA_{hum} reduced the number of lymphocytes rosetting in both systems. This again indicates that both EA systems are binding to the same lymphocytes.

The correlation between the EA tests was observed not only in healthy individuals but also in patients with HD and NHL of high-grade malignancy. The following correlation coefficients were observed: EA_{hum} rosettes *vs.* EA_{ra} rosettes in

Table 1

Depletion of lymphocytes forming EA_{hum} and EA_{ra} rosettes

Exp.	Depletion method	Rosettes, %			
		Pre-depletion		Post-depletion	
		EA_{ra}	EA_{hum}	EA_{ra}	EA_{hum}
I	EA_{ra}	35	32	1.5	0
II	EA_{ra}	28	25	5	0.5
III	EA_{hum}	30	22	5	2
IV	EA_{hum}	27	23	3.5	1

HD was $r = 0.64$, in NHL of high-grade malignancy $r = 0.87$ (Figs 3, 4). The means were within the normal range (Fig. 2). The correlation was not observed in CLL patients and in patients with NHL of low-grade malignancy which was mainly

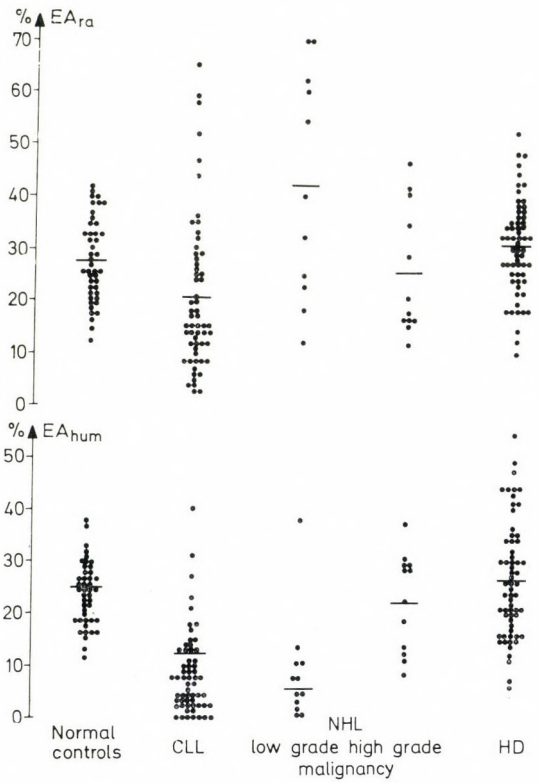

Fig. 2. Percentage of rosettes obtained in EA_{hum} and EA_{ra} tests in healthy persons and in lymphoproliferative diseases

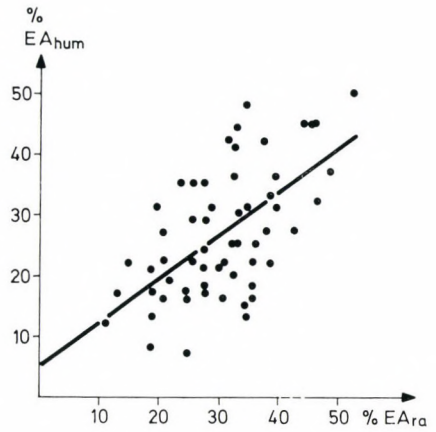

Fig. 3. Percentage of rosettes in the EA_{hum} and the EA_{ra} test in Hodgkin disease

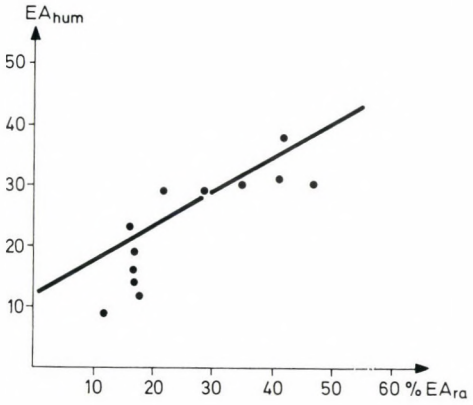

Fig. 4. Percentage of rosettes in EA_{hum} and EA_{ra} tests in non-Hodgkin lymphoma of high grade malignancy

immunocytoma. The correlation coefficients were: $r = 0.04$ and $r = -0.46$ (Figs 5, 6). The mean values were outside the normal range (Fig. 2).

The CLL patients fell into two groups (Table 2). The first group (17 patients) with a normal or elevated percentage of EA_{ra} rosettes and with EA_{hum} rosettes far below the normal amount (< 10 per cent) gave a result similar to that observed in NHL of low-grade malignancy (Fig. 2). In the second group (18 cases) the rosettes were less than 15 per cent in both tests. The different pattern of EA tests in both groups was not related to the period of disease.

Table 2

EA_{hum} and EA_{ra} tests in CLL patients

Clinical staging	No. of patients with	
	EA_{ra}% normal or elevated EA_{hum}% < 10	EA_{ra}% < 15 EA_{hum}% < 15
0	1	2
I	6	5
II	5	4
III	3	4
IV	2	3

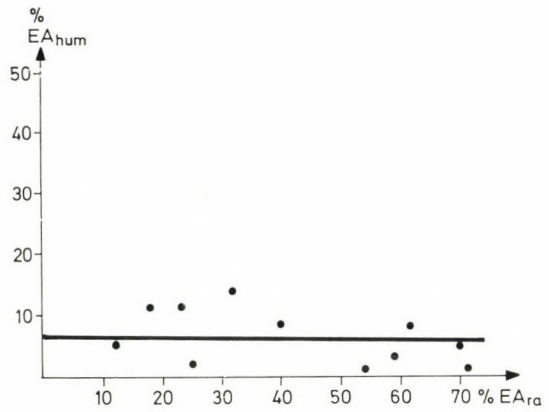

Fig. 5. Percentage of rosettes in EA_{hum} and EA_{ra} test in non-Hodgkin lymphoma of low grade malignancy

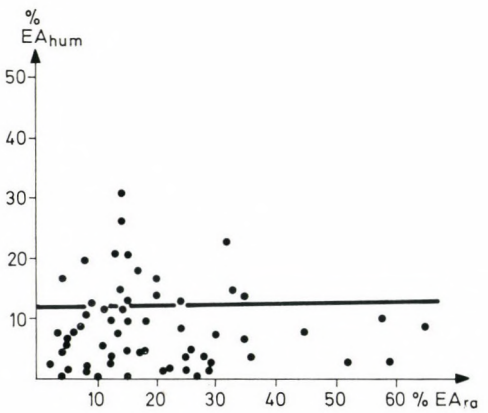

Fig. 6. Percentage of rosettes in EA_{hum} and EA_{ra} test in chronic lymphocytic leukaema

Discussion

The observation that both EA systems (SRBC sensitized with rabbit and HRBC sensitized with human serum) gave a similar number of rosettes and that depletion of EA_{hum} or EA_{ra} rosetting cells caused a reciprocal depletion of cells binding to the other, confirmed the results of Dunne et al. [6], who showed that rabbit and human antibodies were bound to the same lymphocytes. The difference was that they used ox erythrocytes instead of sheep red cells.

In B-CLL patients and in patients with immunocytoma, however, a discrepancy was observed between the two EA tests. In about 50 per cent of patients with B-CLL and in all patients with IM, the percentage of rosettes in the EA_{ra} test was within or even above the normal range, but in the EA_{hum} test the percentage was very low. In the remaining 50 per cent CLL patients both EA tests gave few rosettes. These observations suggest that in some patients with CLL and with IM both EA tests discover lymphocytes that differ in Fc-receptors.

Our observations are difficult to compare with other reports because there are few data concerning Fc-receptor-bearing lymphocytes in CLL; besides, they vary with the different investigators and depend on the marker substrate used (IgG sheep EA, IgG human EA, IgG ox EA, aggregated IgG) [2, 5, 7, 24]. The observations by Dickler et al. [5] also suggested that the quantity of Fc-receptors expressed on CLL cells differ from patient to patient while that was not the case in normal subjects. Also, according to the C3 receptors, CLL patients can be divided into two groups (with C3d and C3b + C3d), while normal individuals have always both receptors on their lymphocytes [13].

The question is to what extent the CLL or IM cells differ from normal lymphocytes and why they differ in CLL from one patient to the other. Our data suggest that the differences between patients were not connected with the clinical staging. This would be in agreement with observations of Foa et al. [8] who found that throughout the different stages in CLL immunological markers remained unchanged.

The differences might rather be connected with the stage of lymphocyte maturation. The nature of lymphocytes in B-CLL is not clear, but it has been suggested that they are arrested in the non-secretory stage of differentiation. The small amount of SIg on lymphocytes and their capacity to form mouse E rosettes, allow to interprete CLL as a proliferation of cells that are blocked at an early stage of maturation [4, 19]. It is not certain whether the arrest of maturation always occurs at the same stage. Although it is thought that in most cases the cells in B-CLL derive from B1 lymphocytes, one cannot exclude the possibility of a B2 cell origin in some cases. Lymphocytes of B2 origin probably form an immunocytoma, such cells being able to mature to the secretory stage [19].

Taking into consideration our knowledge about the cells in CLL and IM together with our observations concerning EA tests, there might be some differences in Fc-receptors that depend on the maturation of lymphocytes. Such discrepancies might be related to the different proportions of Fc-receptors of high and low avidity

or density during the process of cell differentiation. It might be supposed that in the early stage of maturation, lymphocytes have only few receptors, and that is why the percentage of rosettes is low in both EA tests. More mature lymphocytes would have already Fc-receptors of low avidity or density, which are detected in the EA_{ra} but not in the EA_{hum} test. Finally, the mature, normal lymphocytes would have Fc-receptors of high and low avidity or density and that is why the amount of rosettes is similar in both tests.

The heterogeneity of Fc-receptors on human peripheral normal mononuclear cells has been suggested by several authors [1, 10, 13, 22]. Sándor et al. [22] and Gergely et al. [10] found lymphocytes with low and high cellular avidity by means of shedding easily and not shedding Fc-receptors under similar conditions. Different Fc-receptors seem to appear during maturation of the cell, considering the observations that surface markers develop on lymphocytes during their differentiation [11], especially in view of the observations of Gathings et al. [9] that Fc-receptors are lacking on most of the pre-B cells.

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Abstracts

Accumulation of phosphate esters and decline of ATP in red cells incubated in vitro is caused by lack of pyruvate. I. Rapoport, S. Rapoport and R. Elsner (Institut für Physiologische und Biologische Chemie der Humboldt-Universität zu Berlin, Berlin, DDR). *Acta Biol. Med. Germ. 40*, 115 (1981).

Addition of pyruvate to human red cells incubated *in vitro* at alkaline pH values inhibits the accumulation of phosphate esters otherwise observed as well as the decrease of ATP and the corresponding increase of ADP and AMP. The increase of glycolysis at alkaline pH values is only partly due to a pH effect *per se*. About one-half of it is caused by a partial release of the inhibition of phosphofructokinase by a lowering of the ATP concentration and particularly by the increase of AMP. Only in the presence of pyruvate there occurs an increase of 2,3-diphosphoglycerate, such as is observed *in vivo*, which is balanced by the consumption of pyruvate. At alkaline pH values in the absence of pyruvate there is a low movement from the fructose-1,6-diphosphate + triosephosphates pool to the components of the pentose phosphate pathway, mostly by way of recombination from glyceraldehyde-phosphate.

B. Sarkadi

Ca^{2+}-induced biochemical changes in human erythrocytes and their relation to microvesiculation. D. Allan and P. Thomas (Department of Experimental Pathology, School of Medicine, University College London, London, U. K.). *Biochem. J. 198*, 433 (1981).

Human erythrocytes were treated with Ca^{2+} and ionophore A23187 and measurements were made of K^+ efflux, polyphosphoinositide breakdown, 1,2-diacylglycerol accumulation, phosphatidate synthesis, changes in membrane polypeptide pattern and release of microvesicles. It was shown that neither transamidase-mediated protein cross-linking, proteolysis of polypeptides 2.1 (ankyrin) or 4.1, nor accumulation of diacylglycerol or phosphatidate appeared to be necessary for microvesiculation to occur. Microvesicles were released only under conditions where KCl efflux leading to cell shrinkage occurred and where polyphosphoinositides were broken down. These circumstances were sufficient to cause microvesiculation only in the presence of increased intracellular concentrations of Ca^{2+}.

B. Sarkadi

The effects of Ca^{2+} and Sr^{2+} on Ca^{2+}-sensitive biochemical changes in human erythrocytes and their membranes. D. Allan and P. Thomas (Department of Experimental Pathology, School of Medicine, University College London, London, U. K.). *Biochem. J. 198*, 441 (1981).

The Ca^{2+}-dependency of K^+ efflux, microvesiculation and breakdown of polyphosphoinositides and of ankyrin have been measured in intact human erythrocytes exposed to ionophore A23187 and HEDTA [N'-(2-hydroxyethyl)ethylenediamine NNN'-triacetate]-Ca^{2+} buffers. Half-maximal responses were observed at pCa values of 6.4, 4.1, 5.0 and 4.8, respectively. The Ca^{2+} dependencies of

K^+ efflux and breakdown of polyphosphoinositides and ankyrin measured in erythrocyte ghosts without addition of ionophore showed almost identical values with those seen in whole cells treated with ionophore. It is concluded that ionophore A23187 is able to cause rapid equilibration of extracellular and intracellular [Ca^{2+}] in intact cells and that in the presence of a suitable Ca^{2+} buffer, ionophore A23187 can be used to precisely fix the intracellular concentration of Ca^{2+} in erythrocytes. The relatively high concentration of Ca^{2+} required to produce microvesiculation in intact cells may indicate that microvesiculation could be at least partly dependent on a direct interaction of Ca^{2+} with phospholipid. Results obtained with Sr^{2+} paralleled those with Ca^{2+}, although higher Sr^{2+} concentrations were required to achieve the same effects as Ca^{2+}. Mg^{2+} produced none of the changes seen with Ca^{2+} or Sr^{2+}.

B. Sarkadi

Electron microscopic study of reassociation of spectrin and actin with the human erythrocyte membrane. S. Tsukita, Sh. Tsukita, H. Ishikawa, Sh. Sato and M. Nakao (Department of Anatomy, Faculty of Medicine, University of Tokyo and Department of Biochemistry, Faculty of Medicine, Tokyo Medical and Dental University, Tokyo, Japan). *J. Cell Biol. 90*, 70 (1981).

Reassociation of spectrin and actin with human erythrocyte membranes was studied by stereoscopic electron microscopy of thin sections combined with tannic acid–glutaraldehyde fixation. Treatment of the erythrocyte membrane with 0.1 mM EDTA (pH 8.0) extracted >90 per cent of the spectrin and actin and concomitantly removed filamentous meshworks underlying the membranes, followed by fragmentation into small inside-out vesicles. When such spectrin-depleted vesicles were incubated with the EDTA extract (crude spectrin), a filamentous meshwork, similar to those of the original membranes, was reformed on the cytoplasmic surface of the vesicles. The filamentous components, with a uniform thickness of 9 nm, took a tortuous course and joined one another often in an end-to-end fashion to form an irregular but continuous meshwork parallel to the membrane. Purified spectrin was also reassociated with the vesicles in a population density of filamentous components almost comparable to that of the crude spectrin-reassociated vesicles. However, the meshwork formation was much smaller in extent, showing many independent filamentous components closely applied to the vesicle surface. When muscle G-actin was added to the crude spectrin- or purified spectrin-reassociated vesicles under conditions which favor actin polymerization, actin filaments were seen to attach to the vesicles through the filamentous components. Two modes of association of actin filaments with the membrane were seen: end-to-membrane and side-to-membrane associations. In the end-to-membrane association, each actin filament was bound with several filamentous components exhibiting a spiderlike configuration, which was considered to be the unit of the filamentous meshwork of the original erythrocyte membrane.

G. Gárdos

The action of organic mercurials on the erythrocyte membrane. G. B. Ralston and E. A. Crisp (Department of Biochemistry, University of Sydney, Sydney, Australia). *Biochim. Biophys. Acta 649*, 98 (1981).

The solubilisation of proteins from erythrocyte membranes by treatment with organic mercurials has been studied with different species. The marked solubilisation previously reported for human membranes does not seem to be a general phenomenon. All of the other species examined showed less than 50 per cent of the solubilisation shown by human membranes. The protein-solubilizing effect seems to be dependent on hydrophobic mercury derivatives carrying a net negative charge. Uncharged compounds like phenylmercuric acetate blocked the effect, although N-ethylmaleimide and iodoacetamide did not. With the aid of radioactively labelled compounds, and of atomic absorption spectrophotometry, the proteins reactive towards the mercurials were identified. The major integral protein, band 3, was the major protein capable of binding the mercurial. Reaction with the mercurial appears

to disrupt interaction of band 3 with bands 2.1 and 4.2, allowing dissociation of the cytoskeleton from the membrane. In addition, band 4.9 was also found to react with the mercurials, possibly resulting in disruption of the cytoskeleton.

G. Gárdos

Biosynthesis of the erythrocyte anion transport protein. W. A. Braell and H. F. Lodish (Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA). *J. Biol. Chem. 256*, 11 337 (1981).

The biosynthesis of the erythrocyte anion transport protein (Band III) was studied in erythroid precursor cells obtained from the spleens of anemic mice. Newly synthesized Band III was inserted during or immediately after translation into rough endoplasmic reticulum membranes. The asymmetric orientation of Band III in these membranes resembled that of mature Band III in erythrocyte membranes, with the NH_2-terminal portion of the molecule facing of cytoplasm. At this stage Band III contained a high mannose core oligosaccharide, which was susceptible to cleavage by endoglycosidase H. During the next 20 to 30 min, this oligosaccharide was processed to a form resistant to endoglycosidase H degradation, presumably in the Golgi complex. The processed Band III was subsequently expressed on the cell surface, at about 30 to 45 min after synthesis. In many respects, therefore, the biosynthesis of Band III resembles that of cotranslationally inserted proteins whose NH_2-terminal portions are exposed on the exterior of the cell, like VSV glycoprotein, HLA-A antigens, and glycophorin.

G. Gárdos

Internal potassium stimulates the sodium-potassium pump by increasing cell ATP concentration. J. R. Sachs (Department of Medicine, State University of New York at Stony Brook, Stony Brook, N. Y., USA). *J. Physiol. 319*, 515 (1981).

Intracellular K increases the ouabain-sensitive Na—K exchange in human red blood cells. Pump rate increases hyperbolically with internal K with a $K_{1/2}$ for K of 2.58 mmol/l red blood cells. Li also stimulates the pump rate, but with a much higher $K_{1/2}$. The stimulation does not result from a change in the affinity of the pump for its substrates Na, K or Mg or for the product, phosphate. The effect of cell K on the Na—Na exchange is biphasic. At low concentrations it decreases the exchange rate but then the exchange increases linearly with cell K concentration. Stimulation of the pump rate by internal K can be demonstrated in reconstituted ghosts but only if the ratio of the volume of cells to that of solution at the time of haemolysis is high. Stimulation is not observed if the ghosts contain an efficient system for rephosphorylating ADP to ATP, such as creatine phosphate and creatine kinase, or if the measurements are made with iodoacetamide which inhibits rephosphorylation of ADP by inhibiting the enzyme glyceraldehyde-3-phosphate dehydrogenase. Cells with low internal K and Li have low ATP concentrations, and ATP increases hyperbolically with internal K or Li with the same $K_{1/2}$ as does the pump rate. In cells depleted of substrate intracellular K does not stimulate the pump rate. The effect of K and Li on the pump rate probably does not result from enhanced activity of any of the enzymes below phosphofructokinase in the glycolytic pathway.

G. Gárdos

Familial aggregation of cation transport abnormalities and essential hypertension. D. Cusi, C. Barlassina, M. Ferrandi, P. Lupi, P. Ferrari and G. Bianchi (Medical Clinic I, School of Nephrology, University, Milano, and Farmitalia Carlo Erba Research Center, Nerviano, Milano, Italy). *Clin. Exp. Hypertension 3*, 871 (1981).

The maximal rate of activity of Na extrusion by the Na pump, Na—K outward cotransport, Na—Li countertransport and the rate constant for passive permeability to Na, K and Li were measured in the RBC of 24 normotensive subjects with both parents normotensive, 45 hypertensive subjects and 24 of their normotensive offspring. The Na extrusion by the Na pump and the passive

permeability to Na, K and Li are equal. The hypertensives have significantly greater Na—Li countertransport and smaller Na—K cotransport when compared to the normotensives. Na—K cotransport and Na—Li countertransport are positively correlated, thus suggesting some relationship between the two systems. When arbitrary normal limits are set the hypertensives are divided in three groups: normal cotransport and countertransport (22.2 per cent), high countertransport (31.1 per cent) and low cotransport (44.4 per cent). In nine hypertensive families studied if either alteration was observed in a hypertensive propositus, this was of the same kind as the one in case observed in any first degree relative, whether already hypertensive or young normotensive. The observed alterations are primitive to the development of hypertension and possibly related to its pathogenesis.

Ilma Szász

Roles of charged groups on the surface of membrane lipid bilayer of human erythrocytes in induction of shape change. A. Tamura and T. Fujii (Department of Biochemistry, Kyoto College of Pharmacy, Yamashina-Ku, Kyoto, Japan). *J. Biochem. 90*, 629 (1981).

Shape changes of human erythrocytes were induced by partial hydrolysis of phospholipids in the membrane lipid bilayer outer leaflet as a result of the action of exogenous phospholipase A_2 or D at pH 7.4 under non-hemolytic conditions. The extent of the shape change caused by phospholipase A_2 or D altered drastically when the cells were put into a medium of more alkaline or acidic pH, whereas the shape of untreated cells and cells treated with phospholipase C showed only a mild dependence on pH. These pH-dependent shape changes of intact and phospholipase-treated cells were reversible. Removal of the majority of exposed sialic acid residues from the membrane outer surface by neuraminidase treatment had no influence on the cell shape, or on the pH-dependence of the shape change. These results suggest the importance of the electric charges of polar groups located directly on the surface of the membrane lipid bilayer in inducing cell shape changes.

Ilma Szász

Effect of local anaesthetics on lymphocyte capping and energy metabolism. C. Montecucco, S. Ballardin, G. P. Zaccolin and T. Pozzan (C. N. R. Unit for Physiology of Mitochondria and Laboratory of Biophysics and Molecular Biology, Institute of General Pathology, University of Padova, Italy). *Biochem. Pharmacol. 30*, 2989 (1981).

Several local anaesthetic and antipsychotic drugs have been tested for their ability to inhibit the capping of s-Ig in mouse spleen lymphocytes and for their effect on the cellular ATP level. Drug concentrations which inhibit capping also lower cellular ATP content below the minimum amount required for a lymphocyte to cap. The two effects show similar kinetics. The ATP depletion caused by these drugs may help to explain their inhibition of lymphocyte capping as well as other effects they cause on living cells.

Ilma Szász

Asymmetry of lipid dynamics in human erythrocyte membranes studied with impermeant fluorophores. U. Cogan and D. Schachter (Department of Physiology, Columbia University College of Physicians and Surgeons, New York, New York, USA). *Biochemistry 20*, 6396 (1981).

The synthesis, purification, and application of five membrane-impermeant derivatives of pyrene are described. Each probe consists of a membrane-impermeant moiety, either an oligosaccharide or glutathione, linked to pyrene *via* a connecting arm. Intact human erythrocytes and leaky ghost membranes prepared from them were treated with the probes to label, respectively, the outer membrane leaflet and both leaflets. Motional freedom of the pyrene fluorophores in the membrane was assessed by estimation of the steady-state polarization of fluorescence, the excited-state lifetime, and the excimer/monomer fluorescence intensity ratio. The fluorescence anisotropy of each impermeant derivative was lower in the outer as compared to the inner hemileaflet, whereas the corresponding excited-state lifetimes were similar. Excimer formation was consistently greater in the outer leaflet. The results demon-

strate that the impermeant fluorophores experience greater motional freedom ('fluidity') in lipid domains of the outer as compared to the inner leaflet of the human erythrocyte membrane.

Ilma Szász

Purification of a trypsin-insensitive fragment of spectrin from human erythrocyte membranes. M. Hanspal and G. B. Ralston (Department of Biochemistry, University of Sydney, Sydney, Australia). *Biochim. Biophys. Acta 669*, 133 (1981).

When spectrin is treated with trypsin, a series of polypeptide fragments is generated. One particular fragment having an approximate molecular weight of 80 000 constitutes 18 per cent of the trypsin-digested mixture and is trypsin-insensitive. This fragment has been isolated and purified by gel filtration followed by ion-exchange chromatography. The molecular weight of the fragment, as seen from sedimentation equilibrium measurements and from gel electrophoresis, both in the presence and absence of detergent, is close to 80 000. There was no evidence of self-association under the conditions used. Changes in the specific rotation at 365 nm were used to detect temperature-dependent conformation changes in the fragment and to compare these changes with those in the intact spectrin molecule. The fragment undergoes temperature-dependent transitions centered at 46 and 58 °C, similar to those in intact spectrin (49 and 55 °C). Although the thermal transitions exhibited by intact spectrin are markedly salt-dependent, those shown by the fragment are not. ORD (optical rotary dispersion) measurements indicate 53 per cent apparent α-helix in the fragment, compared to 68 per cent in intact spectrin. Antibodies raised against the fragment cross-react only with band 1, the largest polypeptide of spectrin, indicating that the fragment is derived from band 1.

Ágnes Enyedi

Effect of piracetam on sickle erythrocytes and sickle hemoglobin. T. Asakura, S. T. Ohnishi, K. Adachi, M. Ozguc, K. Hashimoto, M. T. Devlin and E. Schwartz (Division of Hematology, The Children's Hospital of Philadelphia, and Departments of Pediatrics, Biochemistry and Biophysics and Human Genetics, University of Pennsylvania, Philadelphia, and Biophysics Laboratory, the Department of Anesthesiology, Hahnemann Medical School, Philadelphia, Pennsylvania, USA). *Biochim. Biophys. Acta 664*, 397 (1981).

Piracetam, 2-oxo-1-pyrrolidine acetamide, inhibits sickling of red cells containing sickle hemoglobin (Hb S). The concentration required for 50 per cent inhibition is about 300 mM. Addition of piracetam into the supersaturated Hb S solution in concentrated phosphate buffer prolongs the delay time prior to gelation. Piracetam shifts the oxygen equilibrium curves of blood toward the right, with a stronger effect at higher piracetam concentrations. Piracetam increases the viscosity of oxygenated cells but reduces the relative viscosity of deoxygenated sickle cells. The mechanism for the antisickling effect of piracetam is discussed.

Ágnes Enyedi

Abnormalities in membrane phospholipid organization in sickled erythrocytes. B. Lubin, D. Chiu, J. Bastacky, B. Roelofsen and L. L. M. Van Deenen (Bruce Lyon Memorial Research Laboratory and Department of Haematology/Oncology, Children's Hospital Medical Center, Oakland, California, and Donner Laboratory, University of California, Berkeley, California, USA and Department of Biochemistry, State University of Utrecht, Utrecht, The Netherlands). *J. Clin. Invest. 67*, 1643 (1981).

In contrast to the wealth of information concerning membrane phospholipid asymmetry in normal human erythrocytes, very little is known about membrane phospholipid organization in pathologic erythrocytes. Since the spectrin-actin lattice, which has been suggested to play an important role in stabilizing membrane phospholipid asymmetry, is abnormal in sickled erythrocytes, the effects of sickling on membrane phospholipid organization were determined. Two enzymatic probes were used: bee venom

phospholipase A_2 and *Staphylococcus aureus* sphingomyelinase C, which do not penetrate the membrane and react only with phospholipids located in the outer leaflet of the bilayer. The results suggest that the distribution of glycerophospholipids within the membrane of sickled cells is different from that in non-sickled cells. Compared with the normal erythrocyte, the outer membrane leaflet of the deoxygenated, reversibly sickled cells (RSC) and irreversibly sickled cells (ISC) was enriched in phosphatidyl ethanolamine in addition to containing phosphatidyl serine. These changes were compensated for by a decrease in phosphatidyl choline in that layer. The distribution of sphingomyelin over the two halves of the bilayer was unaffected by sickling. In contrast to ISC, where the organization of phospholipids was abnormal under both oxy and deoxy conditions, reoxygenation of RSC almost completely restored the organization of membrane phospholipids to normal. These results indicate that the process of sickling induces an abnormality in the organization of membrane lipids in RSC which becomes permanent in ISC.

Ágnes Enyedi

News Items

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*

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Contents

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Bone Marrow Biopsy in Clinical Medicine: An Overview

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Bone marrow biopsies are now employed in the investigation of many disorders in haematology, oncology and internal medicine. This review provides a survey of the recent literature and a summary of observations made on undecalcified bone marrow biopsies embedded in plastic. The conditions investigated include osteopathies, myelopathies, haematologic and non-haematologic malignancies in the bone marrow. The interrelationship and interdependence of bone and bone marrow have been emphasized, and examples of the effects of diseases of bone on marrow, and of disturbancies of marrow function on bone, have been given. In the myelo- and lymphoproliferative disorders bone marrow biopsies contribute to diagnostic evaluation and classification, as well as to provide factors of prognostic significance. In the investigation of patients with solid tumours bone marrow biopsy may detect metastases in 20 per cent (bronchus), 35 per cent (prostate), 40 per cent (breast), to 80 per cent (unknown primaries) of the patients. Bone marrow biopsy constitutes an additional investigative parameter capable of providing valuable information in many different clinical situations.

Key words: biopsy, bone lymphoproliferation, marrow, metastases, myelodysplasia, myeloproliferation, osteodysplasia

Introduction

The function of the skeleton is at least 3 fold: 1. it constitutes the means for direct movement and locomotion, providing support and a protective covering for softer vulnerable tissues; 2. it participates in the homeostasis of minerals, and 3. it houses the bone marrow which supplies the formed elements of the blood. Although it has long been known that the bone marrow is affected in certain diseases of bone (anaemia in osteopetrosis and in Paget's disease), and that osseous changes may occur in various disorders involving primarily haematopoiesis (e.g. hair-on-end

skull in thalassaemia and osteolysis in multiple myeloma) the full extent to which these two tissues are interdependent and interact for the proper fulfilment of their normal functions has only recently been appreciated [1, 2]. Diseases of elements of connective tissue may affect both bone marrow and bone, e.g. hypoplasia of marrow and osteoporosis in association with angiopathy. Moreover, profound alterations in the function of cells of bone and marrow may result from pathologic changes elsewhere, such as anaemia and osteodystrophy in renal disease and erythrocytosis in pulmonary disorders.

Major factors contributing to the increased understanding of disorders of both bone and blood were the development of simple instruments for taking the biopsies and the introduction of plastics for embedding the undecalcified cores. These advances permitted the evaluation of bone and bone marrow with their architecture undisturbed and in their normal spatial relationships, and thus also enabled detailed histomorphometric measurements of haematopoietic tissues and of bone formation and resorption in health and disease. An extensive literature has now been published on bone and bone marrow biopsies in haematology, oncology, internal medicine and osteology since the early publications of Burkhardt [3], Duhamel [4] and Block [5].

The aim of this paper is to summarize some of the observations made in over 25 000 biopsies in the authors' laboratories, and to provide a review of the literature; it will, however, be possible to cite only the most recent articles containing references to previous work on the particular subject.

Indications

Haematology. Bone and bone marrow biopsy (BMB) is indicated for the differential diagnosis of hypoplasias and aplasias; for the differential diagnosis, classification (in the bone marrow) and evaluation of factors of prognostic significance in the myelo- and lymphoproliferative disorders, for monitoring therapy and the evolution of the disease processes, for detection of residual foci of malignant cells and for assessment of haematopoietic tissues before and after autografts and transplants [6–9].

Internal medicine. BMB is useful in many endocrine disorders [10], in disturbances of renal function, for detection of amyloidosis [11], in auto-immune diseases for assessment of the walls of blood vessels, for the diagnosis of granulomatous disorders, and many other non-specific reactions.

BMB is diagnostic in storage diseases such as Gaucher's, and may be so in histoplasmosis, toxoplasmosis, Q fever, in giant cell arteritis, in mesenchymal and collagenous disorders, and in rare metabolic disturbances such as hypophosphatasia [12–15].

Oncology. BMB constitutes an additional tool for the detection of metastases [16, 17], for evaluation of marrow function and reserve before, during and after

treatment, and for early recognition of dysplastic changes which may presage the development of a haematologic malignancy as a consequence of cytotoxic drugs.

Osseous disorders. BMB is indicated in any condition involving alterations in the formation and resorption of bone which may occur in a wide variety of diseases either primarily or secondarily, e.g. Paget's disease, osteoporosis in intestinal malabsorption, in vitamin and mineral deficiencies, in congenital disorders, and in malignancies [18–21].

Clearly, the indications given above are not exhaustive and many more could be listed. Clearly also, not all indications can be dealt with in this overview, therefore only some of the major entities with particular implications for haematology will be considered under the following headings: osteopathies, myelopathies, myelo- and lymphoproliferative disorders and non-haematologic malignancies in the bone marrow.

Biopsy

Site. BMB is most frequently taken from the anterior or the posterior iliac crests; this site reflects the state of the normal marrow in health and is also affected in most disease processes which involve the red haematopoietic marrow primarily: haematologic diseases or secondarily (metastatic cancers). Iliac crest biopsies also provide samples of cortical and cancellous bone [22]. The representativity of the tissues at this site has been considered in previous publications. However, whether the amount of tissues contained in one biopsy, which is about 0.3 ml for a large core, can adequately reflect an organ of about 3000 ml is still under investigation.

Methods. The needle most widely employed is the Jamshidi cannula [23] with which biopsies may be taken from the anterior or the posterior iliac crests. Two biopsies may be taken from adjacent spots on the same site if more material is required (Fig. 1*a*). For larger single biopsy cores (Fig. 1*b*) and for histomorphometric measurements of both cortical and trabecular bone, vertical biopsies are taken from the anterior iliac crest by means of an electric drill [3], or horizontally – transileal biopsies – by means of a manual trephine [6]. Bone biopsies are routinely taken under local anaesthesia. Needle biopsies have proved to be safe, simple to perform and without significant complications. Bone biopsies are (relatively) contra-indicated in patients with bleeding disorders; if unavoidable the same precautionary measures must be taken as for other operations in such patients.

Technical Methods

Biopsy cores with large diameters may be halved longitudinally and used for immuno-histology by means of FITC labelled antibodies (Hyland, Behring), for enzyme cytochemistry in fresh frozen sections, as well as for histology, pathology and for electron microscopy [24, 25].

Various plastic resins are used for embedding without prior decalcification; some have the advantage of permitting subsequent demonstration of enzyme activities [26]. The details of a relatively simple, fast and reliable technique are given in the appendix. Using this method, stained sections of undecalcified biopsies may be examined within 3 days of their receipt.

The majority of the biopsies on which our own observations are based, was taken with the myelotomy drill, the rest with the Jamshidi needle. Informed consent was obtained in all cases. The undecalcified biopsies were processed into plastic (see appendix) and sections cut at 3 μm were stained with (i) Gallamine Blue Giemsa for cytologic detail; (ii) Gomori's stain for reticulin fibres; (iii) periodic acid Schiff (PAS) for glycoprotein; (iv) Ladewig's stain for calcified bone and osteoid and (v) Berlin Blue for iron [3, 6]. A multivariate computer based analysis of both clinical and histologic data was performed to evaluate their relationship to survival of the patients from the time of the first biopsy to date of last contact or death (Table 5). Selected BMPD computer programs were utilized for statistical analysis of the data [27].

Evaluation of BMB

Normal bone and bone marrow: values for the components of normal bone and bone marrow derived from iliac crest biopsies are given in Table 1 and their topography is illustrated in Fig. 1*c*. The haematopoietic tissue is distributed in the bone marrow spaces in the extravascular compartment: erythropoietic islands and megakaryocytes are associated with the marrow sinusoids, early myeloid precursors lie close to the endosteal surfaces and arterioles, and the more mature granulocytes

Table 1

Histomorphometry of normal bone and bone marrow*

Variables	Mean value (St. dev.)	Dimension	Correlation with age
Haematopoietic tissue	40 (9)	vol. %	−0.039
Fatty tissue	28 (8)		0.163
Trabecular bone	26 (5)		−0.083
Osteoid	0.3 (0.2)		−0.091
Sinusoids	4.5 (2.1)		−0.082
Mast cells	2 (1)	per mm^2 bone marrow	
Megakaryocytes	8 (4)		
Macrophages (containing iron)	16 (10)		
Plasma cells	21 (18)		

* These values were derived from 158 biopsies of normal healthy individuals

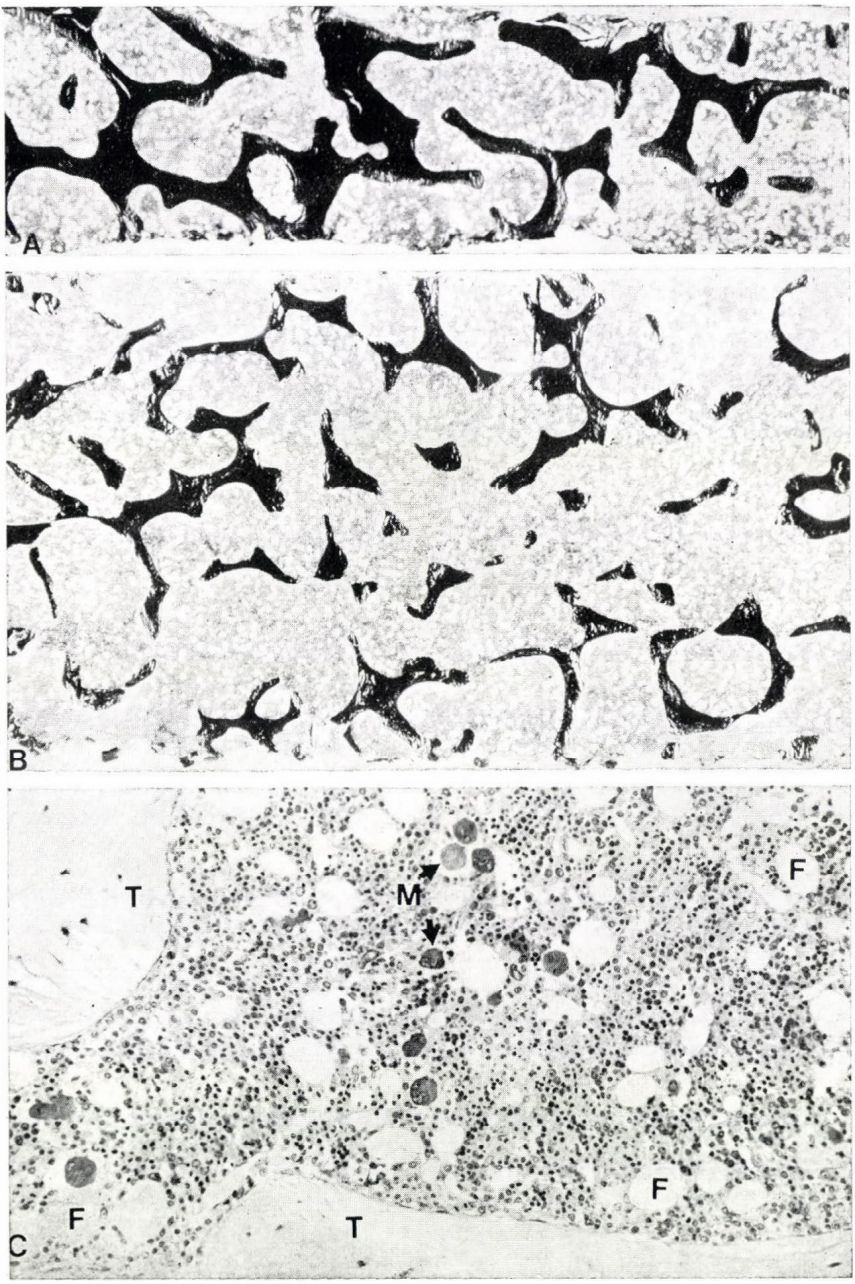


Fig. 1. (*a*) Biopsy taken with 2 mm wide Jamshidi needle, and (*b*) biopsy taken with 4 mm wide myelotomy drill. Gomori's stain, both at × 15; (*c*) histotopography of haematopoietic tissue in the bone marrow, trabeculae (T), megakaryocytes (M), fat cells (F), Giemsa stain, × 100

are found in the central intertrabecular regions. The stromal tissues of the bone marrow form a highly specific micro-environment which is required for the normal recruitment and maturation of the cells of the blood [28, 29]. Reticulum cells, endothelial cells, fibroblasts, fibres and fat cells contribute to the micro-environment which when defective may play an important role in some cytopenias. The single layer of endothelial cells which forms the interface between the extra-vascular and the intravascular compartments must be traversed by the blood cells for entry into the circulation. Possibly, there is also a reverse traffic across this barrier as by

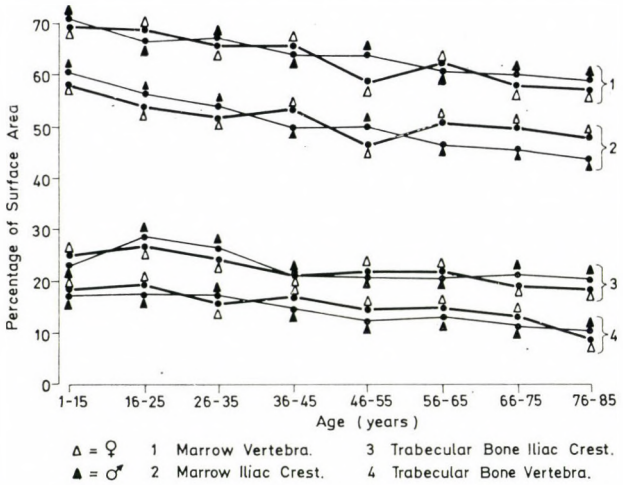


Fig. 2. Volume percentages of marrow and trabecular bone in vertebral bodies and iliac crest at different ages. Note reduction in trabecular bone with advancing age

lymphocytes migrating into the bone marrow [30]. The trabecular bone surfaces are covered by a layer of endosteal lining cells, continuous with osteoblasts derived from the stromal (mesenchymal) tissues.

Osteocytes (originally superficial osteoblasts enclosed within the bone as it is laid down) are connected with each other and with the endosteal layer by means of long processes within the osseous canaliculi. Osteocytes participate in the turnover of minerals, particularly the homeostasis of calcium.

Osteoclasts arise from a haematopoietic cell line and are normally present only on resorption surfaces [31]. In health there is a delicate balance between bone formation and resorption whose regulatory mechanisms are not known.

Age [32] and sex related quantitative changes, which must be kept in mind when evaluating any given biopsy section, have been documented for both haematopoietic tissues and bone (Fig. 2). There is a decline in the volume percentages of trabecular bone and haematopoietic tissues and a corresponding rise in fat cells.

Osteopathies

Anomalies of bone formation, resorption and mineralization may be diagnosed by bone biopsy well before their effects are evident on X-rays.

Osteoporosis. This is defined as osteopenia plus clinical symptoms and it is the commonest of the skeletal disorders [33]. It occurs as a secondary event in a wide variety of conditions (more than 20 have been listed in a recent review [19]). Osteoporosis is characterized by a reduction in trabecular bone volume (Fig. 3*a*), and to a lesser extent also in that of cortical bone. Quantitative estimates of histologic parameters in iliac crest biopsies have shown that age-related (primary, postmenopausal or senile) osteoporosis is due to increased bone resorption which especially in patients with pathologic fractures, is accompanied by decreased formation [34, 35]. Osteoporosis may also occur in some metabolic disorders such as diabetes, in the presence of vascular changes (microangiopathies) of the marrow vessels; and in the so-called myelogenous osteopathies in which a reduction in total trabecular bone volume is due to alterations in marrow cellularity. Examples are the cancellous rarefaction (osteopenia) found in haematologic disorders associated with hypercellularity as in the myelo- (MPD) and the lympho- (LPD) proliferative disorders. In a recent survey of 892 biopsies of patients with MPD, and 559 biopsies of patients with LPD, we found osseous changes in 61 per cent and 56 per cent respectively, with osteoporosis occurring in approximately half of the cases. X-rays of the skeleton were, however, normal in 77 per cent of 230 patients with MPD, and in 72 per cent of 343 patients with LPD showing that BMB is an earlier and more sensitive indicator of osseous changes than are routine X-rays.

Thalassaemia provides an example of the close relationship between hyperplastic erythropoiesis and bone metabolism: a multiparameter investigation in thalassaemic patients has documented the trabecular bone status before and after transfusion [36]. When erythropoiesis was ineffective and hyperplastic, there was osteopenia with decreased mineralized bone and increased osteoid and normal or increased bone turnover in spite of decreased parathyroid hormone levels. The osseous abnormalities were reversed by transfusion, indicating the possibility of a direct effect on the bone of the hyperplastic marrow.

A reduction in trabecular bone volume also occurs in hypoplastic conditions such as aplastic anaemia, probably due to atrophy of the nutrient blood vessels [6].

Osteomalacia. This condition arises when newly formed bone matrix is inadequately mineralized (Fig. 3*c*). Increased osteoid seams and width, and augmented remodelling together with anomalies of tetracycline labelling as revealed by BMB are generally required for diagnosis [37]. Osteomalacia may ensue due to vitamin deficiencies resulting from hepatic, renal or intestinal disease, from dietary insufficiencies and from drugs [38]. In many cases the direct cause is a decrease in serum calcium or phosporus or both. Since X-rays cannot distinguish osteoid, a bone biopsy is mandatory if osteomalacia is suspected on biochemical or clinical grounds.

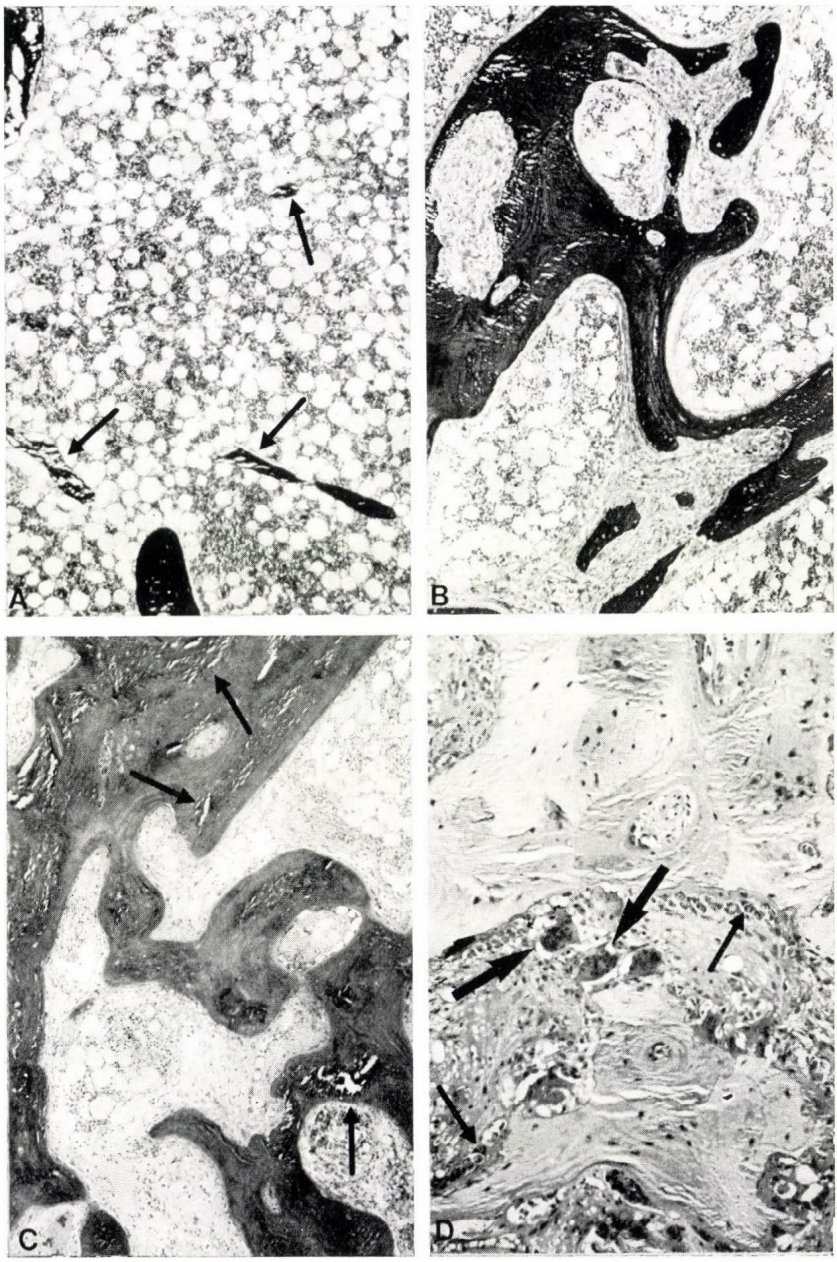


Fig. 3. (*a*) Osteoporosis, note attenuated trabecular profiles (arrows); (*b*) osteodystrophia fibrosa generalisata, primary hyperparathyroidism, note cystic excavations and paratrabecular fibrosis, both Gomori's stain, × 100; (*c*) osteomalacia with concomitant osteodystrophy due to malabsorption, mineralized bone at arrows, most of the trabeculae consist of uncalcified osteoid, Ladewig's stain, × 100; (*d*) osteitis deformans Paget; note mosaic pattern of trabeculae and giant osteoclasts (arrows), osteoblasts on trabecular surfaces (small arrows) and fibrosis of marrow cavities. Giemsa stain, × 250

Osteodystrophy. Classic examples are the changes which occur in primary hyperparathyroidism (*osteitis fibrosis generalisata*, Fig. 3*b*) and the osteodystrophy seen in patients with impaired renal function and on haemodialysis [19, 39–42]. All these forms of augmented remodelling exhibit various degrees of paratrabecular fibrosis in the affected areas, and a reduction in haematopoiesis. In severe cases, the fibrosis may even replace the blood forming elements. In primary hyperparathyroidism the greatly increased osteoclastic remodelling usually outpaces osteoblastic bone production, so that the trabeculae may become excavated with cystic formations as well as showing rarefaction and widened osteoid seams. Occasionally, however, osteoblastosis and fibrosis may predominate. Moreover, two types of bone change have been distinguished: striking lesions in patients without renal calculi, and minimal effects in those with kidney stones [41]. Renal osteodystrophy presents a variable picture which may include osteoporosis, osteomalacia and the effects of secondary hyperparathyroidism. As the bone marrow is also involved (atrophy, oedema, angiopathy) a better term might well be renal osteomyelopathy. Different aspects of osteodystrophy may predominate in any given patient, requiring identification of the type by bone biopsy, so that appropriate therapy may be instituted.

Paget's disease. This disease is an intrinsic disorder of osteoclasts [43] which show increases in number, in size, in nuclei and in activity. The size in particular is affected – it may reach many times that of the normal osteoclasts. There is an accelerated rate of bone resorption which in turn stimulates rapid bone formation. This is accompanied by vascular hyperplasia and fibrosis which together with the increased bone formation encroach on the marrow cavities and cause anaemia which frequently occurs in these patients. Both cortical and cancellous bone are involved and the final result is a structurally altered and abnormal bone which gives rise to the typical deformities in the severely affected areas. Microscopically osteoblasts and osteocytes show no abnormality, though the bone which is produced may be both woven and lamellar and has the mosaic pattern pathognomonic for Paget's disease (Fig. 3*d*). The osteoclasts exhibit various features, of which some are specific. The number of nuclei per cell varies greatly, as many as one hundred have been counted and the nucleoli are also large and prominent. Inclusion bodies, similar to the nucleocapsids of the measles virus group, are found in both nucleus and cytoplasm; indicating that Paget's disease may be due to a viral infection. There is generally a focal involvement of the pelvis, femur, skull, tibia, clavicles and ribs (in decreasing order of frequency). The most common symptom is bone pain. With the introduction of calcitonin and more recently the diphosphonates effective modalities of treatment with only moderate detrimental side effects appear to be available [44]. BMB is therefore indicated both for establishing the diagnosis and to monitor the effects of therapy. The incidence of Paget's disease is quite high, an estimated 4 per cent of the general population in some countries, with low levels in young adults but increasing numbers in the older age groups. Since the pelvis is nearly always involved, a bone biopsy is diagnostic in a very high proportion of the patients.

Myelopathies

Cytopenias. For the proper assessment of marrow cellularity a BMB of adequate size is required (minimum 15 to 20 mm × 2 mm/needle biopsy), because the subcortical marrow cavities are often entirely occupied by fat cells, especially in the older age groups, and can therefore be misleading. BMB reveals both quantitative and qualitative alterations. Hyperplasia is manifest as an increased cellularity with a corresponding decrease in fat cells which may be completely replaced. Hyperplastic BMB is seen in conditions with ineffective erythropoiesis, including some haemolytic states. Hypoplasia is manifest as a drastic reduction in haematopoiesis with a corresponding increase in fat cells (see also Fig. 7*a*).

Though local structural alterations, age and previous therapy (e.g. radiation to the pelvic area) must be taken into account, nevertheless a decrease in the erythropoietic compartment in the BMB suggests a decreased marrow reserve. Reduction in red cell precursors is accompanied by an increase in fat cells, mast cells and histiocytes; when lymphocytes are also present the anaemia may have an immunologic component. Iron stores in BMB depict reproducibly overload, depletion or normal storage in a more representative fashion than is observed in smears of aspirates [6].

Aplastic anaemias. A BMB (preferably large scale) is mandatory in suspected aplastic anaemia to exclude other causes that might be responsible. Among these are: refractory anaemias, malignant lymphomas, hairy cell leukaemia, myelofibrosis and metastatic carcinoma which in our series were the most frequent diseases revealed by BMB in 112 patients suspected clinically of aplastic anaemia. In aplastic anaemia (see Fig. 7*a*) the bone marrow picture of hypoplasia noted above may be relieved by a few isolated erythropoietic islands – hot spots – which have remained; usually close to the sinusoids and which show maturation arrest. Megakaryocytes may be absent or there may be one or two clusters. Groups of myeloid precursors may also be present as well as scattered neutrophils, lymphocytes, plasma and mast cells. In some early cases oedema and necrotic cells and blood vessels may be found. Though characteristics of prognostic significance have been sought in BMB, the evidence so far is inconclusive and no features have yet been identified which accurately predict the eventual outcome. Nevertheless, it appears that lymphocytic infiltrations and capillary necroses were usually absent or minimal in those cases which later recovered [6]. Meticulous investigation of BMB is warranted in aplastic anaemia; since a considerable number of patients is above the age at which transplantation can be considered, other modalities of treatment must be sought for, applied and monitored [45, 46].

Pure red cell aplasia. In this condition the marrow may show a patchy replacement by fat cells together with areas of normal cellularity which on closer inspection contain no erythropoietic islands or precursors. There are considerable iron deposits in the stromal cells, and aggregates of lymphocytes are also frequently present in addition to macrophages containing cellular debris, mast cells, plasma cells and possibly some isolated erythroblasts (Fig. 4*c*). Pure red cell aplasia has been de-

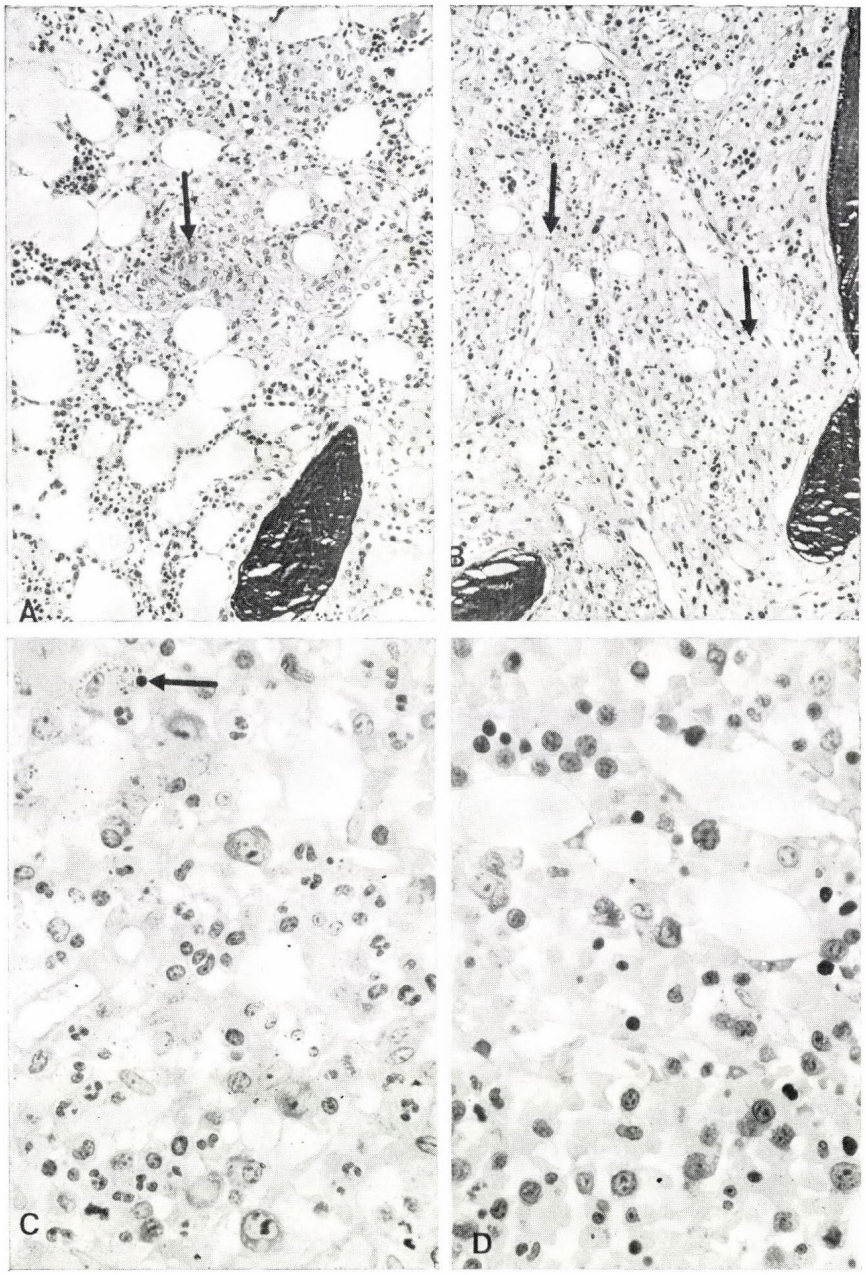


Fig. 4. (*a*) Sarcoidosis, epithelioid cell granuloma in the bone marrow (arrow), (*b*) sclerosing myelitis (arrows) of unknown aetiology, both Gomori's stain, × 100; (*c*) pure red cell aplasia, note absence of erythropoietic islands; granulocytes and reticulum cell with iron (arrow); (*d*) agranulocytosis, note erythroid precursors, absence of myeloid precursors. Both Giemsa stain, × 250

scribed in lympho- and myeloproliferative disorders in addition to other cases with an immunologic background or obscure aetiology [6].

Refractory anaemias. These are characterized by a hypercellular bone marrow in spite of a peripheral cytopenia [47], indicating ineffective erythropoiesis with dyserythropoiesis, variable numbers of sideroblasts and iron overload [6]. These conditions are not well demarcated from the myelodysplastic syndromes (preleukaemic states) and are in fact included among these by many authors. Maturation inhibition leads to accumulation of early precursors, usually in clusters.

Dysmyelopoiesis (myelodysplasia, preleukaemia). These conditions represent stem cell disorders and up to 50 per cent of the cases are characterized by chromosomal aberrations [48]. Abnormalities occur in all three cell lines and are observed in smears of aspirates, in sections of BMB and by electron microscopy [49]. There is usually a single or combined cytopenia and the bone marrow may be normo-, hypo- or hyper-cellular. Erythroid precursors show asynchronous maturation with megaloblastoid to megaloblastic features, sideroblasts, multinuclearity and other nuclear anomalies including chromatin bridges and atypical mitotic figures. White cell precursors also exhibit disassociation of nuclear and cytoplasmic development, frequently with a bi-lobed nuclear configuration (pseudo-Pelger-Huet anomaly). There may be hypo- or hyper-granularity, or very large granules. The maturation inhibition which characterizes these myelodysplastic conditions leads to accumulation of early precursors usually in clusters. Megakaryocytes are often increased with polymorphism and microforms, i.e. small megakaryocytes with one or two small, round nuclei, but mature cytoplasm. When there is hypercellularity of the monocytic series plus an increase in granulocyte precursors and an elevated monocyte count in peripheral blood, the condition is that of smouldering chronic myelomonocytic leukaemia. In the bone marrow there is frequently an increase in eosinophilic myelocytes and mast cells [50].

In a recent study of 93 patients with myelodysplasia (preleukaemia) 27 developed overt leukaemia [51] but the findings in their bone marrows were not initially distinguishable from those of the patients who did not develop leukaemia. Recent work, however, indicates that when there are over 10 per cent micromegakaryocytes in the megakaryocyte population in the bone marrow, overt leukaemia is likely to develop [52].

Granulocytopenia and thrombocytopenia. Studies on BMB in the acute stage of these conditions are rare, particularly when due to toxic or allergic reactions to drugs. In recent reviews [53] over 100 drugs have been listed which have been associated with the development of agranulocytosis; and about 60 for a similar association with thrombocytopenia. Included among these drugs are the cytotoxic agents which have been implicated in leukaemogenesis, especially in patients who received immunosuppressive or cytotoxic treatment for non-neoplastic diseases [54, 55]. The appearance of frank leukaemia is often preceded by a period of peripheral pancytopenia and dysmyelopoiesis (as described above). BMB findings in the acute forms are variable, ranging from hypocellularity with extensive oedema and extravasation of erythrocytes, to a reduction in the precursors of the particular

cell line affected, and/or maturation inhibition (Fig. 4*d*). Especially in old people other causes of a single or a combined cytopenia may include the insidious onset of a myelo- or lymphoproliferative disorder. For example, granulocytopenia may be due to a smouldering leukaemia and thrombocytopenia to a megakaryocytic myelosis, and a BMB should be carried out to establish the diagnosis.

Inflammatory and other bone marrow reactions in patients with cytopenia. In a recent study of 520 BMB [14] the following types of inflammatory reaction were distinguished: proliferative in 215 cases; granulomatous in 129 cases (Fig. 4*a*), exudative in 95 cases and fibrotic in 81 cases (Fig. 4*b*). In the proliferative type the bone marrows were cellular with a marked increase in either plasma cells or mast cells, or eosinophils or megakaryocytes. In the granulomatous type there were single or multiple granulomata. The exudative inflammatory changes were characterized by a reduction in cellularity, increase in fat cells, some degree of oedema and infiltration by lymphocytes, plasma cells and histiocytes. In the fibrotic group, in addition to haematopoietic hypoplasia with maturation arrest, there was oedema and fibrosis, with increase in fat cells and in iron-containing histiocytes. The underlying clinical conditions for all types included infections, diseases of collagen and sarcoidosis. Additional causes were Hodgkin's disease and other malignant lymphomas as well as extramedullary cancers all without direct bone marrow involvement in the biopsy. No aetiology or concomitant disease was, however, discovered in 169 cases in spite of intensive investigation. Aplastic anaemia supervened in 27 of these patients. A BMB is almost invariably diagnostic in storage diseases such as Gaucher's, in Fabri's and Hand–Schüller–Christian's disease, in giant cell arteritis, and frequently so in amyloidosis.

Haematologic Malignancies

Some subtle but significant changes have been evolving in the concepts of these neoplasias over the past decade or so. One has been the shift in emphasis from a carcinogenic event(s) occurring at the single cell level, to the recognition that abnormal accumulations of cells may be due to a shortage or inadequate function of cells at some stage of maturation which leads to stimulation of increased production at another stage and thus to progressive expansion of the compartment involved. Cells of a specific type might also accumulate slowly over a given period of time, without an increase in their rate of proliferation, if loss by cell death or movement into the next maturation compartment is inadequate. In other words this implies an inseparable linkage of proliferation and differentiation and an imbalance could be caused by any one (or a number of) factors or cells which advance or inhibit maturation at any stage of the differentiation pathway. Hypotheses on the equilibrium between cell production and loss, factors which govern the mass of a tissue and thereby control mitotic division on the one hand and maturation and cell death on the other, were put forward by Bullough as the theory of tissue-specific

chalones [56], and evidence for these has lately been forthcoming for normal as well as for neoplastic growths.

Such concepts have spear-headed the advances made in the lymphoid malignancies so that their characterization now requires phenotypic 'differentiation' markers which assist in their assignation to a defined stage in the maturation pathway [57]. These insights have profoundly influenced the approach to therapy in the malignant lymphomas, and are beginning to make an impact on the myeloproliferative disorders, too.

Chronic myeloproliferative disorders (MPD)

The classification of the MPD as proposed by Dameshek in 1951 [58] was widely adopted with the consequence that the uniform terminology and more critical criteria applied led to the definition of variants and transitional forms as well as to a more precise comprehension of the evolution of the disease process. It has since been established that the MPD are clonal proliferations [59] and that the fibrosis is reactive and secondary. The variable nature of the major disease entities could then be explained by assuming that the change or damage had occurred at different levels in the differentiation pathway. When a very early precursor is affected to such an extent that further differentiation is blocked, it will be morphologically unidentifiable and require marker techniques [57] for its characterization as in the blastic crises of chronic myeloid leukaemias (CML). Moreover, the earlier the affected precursor, the more subsequent cell lines might be involved. For example, in both polycythaemia vera (PV) and in CML iso-enzyme studies of glucose-6-phosphate dehydrogenase have shown that the erythrocytes, granulocytes and platelets are all derived from the same clone [59]. Nevertheless a large proportion of the three cell lines shows features of normal differentiation for variable periods of time, both morphologically and functionally: red cells carry oxygen, neutrophils combat infections and platelets participate in haemostasis and coagulation. Recognition of such cells as derived from a malignant clone may present considerable difficulties. As the source of myeloid cells is the bone marrow it would seem reasonable to include a BMB in the investigation of all the disorders classified under the heading MPD and not only those cases in which a dry tap is obtained. Moreover, the criteria of the Polycythaemia Vera Study Group cannot always be met, so that early, borderline, variant and transitional cases may not only be excluded from classification but also from consideration in therapeutic protocols, in comparative studies and clinical trials.

The chronic MPD comprise PV, CML, idiopathic thrombocythaemia (IT), myelofibrosis (MF), osteomyelosclerosis (OMS) and the variant and transitional forms. On the basis of about 1500 BMB of 1200 untreated patients with MPD diagnosed clinically by the established criteria, the interrelationships between them could be represented, as outlined in Table 2.

Table 2

Histologic classification of MPD

Clinical entities	Proliferative cell line(s)			Frequency, %	Median survival months**	Predominant metamorphosis
PV (191)*	ERY	—	—	3	115	0
	ERY	GRAN	—	3	99	0
	ERY	—	MEG	27	84	MF/OMS (58%)
	ERY	GRAN	MEG	22	79	MF/OMS (74%)
MegM (53)	—	—	MEG	14	63	MF/OMS (61%)
CML (114)	—	GRAN	MEG	17	27	MF/OMS (76%)
	—	GRAN	—	14	18	blast crisis (79%)

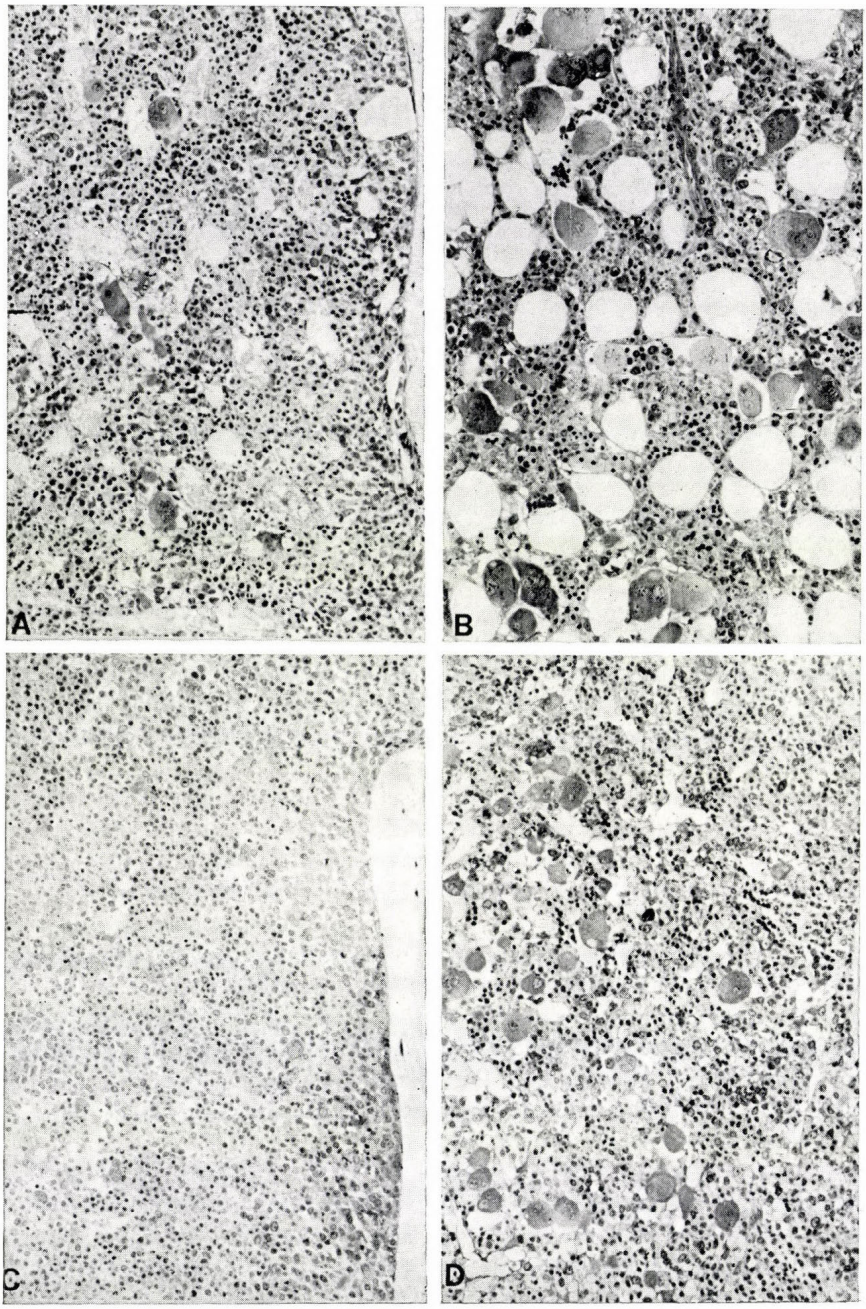
* No. of patients

** Survival time was measured from the time of the biopsy to death or date of last contact

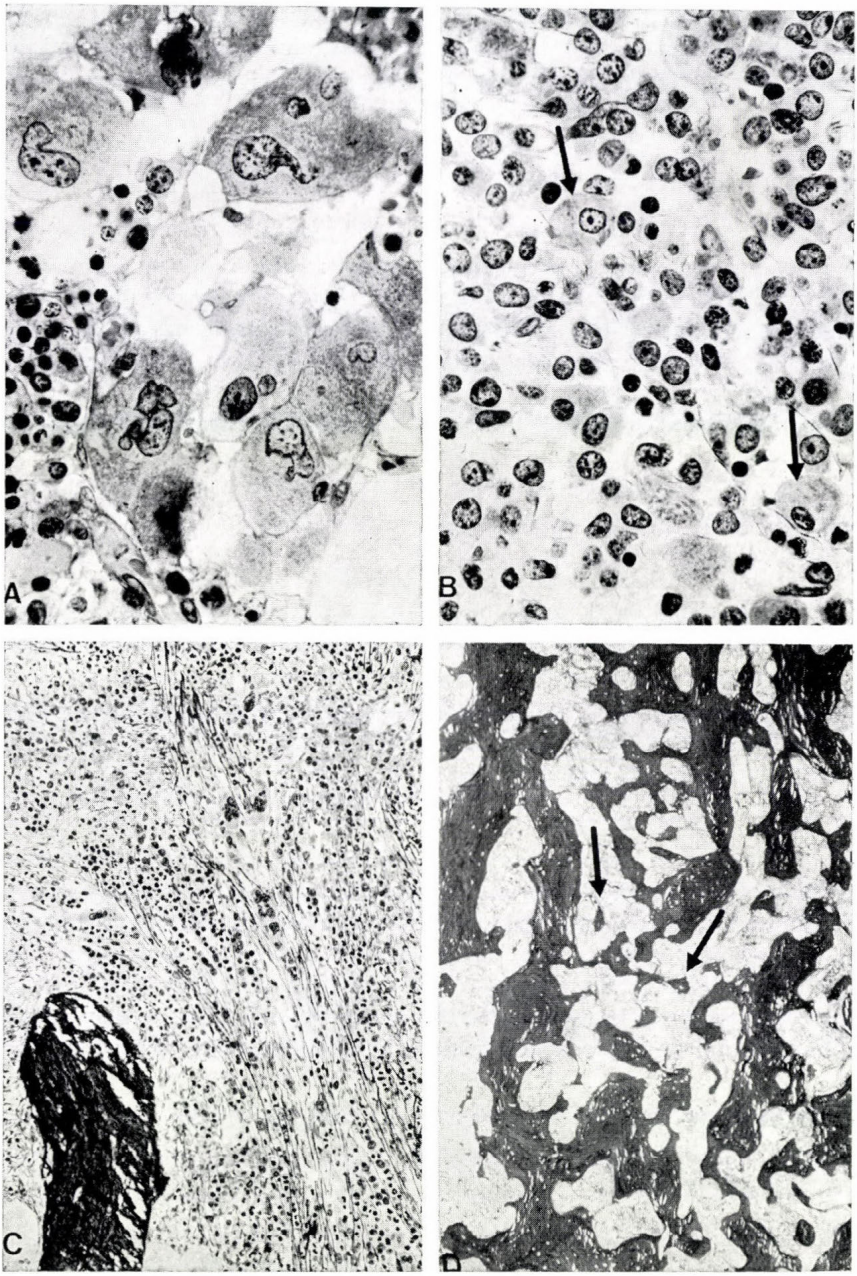
Polycythaemia vera (PV)

In most cases the bone marrow histology in our series fell into one of 4 categories: 1. the classic tri-linear, in which the erythrocytic, granulocytic and the megakaryocytic cell lines are all involved; 2. the erythrocytic and megakaryocytic; 3. the erythrocytic and granulocytic; 4. the mainly erythrocytic. In addition to the hyperplasia of the affected cell line(s), there is marked polymorphism and necrosis of megakaryocytes in the first and third groups (Figs. 5*a*, *b*). In all groups there is a reduction in fat cells, hyperplasia of vessels and hyperaemia of the sinusoids, depletion of iron stores and variable infiltration with plasma cells, slight reticulin fibrosis of the stroma and rarefaction of the osseous trabeculae. Lymphoid aggregates in the bone marrow were observed in 15 per cent of the patients. Some BMB of patients with clinically established disease could not be assigned to any of these 4 groups, such patients usually represented intermediate stages or variants. In secondary erythrocytosis there are no quantitative or qualitative alterations of megakaryocytes, reduction in fat cells or depletion of iron stores. Transitions from one of the 4 types to another were observed in 12 cases followed by sequential biopsies in our series. Patients with the isolated erythrocytic form had the longest survival and those with the classic tri-linear form the shortest (Tables 2 and 5).

A variable increase in the reticulin network is part of the disease, but extensive myelofibrosis occurs almost exclusively in cases with marked proliferation of megakaryocytes, that is types 1 and 3. Fifty-two per cent of our patients with PV developed MF/OMS (see below). Though acute leukaemia may rarely supervene during the natural course of the disease, an increase in its occurrence has been linked to therapy with chlorambucil, so that this has now been abandoned [60]. The incidence of malignant lymphomas is higher in patients with MPD than in the general population, and regular follow-up biopsies may lead to their early de-



Figs 5 and 6. Myeloproliferative disorders — 5. (*a*) PV trilinear type, (*b*) PV bilinear erythrocytic and megakaryocytic type, (*c*) CML granulocytic type, (*d*) CML mixed granulocytic and megakaryocytic type. All Giemsa stain, × 100



6. (*a*) MegM mature type, cluster of polymorphous megakaryocytes within a sinusoid; (*b*) MegM immature type, note numerous small promegakaryocytes, (arrows) both × 250; (*c*) MF, note longitudinal alignment of fibres, cells and clusters of megakaryocytes; (*d*) OMS, note increase in bone and woven bone (arrows), both × 60. All Gomori's stain

tection. The following were observed in our series of 58 patients with PV: malignant lymphomas: 5 cases; CML: 7 cases; and megakaryocytic myelosis: 6 cases. The PV Study Group has recommended that a BMB be obtained at initial diagnosis and once annually thereafter [61].

Chronic myeloid leukaemia (CML)

Studies of BMB of patients with clinically established CML have shown that it may be divided, broadly speaking, into 2 groups: the granulocytic and the mixed granulocytic and megakaryocytic groups (Figs 5*c*, *d*). In the former there is hyperplasia of the white cell series only, in the latter the megakaryocytes are also affected and show hyperplasia, polymorphism and heterotopia [6, 62]. In both types the fat cells are decreased in number and reticulin is increased to a variable extent. There may be numerous macrophages containing crystalloid material, as well as plasma cells and mast cells dispersed throughout the marrow and in proximity to blood vessels. Lymphocytic infiltrations and nodules are also found. Ineffective erythropoiesis and maturation arrest occur in many patients with CML. There may be rarefaction of the osseous trabeculae as well as osteolytic lesions [63]. In leukaemoid reactions the bone marrow shows little decrease in fat cells, no hyperplasia of megakaryocytes, and the granulocytic precursors are perivascular rather than paratrabecular. In CML (as in PV) the nature of the initial bone marrow histology influences the course of the disease. Patients with increases in megakaryocytes have a tendency to develop MF which in CML has a favourable effect on prognosis; while those with the purely granulocytic form are more likely to undergo transformation to a blastic crisis which shortens survival (Table 5). In our series of 42 patients with CML 46 per cent evolved MF/OMS and 36 per cent blastic crisis. Histologic studies of the bone marrow in CML in blast transformation, after ablative therapy and after bone marrow autografting [9] have shown that a BMB is required for differential diagnosis, for detection of residual blasts, for assessment of overall cellularity and to monitor the regeneration of haematopoiesis. There is disagreement in the literature concerning the prognostic significance of different clinical and biopsy findings at the time of diagnosis but identification of subgroups of patients with greater risks for early blastic transformation is required so that individualized treatment may be considered, including more aggressive chemotherapy and autografting [64, 65]. A BMB is necessary in the evaluation of marrow function for these purposes.

Megakaryocytic myelosis

This term refers to a proliferation of the megakaryocytic cell line in the bone marrow (Fig. 6*a*). When the cells are mature and produce platelets, in peripheral blood they can reach very high numbers and the condition is known as idiopathic thrombocythaemia. The bone marrow is characterized by numerous clusters of highly polymorphic megakaryocytes, various degrees of reticulin fibrosis, few or no

fat cells, and a reduction in the precursors of red and white cells. In our material 78 per cent of these cases developed into MF/OMS and only 2 per cent into an acute phase. When, however, there is a majority of very immature cells (Fig. 6*b*) in the hyperplastic population in the bone marrow then it is called megakaryoblastic myelosis [66] and is characterized by thrombocytopenia and has a greater likelihood (19 per cent in our series) to terminate in blastic crisis. An increase in megakaryocytes without polymorphism may occur as a reaction to a malignancy somewhere in the body, and in many other conditions.

Malignant (acute) myelofibrosis

There are still disparate views in the literature concerning the histologic findings in acute or malignant myelofibrosis [6]. The clinical criteria recently proposed are: peripheral pancytopenia, minimal or no splenomegaly and a rapid downhill course [6]. Bone marrow histology shows a preponderance of the precursors (maturation arrest) of all these cell lines. There is oedema, an increase in reticulin fibres, and variable infiltration of lymphocytes, plasma and mast cells. On the other hand 8 patients with a condition termed acute myelodysplasia with myelofibrosis have recently been described [67]: in 4 the condition was idiopathic and in 4 it was secondary to cytotoxic therapy. The condition itself was unresponsive to chemotherapy and was rapidly fatal. Histologically the bone marrow manifestations were identical in all cases and consisted of increased cellularity with morphologic abnormalities in red and white cell precursors, polymorphic and micromegakaryocytes, the presence of unidentifiable blasts, and an increased reticulin fibre network. These myelodysplastic syndromes represent myeloid stem cell disorders and as such may show a disturbance in maturation of all 3 cell lines in the bone marrow. In this sense the conditions described under the headings of acute or malignant myelofibrosis and acute myelodysplasia with myelofibrosis are probably similar, if not identical.

Myelofibrosis/osteomyelosclerosis (MF/OMS)

Fibrosis of the bone marrow occurs in response to different kinds of injury and toxic agents, and in association with or as a consequence of malignant processes: the myeloproliferative and lymphoproliferative disorders and metastatic carcinomas [6, 68–70]. Two pathogenic mechanisms (which probably act in concert) have been proposed: 1. an autoimmune reaction mediated by circulating immune complexes and indicated by the presence of lymphocytes, plasma cells and mast cells in the bone marrow undergoing fibrosis [68]; and 2. the production of fibroblast stimulating growth factor by megakaryocytes and platelets. In support of this latter theory, deposition of platelets into the interstitial spaces instead of into the *sinus lumina* and accumulations of necrotic megakaryocytes have both been observed in areas of incipient fibrosis [6]. In the MPD, as mentioned above, MF/OMS develops in those cases in which megakaryocytic proliferation was present from the outset, so that the diagnosis should MF/OMS secondary to PV or CML or

MegM, and the term idiopathic myelofibrosis (or agnogenic myeloid metaplasia) would be used only when the patient presented with the full blown picture from the start and no cause could be determined. OMS may or may not develop out of MF, while a minority of cases presents as OMS.

Three types of bone marrow histology have been described in myelofibrosis: initially there is haematopoietic hyperplasia of all three cell lines, then a patchy hyperplasia (Fig. 6*c*) alternating with fibrosis, finally an obliterative fibrosis and sclerosis. This progression has, however, only been documented for the cases developing from previously diagnosed PV or CML. Nor is this necessarily a temporal sequence affecting the whole bone marrow equally. We have observed cases with dense myelofibrosis without any haematopoietic cells whatever in the iliac crest BMB at initial presentation and a second biopsy taken from the ipsilateral iliac crest a few months later (without any therapy having been administered in the interval) showed the typical myelofibrosis described below. Thus, there may be discordance in the evolution of the disease process between different parts of the haematopoietic marrow in different areas of the skeleton. Foci of hyperplasia with osteolytic lesions in the long bones, together with MF in the iliac crest biopsy have also been observed.

The bone marrow picture in early MF is fairly typical: there is hypercellularity with increase of capillaries and disappearance of fat cells, there are clusters of megakaryocytes, polymorphic and occasionally necrotic, interstitially deposited platelets and infiltration with lymphocytes, plasma cells and mast cells. There is a network of reticular fibres as well as bundles of collagen near the blood vessels, and sclerosis of the sinus walls. Haematopoiesis is progressively diminished till complete replacement by fibrous connective tissue has supervened, and only a few islands of haematopoiesis are found in the sinuses. In OMS the formation of irregular spicules of woven bone together with progressive widening of the trabeculae add to the obliteration of the marrow cavities (Fig. 6*d*). In fibrosis of the marrow due to causes other than the MPDs there is no hyperplasia or polymorphism of megakaryocytes. OMS appears to have a slightly more favourable course than MF (Table 5). Early splenectomy may have a beneficial effect on survival.

Acute leukaemias

There have been few studies of bone marrow histology in the acute leukaemias as most efforts over the past decade or so have been directed to the identification of leukaemic cells from peripheral blood or bone marrow aspirates [57]. In early cases, the BMB may show a reduction in normal haematopoietic tissue, an increase in fat cells (Fig. 7*a*) and foci of leukaemic blasts dispersed throughout the stroma. As the disease progresses there is almost complete replacement of the marrow by sheets of leukaemic blasts (Fig. 7*c*) together with disruption of the marrow sinusoids. When there is very little, or very slow progression, the picture is that of smouldering leukaemia (Fig. 7*b*).

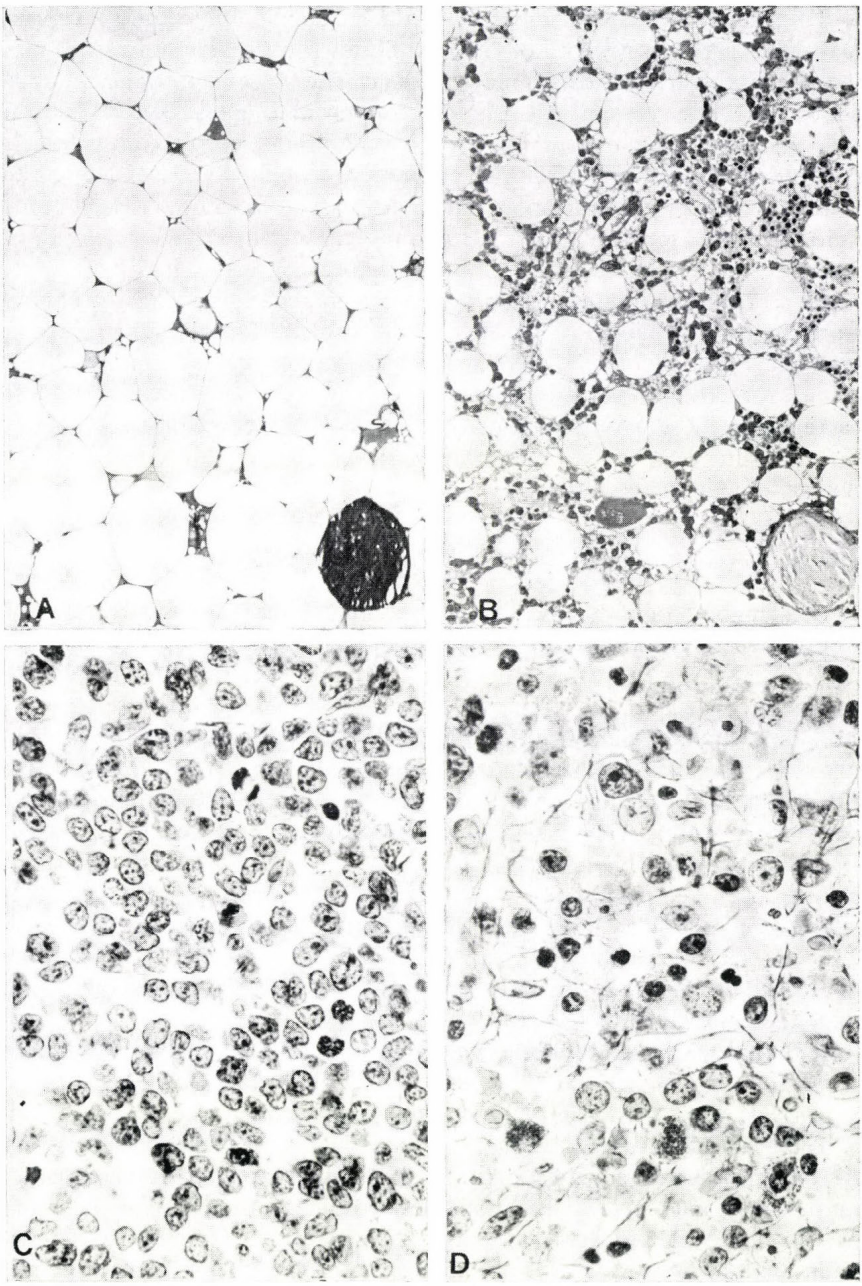


Fig. 7. (*a*) Aplastic bone marrow, acute leukaemia developed subsequently, × 150; (*b*) 'smouldering' leukaemia, myeloblasts dispersed in hypoplastic bone marrow × 100; (*c*) acute leukaemia, replacement of marrow by myeloblasts × 250; d) erythroleukaemia, note precursor cells (arrows) and fine fibrosis, × 250. All Gomori's stain

Necrotic areas may be found when the leukaemic cells undergo very rapid growth [8]. There is a network of reticular fibres (Fig. 7*d*), and numerous macrophages containing nuclear debris may be found. The osseous trabeculae may show osteoporosis, while osteomalacia may develop during therapy [38]. BMB is more reliable than are smears of aspirates for the recognition of early relapse and therefore for monitoring therapy. In adults, progressive reticulin fibrosis is thought to be associated with impending relapse. Late marrow recurrences have been reported in childhood acute leukaemia. These could probably be detected early by BMB at intervals during the period when the patients are most at risk [6, 71–73].

Of particular interest is the timing of leukaemic cell egress from the bone marrow and its relationship to cell surface characteristics. Transmural passage takes place through the endothelial cells and has been compared to the initial step in metastasis formation from solid tumours [30]. In leukaemic cells it may result from the acquisition by immature cells of a cell surface property normally residing in mature cells (asynchronous differentiation?) and is probably facilitated by the disruption of sinus walls.

Lymphoproliferative disorders (malignant lymphomas, ML)

The immunologic identification of the cellular constituents of these disorders, their relative positions in the cell cycle, and in the differentiation pathway [57] have led to great advances in treatment and thus in the prospects of the patients. Moreover, characterization of the lymphoid neoplasias by means of phenotypic markers is expanding almost daily since the introduction of hybridoma monoclonal antibodies [74]. These have already led to the identification of cells belonging to the same clone in the bone marrow and in the peripheral blood of patients with various malignant lymphomas [75]. As pointed out recently, bone marrow involvement occurs in 4 situations: 1. when the neoplasm affects one of the stem cells normally resident in the bone marrow, 2. when the neoplasm involves a cell normally migrating through the bone marrow, 3. when the neoplasm arises elsewhere but spills out and its cells reach the bone marrow and involve it secondarily and 4. the neoplastic clone affects cells that at some point in differentiation also pass into or through the bone marrow [74, 75].

It should, however, be borne in mind that not all lymphoid aggregates in the bone marrow are malignant and the incidence of benign nodules in the marrow has been given as 4 per cent rising to a possible 47 per cent in the older age groups [76]. These nodules are well demarcated from their surroundings and consist of small lymphocytes in a network of reticular fibres and blood vessels. When numerous they may not be distinguishable from ML on morphologic grounds alone. Though some ML (such as hairy cell leukaemia and immunocytoma) may be found in the bone marrow very early in the disease, involvement by others such as the T cell cutaneous lymphomas has not been observed.

The morphologic criteria of lymph node histology for diagnosis and classification of the ML [77, 78] may also be applied to sections of involved bone marrow.

Table 3

Incidence of bone marrow involvement in ML
(Pretreatment patients)

Histologic type	Positive biopsies, %
ML of low grade malignity	77
Lymphocytic	99
Hairy cell	95
Lymphoplasmacytic/cytoid	85
Centrocytic	71
Centroblastic/centrocytic	20
ML of high grade malignity	26
Lymphoblastic (without ALL)	39
Immunoblastic	22
Centroblastic	27
Unclassifiable	24
ML overall	69

In our study of 678 patients with ML (non HD, all types combined) bone marrow involvement was found in 69 per cent (Table 3). Certain histologic criteria proved to be significant prognostic indicators: the type and the maturity of the lymphoid cells as well as the extent and the spatial orientation of the infiltration, i.e. diffuse or nodular [6, 79]. Also prognostically significant were the levels of haemoglobin and the numbers of thrombocytes in the peripheral blood, reflecting the tumour cell burden in the bone marrow. The different entities will be considered briefly.

Chronic lymphocytic leukaemia (CLL)

In early cases there may be only a diffuse infiltration of the normal haematopoietic tissues by small round lymphocytes, with or without nodular aggregates (Figs 8*a* and 9*a*). Early cases may also be diagnosed by means of immunohistochemistry of the bone marrow (Figs 8*c*, *d*). With progression of the disease both forms (diffuse and nodular) encroach on the haematopoietic tissue until it is completely replaced by lymphoid cells. However, terminal transformations of CLL into acute forms (lympho-, mono- and myeloblastic, as well as myelomonocytic and monocytic leukaemias) have also been described [6]. Statistical analysis of the biopsies of 167 untreated patients with the diffuse and nodular patterns has shown that these are associated with significantly different median survival times: 34 and 115 months respectively, indicating that these modes of bone marrow involvement reflect differences in the biologic behaviour of the cells and not different stages in CLL. BMB will reveal the effects of therapy on the malignant cells as well as on the haematopoietic tissues. Fig. 8*a* shows a biopsy section before and Fig. 8*b* two months after therapy with cytostatics.

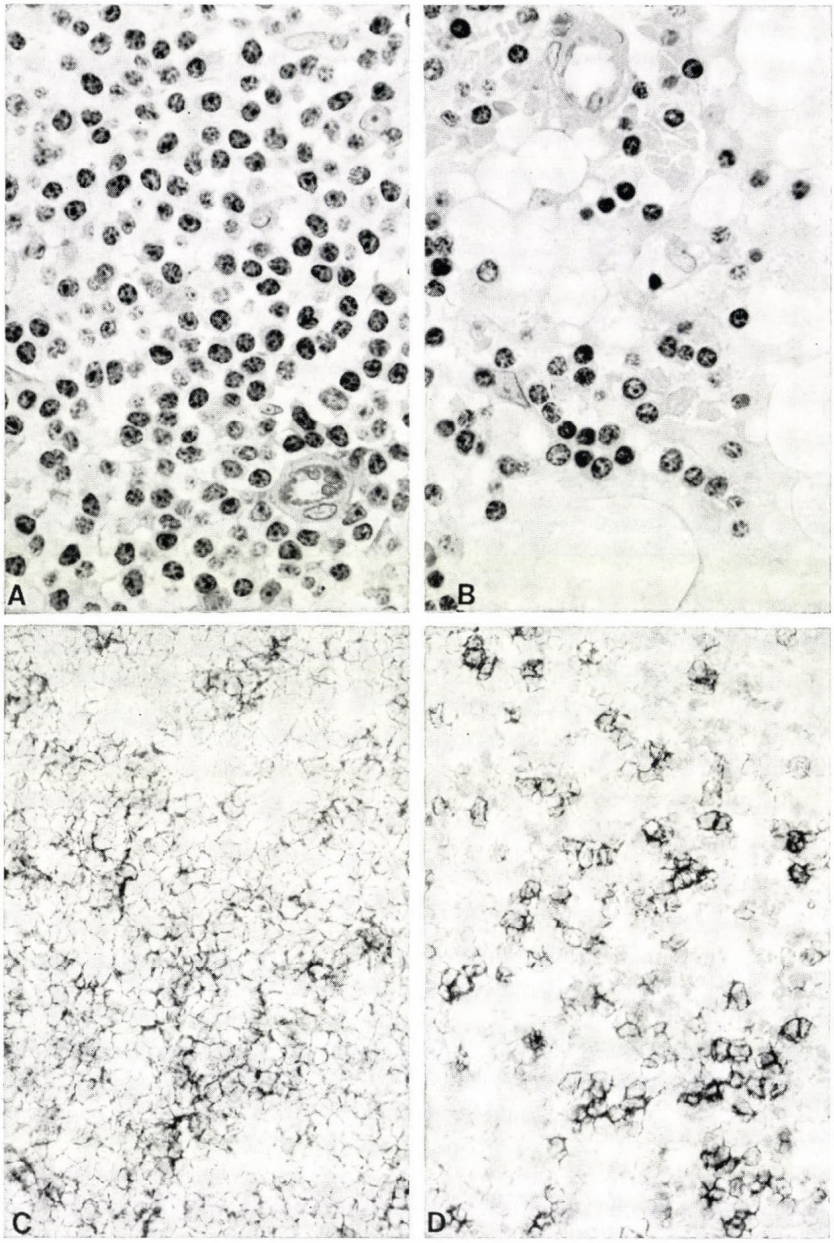


Fig. 8. (*a*) ML lymphocytic, bone marrow before treatment and (*b*) two months after therapy with cytostatics (leukeran and prednisone), note residual lymphocytes in hypoplastic marrow. Both Giemsa stain, × 250. Immunochemistry of bone marrow in CLL fresh frozen sections peroxidase anti peroxidase (PAP) method, (*c*) infiltrating lymphocytes labelled with anti-kappa, (*d*) T lymphocytes labelled with anti-T globulin while the neoplastic B lymphocytes are negative, both × 330

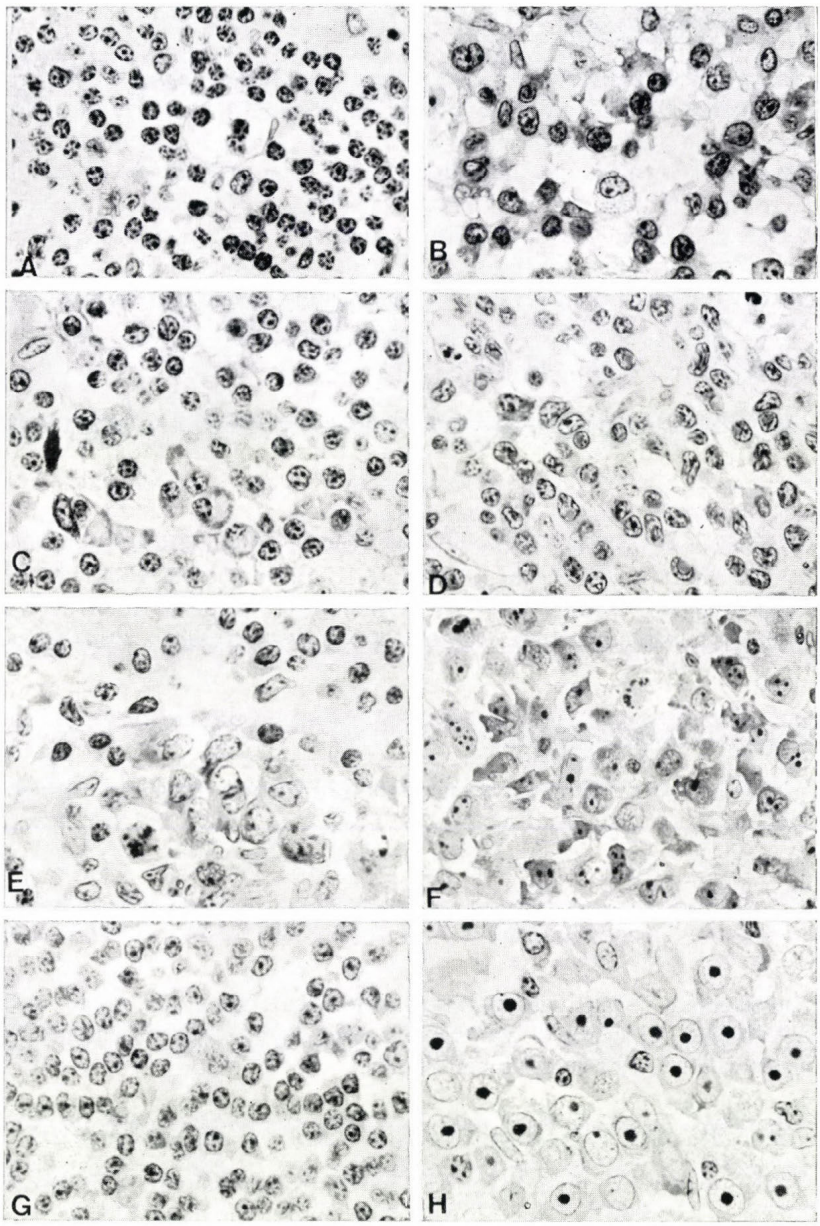


Fig. 9. ML in the bone marrow — (*a*) ML lymphocytic, predominantly small round lymphoid cells; (*b*) ML hairy cell, note variation in nuclear shape, loose chromatin arrangement and abundant cytoplasm; (*c*) ML immunocytic, lymphoplasmacytoid cells and plasma cells; (*d*) ML centrocytic, the lymphoid cells are elongated and 'cleaved'; (*e*) ML centroblastic/centrocytic; (*f*) ML centroblastic, note large nuclei with one to several prominent nucleoli, (*g*) ML lymphoblastic, monomorphic nuclei almost devoid of cytoplasm; (*h*) ML immunoblastic, note high nucleo-cytoplasmic ratio and large single central nucleoli. Giemsa stain, × 250

Hairy cell leukaemia (HCL)

Except in very rare and very early cases the BMB is diagnostic and presents a typical picture. There is a diffuse infiltration plus multiple poorly delineated foci consisting of the typical hairy cells (Fig. 9*b*). In sections these are larger than small lymphocytes, have more abundant cytoplasm, often with lateral extensions and inclusions, and round, oval or kidney shaped, cleaved nuclei whose chromatin shows little clumping. The hairy cells are enmeshed in a reticulin network which also contains plasma and mast cells and numerous erythrocytes. Fat cells and haematopoietic tissues are progressively replaced till only isolated precursors are left.

ML lymphoplasmacytoid (immunocytoma)

Three types have been described: 1. lympho-plasmacytic, often showing a diffuse pattern in the bone marrow (Fig. 9*c*); 2. lympho-plasmacytoid frequently exhibiting a nodular involvement; 3. pleomorphic in which a densely packed marrow may already be present at the initial presentation.

Different proportions of lymphocytes, plasma cells and lymphoplasmacytoid cells are present in the three categories. This lymphoma is also characterized by a considerable mast cell population [80]. These three types are not always clearly distinguishable, as there is some overlap between them. Rarely, a case of CLL with reactive plasmacytosis may present a somewhat similar picture.

ML centrocytic (follicle centre cell)

Typical for this ML is the accumulation of cleaved lymphoid cells (Fig. 9*d*) along the endosteal surfaces of the trabeculae. With progressive widening and confluence they eventually occupy all the intertrabecular spaces to the exclusion of normal haematopoietic tissues.

ML centroblastic/centrocytic

Involvement of the bone marrow is infrequent. When present it consists of centrally located aggregates of lymphocytes, centrocytes, centroblasts (Fig. 9*e*) and histiocytes with or without germinal centres.

ML centroblastic, ML lymphoblastic (without ALL) and ML immunoblastic

When involved in these ML, the bone marrow is densely infiltrated by the corresponding cell type, without a particular spatial orientation (Figs 9*f*, *g*, *h*).

Multiple myeloma (MM)

Plasma cells represent the end stage in the differentiation pathway of B lymphocytes. They are usually resident in the bone marrow and their number may in-

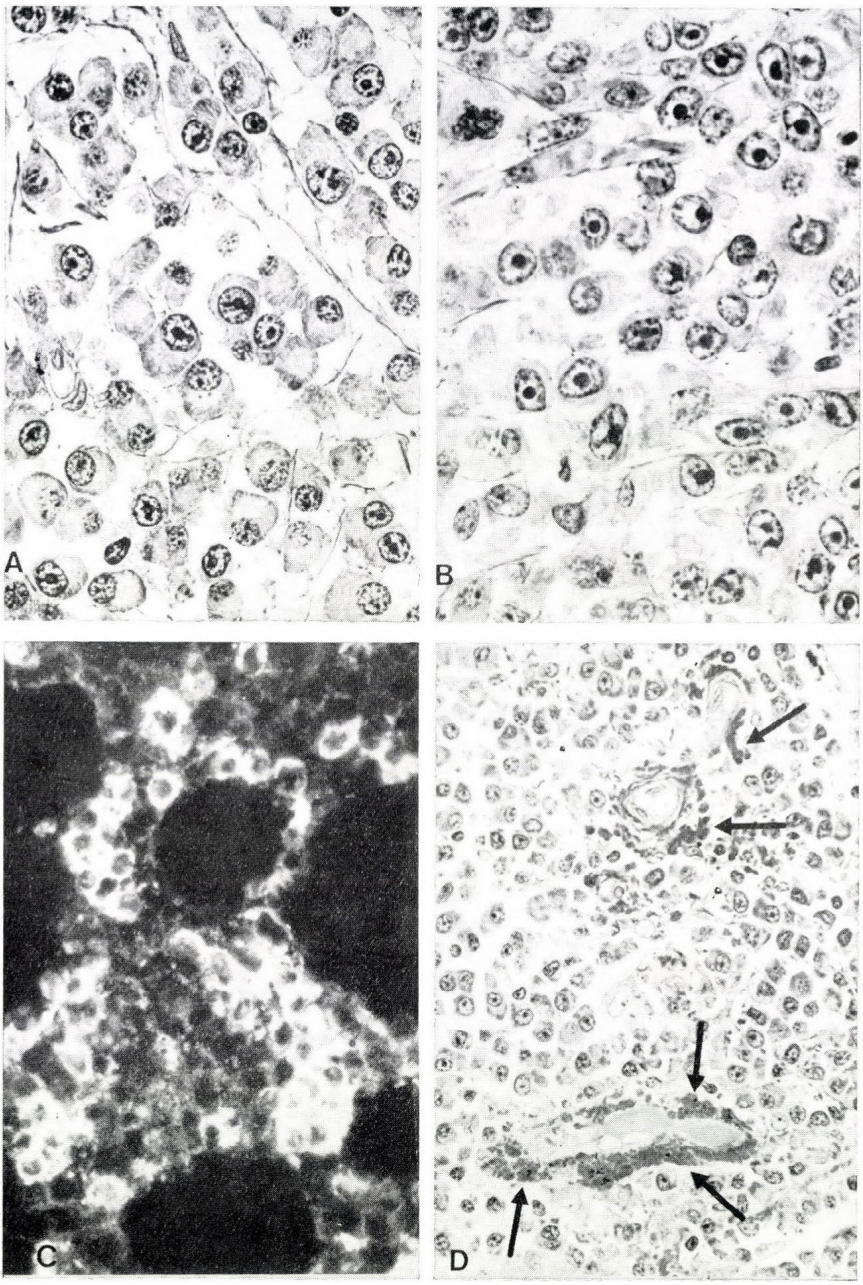


Fig. 10. (*a*) MM plasmacytic and (*b*) MM plasmablastic both Gomori's stain, × 300; (*c*) immunohistology, monoclonal identification of early case of MM: fresh frozen section of bone marrow, FITC, anti-Ig A, × 250; (*d*) amyloidosis in MM, note perivascular deposits of amyloid (arrows) and diffuse plasmacellular infiltration. Giemsa stain, × 100

crease considerably in many conditions – reactive plasmacytosis. There is no single morphologic characteristic of plasma cells which is found exclusively in the cells of a malignant clone, but a high incidence of certain cytologic and cytochemical features may be strongly suggestive. In addition, their topographic distribution in the bone marrow may also distinguish benign from neoplastic plasma cells. In our study of BMB of 220 cases of multiple myeloma the following features were found to indicate malignancy: 1. plasma cell clusters in the haematopoietic tissue in the intertrabecular spaces, as well as in the paratrabecular and perivascular regions; 2. a high nucleocytoplasmic ratio and asynchrony of nucleus and cytoplasm; 3. increased osseous remodelling and 4. monoclonality of the plasma cells (performed on fresh frozen sections only) (Fig. 10*c*). Of prognostic significance were the maturity of the plasma cells, i.e. plasmacytes (Fig. 10*a*) or plasmablasts (Fig. 10*b*), and the extent of the infiltration, that is, the volume percentage in the bone marrow.

Noteworthy also was the observation that in some of the biopsies there was a reduction in haematopoietic tissue and increase in fat cells in the vicinity of the infiltrates. In addition, amyloidosis was found in association with the plasmacytosis in some biopsy sections (Fig. 10*d*).

Hodgkin's disease (HD)

The Ann Arbor (1971) criteria of bone marrow involvement in HD were applied [81]. For primary diagnosis, Reed–Sternberg cells within a granulomatous stroma were obligatory (Fig. 11*a*). When HD was already established elsewhere, atypical mononuclear cells within a suitable background were sufficient [81]. Bone marrow manifestations in HD varied in size from a single small focus to multiple foci or large areas (Figs 11*c*, *d*). The overall incidence of HD in the bone marrow reported in the literature ranges from 2 per cent to 32 per cent. The rate of detection is influenced by 1. unequal proportions of patients in the different clinical stages when the biopsies were taken; 2. varying biopsy techniques (Table 4) and histologic preparations; and 3. differences in interpretation of the histologic findings.

Our study of 491 untreated patients has shown an overall incidence of 10 per cent with the following distribution: 168 patients in clinical Stage I: 1 per cent involvement; 212 in clinical Stage II: 2 per cent involvement; 72 in clinical Stage III: 25 per cent involvement; and 28 in clinical Stage IV: 45 per cent involvement of the bone marrow. When the patients were assigned according to lymph node histology, 8 per cent of 53 patients with lymphocytic predominance had a positive bone marrow; 4 per cent of 197 patients with nodular sclerosis; 9 per cent of 173 patients with mixed cellularity; and 22 per cent of 47 patients with lymphocytic depletion.

Involvement was found in 25 per cent of BMB of treated patients. Unlike the lymph nodes, the bone marrow showed only 2 histologic types, lymphocytic predominance and lymphocytic depletion, with favourable and unfavourable prognostic significance, respectively (Table 4). Also of clinical importance was the fact

Table 4

a) Incidence of bone marrow involvement in HD according to different biopsy techniques — Own series

Techniques*	Patient No.	Mean section area, mm^2	Positive biopsies, %
Jamshidi unilateral	210	29	9
Jamshidi bilateral	58	54	14
Burkhardt drill unilateral	832	66	15

* Incidence of clinical stages III and IV in the patients with Jamshidi unilateral (51%), with Jamshidi bilateral (54%) und with Burkhardt drill (52%)

b) Incidence of bone marrow involvement in HD according to the clinical stage and histologic lymph node type

	Patient No.	Positive bone marrow biopsies %
Clinical stage		
I	168	1
II	212	2
III	72	25
IV	28	45
Lymph node histology		
Lymphocyte predominance	56	8
Nodular sclerosis	205	4
Mixed cellularity	180	9
Lymphocyte depletion	49	22
HD overall	491	10

that 335 untreated patients (79 per cent of those with negative biopsies) had various nonspecific reactions in their bone marrow, and only 21 per cent of those with negative biopsies had a histologically normal bone marrow. These initial findings had a predictive value: hypoplasia and exudative and leukaemoid reactions indicated a poor prognosis, while epithelioid cell granulomas and lymphocytic aggregates constituted more favourable signs. Bone marrow involvement at initial presentation of untreated patients proved to be a highly significant unfavourable prognostic factor, as reported also by others [82].

Angio-immunoblastic lymphadenopathy (AILD)

Bone marrow involvement in AILD indicates systemic spread and a short survival [83]. The infiltrates consist of a heterogeneous population of lymphocytes, plasma cells, histiocytes and immunoblasts within a network of reticulin fibres and

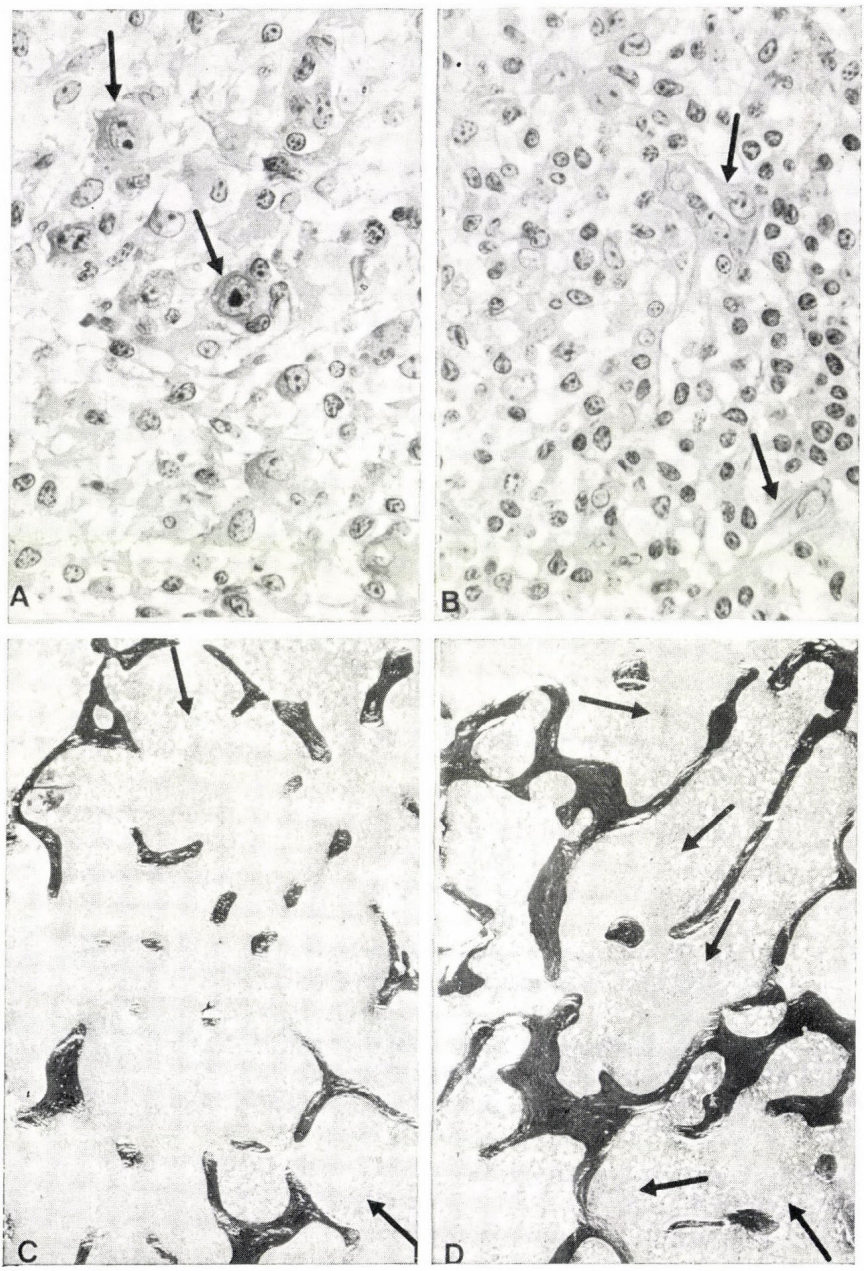
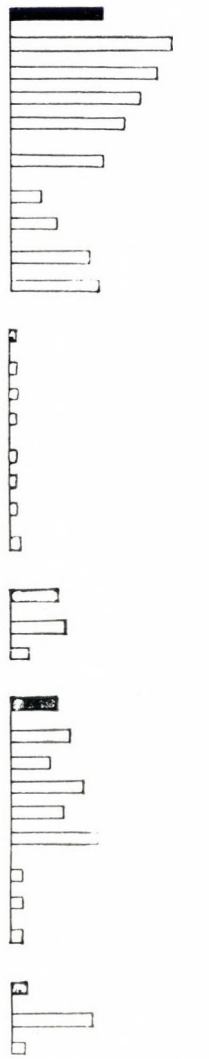


Fig. 11. (*a*) HD in the bone marrow, note Reed–Sternberg and large mononuclear cells (arrows), Giemsa stain, × 250; (*b*) angio-immunoblastic lymphadenopathy in the bone marrow, note histiocytes lymphoid cells and blood vessels (arrow), Giemsa stain, × 250; (*c*) HD in the bone marrow, large focus indicated by arrows; (*d*) HD in the bone marrow, multiple small foci (arrows). Gomori's stain, × 60

Table 5

Histologic classification of haematologic malignancies in the bone marrow, 1302 pretreatment patients

Histologic type	Patients No.	Median survival time, months*
Chronic myeloproliferative disorders (MPD)	466	58
erythrocytic	10	115
erythro/granulocytic	12	99
erythro/megakaryocytic	92	84
erythro/megakaryo/granulocytic	77	79
megakaryocytic	53	63
granulocytic	52	18
granulo/megakaryocytic	62	27
myelofibrotic	52	50
osteomyelosclerotic	56	51
Acute leukaemia (AL)	177	3
myeloblastic	63	3
myelo/monocytic	27	5
monocytic	17	3
promyelocytic	23	3
erythroblastic	15	2
megakaryoblastic	11	4
lymphoblastic	21	8
Multiple myeloma (MM)	220	27
plasmacytic	149	32
plasmablastic	71	8
Malignant lymphomas (ML)	370	28
lymphocytic	132	41
hairy cell	87	21
immunocytic	90	44
centrocytic	20	25
centroblastic/cytic	17	50
immunoblastic	7	4
centroblastic	7	5
unclassifiable	10	5
Hodgkin's disease (HD)	69	8
HD, lymphocyte predominance	19	52
HD, lymphocyte depletion	50	5



* Survival time was measured from the time of the biopsy to death or date of last contact

arborizing vessels (Fig. 11*b*). These lesions may resemble those occurring in rheumatic and allergic conditions as well as in HD, but without RS and mononuclear Hodgkin cells. A histologic classification of the haematologic malignancies in the bone marrow together with the median survival times is given in Table 5.

Non-Haematologic Malignancies

Efforts aimed at the early detection of dissemination are motivated by the realization that most patients who die of cancer succumb to metastases. Therefore, adjuvant systemic chemotherapy has been introduced into the treatment of solid tumours, in some cases before but mostly after surgery and/or radiotherapy. It has long been appreciated that the red well-vascularized haematopoietic marrow is one of the sites most frequently involved by metastatic cancer [84]. Nevertheless, BMB is not routinely done in patients with solid tumours, and the rate of metastatic detection in BMB reported by various authors is generally low [16], probably due to difficulties caused by decalcification and the resulting poor preservation of the bone marrow. In our survey of 1164 unselected patients with solid tumours (using plastic embedding of undecalcified biopsies) involvement was found in 40 per cent (463 patients) (Figs 12*a*–*d*). Other investigators, using similar techniques, obtained an overall incidence of 35 per cent positive biopsies [85], in spite of the fact that many patients in early stages of their disease were included. In both studies the rate of positive cases varied with the (presumed) origin of the primary: in our investigation the incidence of positive biopsies in decreasing order of frequency was: occult primaries 81 per cent of 141 patients who had been thoroughly investigated; unknown primaries 78 per cent of 40 patients not yet fully investigated; breast 44 per cent of 330 cases; prostate 35 per cent of 211 cases; gastrointestinal 32 per cent of 59 cases, pulmonary 19 per cent of 231 cases, genital 42 per cent of 21 cases and all others together 14 per cent of 124 cases. When the patients with known primaries were divided according to the presence or absence of systemic disease the incidence of bone marrow involvement was always higher in those with other indications of dissemination. Nevertheless, appreciable numbers of patients in each group had metastases in the BMB in the absence of other signs of spread as shown by the following figures for well investigated patients: breast 22 per cent of 85 patients, prostate 11 per cent of 37 patients, lung 12 per cent of 69 patients and intestinal tract 17 per cent of 29 patients. Clearly the demonstration of bone marrow metastases in such patients will profoundly influence the choice of the therapeutic approach. Interestingly, there was no correlation between haematologic values in the peripheral blood and the size or type of the metastases in the BMB. In many cases there was complete replacement of haematopoiesis by the metastases (Figs 12*a*–*d*).

These results support previous work indicating that clinical and haematologic methods alone are unreliable for the early detection of bone marrow metastases [16]. Recognition of the skeletal effects of metastatic cells [86, 87] leading to patho-

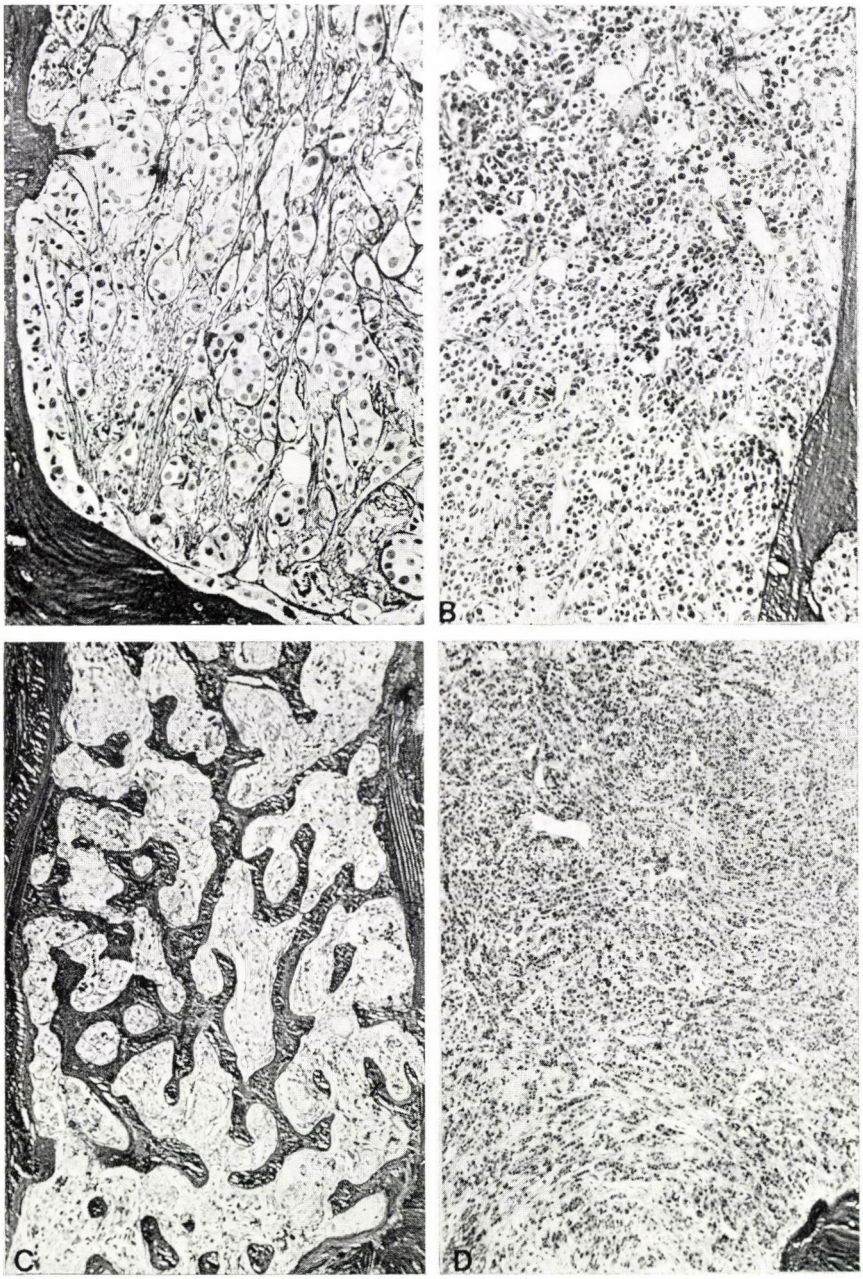


Fig. 12. Metastatic carcinoma in the bone marrow, complete replacement of haematopoietic tissue by tumour. (*a*) Adeno-ca (mammary), (*b*) oat-cell ca (bronchus), both $\times 250$; (*c*) osteosclerotic reaction with production of woven bone to metastatic ca (mammary) and (*d*) osteolysis with metastasis of bronchial ca, both $\times 100$. Gomori's stain

logic fractures, e.g. osteolysis (Fig. 12*d*), and the possibility of their prevention by inhibitors of osseous remodelling [88] has also stimulated the search for early metastases in the bones.

Other information of clinical significance may be derived from BMB [16]. This includes the cellular and stromal host reactions which vary for different tumours and their metastases and thus have implications for therapy. Two aspects are of particular interest in this respect: the production of tumour angiogenic factor (TAF) by the metastases [89], as this is required for their lodgement and growth; and the host cells infiltrating the tumour [90]. It has been shown that these have special antigenic properties in some primaries [91] and their presence has a favourable effect on prognosis in others [92]. Moreover, immunohistochemical techniques may be applied to fresh frozen sections of involved bone marrow to identify the origin of the metastases in patients with unknown primaries [93, 94] which have a high incidence (81 per cent in our material) of skeletal metastases. In addition therapeutic response has been shown to be related to the histologic type of tumour and to its proliferative activity which varies even in different regions of the same primary, and this may be investigated for its metastases by BMB in the involved cases [95].

Conclusions

This overview has aimed at indicating the many areas and disciplines in which a bone marrow biopsy can provide valuable information and contribute to diagnosis. With the growing interest and advances in cell characterization in haematology, more information has also accrued on the influence of the environment on cell development and function. Therefore the need has arisen to study the cells in their natural environment in the organ which produces them, the bone marrow. Fortunately this has come at a time when improvements in technique have made such studies possible, rewarding and in many cases indispensible.

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Appendix
Method for plastic embedding of undecalcified bone biopsies

Fixation. The solution is made up of 50 ml formaldehyde 35–40 per cent; 96 ml absolute methanol; 4 ml glucose phosphate buffer pH 7.4. The stock formaldehyde is previously neutralized by the addition of 50 g/l $CaCO_3$. To make the buffer, 48.0 g $Na_2HPO_4 \cdot (2\ H_2)$, 8.7 g KH_2PO_4, 154.0 g glucose 1 H_2O (mol. wt.

198.17) are mixed in a flask and distilled water is added to 5 litre. The buffer may be sterilized by filtration through a bacterial filter. Store in the refrigerator at 4 °C.

Glass vials, capacity 20–25 ml, with screw caps are used for fixation, dehydration, infiltration and embedding.

The biopsies are immediately placed into the fixation solution and fixed for 16–24 h, with shaking or rotation for the first 2–3 h. A biochemical shaker or roller or mixer is used for this purpose. If tissues have previously been fixed in formalin for more than 24 h, they should be washed in distilled water for ±2 h and then transferred to the fixation solution as above.

Dehydration. Transfer the biopsies directly from the fixation solution to absolute methanol, and change the methanol as follows: 30 min, 30 min, 1 h, 1 h and 1 h. A minimum dehydration time of 4 h with 5 changes of absolute methanol is required for needle biopsies 2 mm wide. Larger biopsies require longer dehydration, i.e. 24 h, with at least 6 changes of absolute methanol. Dehydration should be carried out under gentle agitation on a roller or shaker as above, if possible, for at least the first 2–3 h.

Infiltration. The infiltration mixture is made up of 3 ingredients: methylmethacrylate 180 ml, Plastoid N 25 ml, and benzoyl-peroxide 3.5 g. Proceed as follows: the methylmethacrylate is destabilized by passage through a chromatography column filled with basic aluminium oxide to a height of 15–20 cm; passage through two such columns in turn is recommended. The benzoyl-peroxide is placed in a glass dish and dried in an oven at 37 °C for about 30 min before use. The three ingredients are then mixed in the required proportion and the infiltration mixture is kept in a dark, loosely stoppered bottle in the refrigerator between changes. It is best to make up only the estimated required amount and to use fresh, though the infiltration mixture may be kept in the refrigerator for periods of up to 14 days. Procedure: the last absolute methanol is drained off, and the infiltration mixture is poured into the vials to reach a level just above the biopsy, the glass vials are left open and placed into a desiccator (Bell jar) connected via a Hg-manometer to a vacuum pump. The desiccator has a layer of 'Siccapent' at the bottom with a perforated porcelain plate over it and the glass vials containing the biopsies and the infiltration fluid are placed on this plate.

The jar is closed (the apposed surfaces of lid and jar should first be smeared with vaseline to facilitate opening) and the air is pumped out to a negative pressure of 15–20 mmHg. The entire infiltration procedure should be carried out with the desiccator in a hood at room temperature. After 30 min air is let into the desiccator and it is opened and the glass vials taken out, the infiltration mixture is poured off into an old bottle and replaced by fresh infiltration fluid again just to cover the biopsies. The open glass vials are placed back into the desiccator, which is closed and the air pumped out as before to a negative pressure of 15–20 mmHg. This procedure is carried out at the following time intervals to give a total infiltration time of 5–6 h (or more for larger specimens). (1) 30 min (the first infiltration after abs. methanol), change; (2) 30 min, change; (3) 1 h, change; (4) 1 h, change and fill; (5) 2 (or more) h.

At the last change (4) the vial is filled with the infiltration mixture to approximately a centimeter from the top. The specimen identification number is written with a lead pencil onto a thin strip of paper which is inserted into the vial, and this will be fixed into the plastic block when it hardens. The biopsy core is gently pushed as far as possible to one side at the bottom of the vial, so that it lies horizontally and flat and close to the bottom. After a minimum period of 2 h (at this point the vials are left overnight in the desiccator if the biopsies are large) under negative pressure, the vials are removed and closed very tightly with screw caps and placed in a water bath at 45 °C for 1 h to initiate polymerization and then transferred to a water bath at approximately 34–38 °C overnight for hardening. The level of the water in both baths should reach to just above the level of the fluid in the vials, but not higher; this is to prevent air and water from penetrating into the vials. After overnight incubation the plastic will generally have hardened; if the top of the block in the vial is still soft or even fluid the vial may be returned to the 45 °C oven for an h or so. But if the block is large enough and there is still some fluid plastic at the top, this may be poured off (not into the sink, as it will harden later). The vial is then placed into the refrigerator (or the freezer compartment) for 15–30 min, after which it is taken out and the glass is shattered with a hammer, the shards are removed, and the block is ready for cutting. To facilitate sectioning and mounting, it is advisable to remove that part of the plastic which does not contain the biopsy core: the block is gripped in a vice and the plastic is sawn first vertically to a depth of about 5 mm and then horizontally with a small hand saw, leaving a half circle of plastic with the biopsy inside it. The block is then trimmed and cut at 1 to 5 μm in a heavy duty microtome such as Jung's autocut with a tungsten-tipped knife (Jung, Heidelberg). A soft brush is used to moisten the surface of the block with 30 per cent alcohol between sections. Each section is grasped at its edge with a pair of tweezers as it comes off the knife, and placed into a Petri dish filled with distilled water. Each section is then separately mounted as follows. The section is picked up with tweezers from the distilled water and placed as flat as possible onto a gelatinized slide, and a few drops of 90 per cent alcohol are dropped onto it. After a few seconds the section will soften and it can then be stretched and the folds smoothed out with a soft brush. The section is brushed gently to avoid tearing it, until flat and dry. (This technique requires some practice. Once acquired, mounting is accomplished quite rapidly and even large sections will be flat and free of folds). The section is covered by a small piece of PVC used for wrapping, e.g., boxes of chocolates so that slides may be stacked on top of each other and then placed in an oven at 50 °C for about 2 h or longer, even overnight under a weight, for example a lead weight, paper-weight, piece of marble tile etc.

Before staining, the methyl-methacrylate is dissolved from the sections as follows. The slides are taken from the oven, the PVC slips removed, and the slides placed into benzene (benzol) 1–20 min, benzene 2–10 min, absolute methanol 1–2 min, 96 per cent methanol 2 min, 80 per cent methanol 2 min, methanol ammonia solution 10 min, distilled water 10 min. Alternatively, acetone may be used instead of the benzene. (Methanol ammonia solution: 70 per cent methanol 100 ml, 25

per cent ammonia 10 ml.) The slides are then ready for staining and most stains used in routine histopathology (Giemsa, haematoxylin and eosin, van Gieson's, stains for iron, for reticulin, the PAS reaction, etc.) can be used. Different times are required according to the thickness of the sections and it is best for each laboratory to determine these for itself. For rapid evaluation of sections: place into 0.1 toluidine blue at about 40 °C for 10 to 30 min (the time varies with the thickness of the section), remove from stain, dip into distilled water, dry on filter paper, dip into xylene, mount cover glass with Entellan (Merck) or any similar mounting medium. For additional details see [57].

Reagents. Methyl-methacrylate, Merck–Schuchardt Cat. No. 800 590, FRG; benzoyl peroxide, Merck–Schuchardt Cat. No. 801 611, FRG; Plastoid N, Burnus Ges. Darmstadt, FRG; Soft Brush, Komondor No. 10, Kolinsky–Rotmarder, FRG; Siccapent, Merck Cat. No. 747-8033, FRG.

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Management of Leukaemia-associated Disseminated Intravascular Coagulation (DIC)

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Seven patients presenting with DIC in association with acute promyelocytic and myelomonocytic leukaemia were treated with intensive chemotherapy (IC), intensive platelet support (IPS) and continuous heparinization (CH), or IC + CH but minimal platelet support (MPS), or no chemotherapy, no heparin or MPS. All four patients who received IC + IPS + CH achieved remission (survival time: 516, 400+, 371 and 368 days). The remaining three patients died early (survival time: 11, 7 and 3 days). The results suggest that a combination of IC + IPS + CH is essential in the management of leukaemia-related DIC.

Key words: acute leukaemia, disseminated intravascular coagulation, heparin, platelet transfusion

Introduction

The question of the pathogenesis and management of leukaemia-associated disseminated intravascular coagulation (DIC) remains controversial although some progress has recently been made. For instance, Gralnick and Tan [7] showed that the leukaemic promyelocyte exhibited procoagulant activity which was ten to fifteen times greater than that of the normal promyelocyte.

Furthermore, Gralnick and Abrell [6] and Sakuragawa et al. [18] identified procoagulant and concomitant fibrinolytic activities in subcellular fractions of the lysate of leukaemic promyelocytes. It is therefore understandable why haemostatic failure frequently complicates this type of acute leukaemia. Whatever the mechanism(s) of the leukaemia-associated DIC, an ideal treatment programme should aim at minimizing the leukaemia cell burden and simultaneously restoring and maintaining the haemostatic reserve.

In this communication, we describe our experience of management of 7 patients with acute leukaemia complicated by disseminated intravascular coagulation (DIC).

Abbreviations:

AMML	acute myelomonocytic leukaemia
APROL	acute promyelocytic leukaemia
APTT	activated partial thromboplastin time

(cont'd p. 288).

Patients and Methods

The patients were investigated and managed at two medical centres with markedly different medical facilities: 4 at the Mount Sinai Hospital (MSH) in New York City, USA, and 3 at the University College Hospital (UCH) Ibadan, Nigeria. Acute leukaemia was diagnosed in all cases on the basis of peripheral blood and bone marrow findings: packed cell volume and/or haemoglobin concentration, total and differential white cell count and platelet count. Peripheral blood smears were processed with routine Romanovsky stain. Bone marrow smears were obtained in each case and were processed with May–Gruenwald–Giemsa stain. In addition at MSH special cytochemical stains (periodic-acid-Schiff, peroxidase etc.) were used to characterize the cells. The criteria of diagnosis and subclassification of acute leukaemia were similar to those suggested by Hayhoe and Cawley [11].

The state of the coagulation systems was assessed by the determination of prothrombin time (PT) and activated partial thromboplastin time (APTT) using the routine techniques. Plasma fibrinogen (PF) was estimated at MSH by the heat-precipitation method. The technique which is based on the selective precipitation from citrated plasma of fibrinogen at 56 °C is a simple but reliable method of estimation of fibrinogen (L. Aledort, personal communication). The clot-weight method [5, 10] was used to estimate fibrinogen at the UCH, while Factor VIII coagulant activity (FVIIIC) was determined by the one-stage technique [10] in two UCH patients. At the UCH, fibrin degradation products (FDP) were estimated using the tanned red cell haemagglutination and inhibition immuno-assay (TRCHII) [14].

Bleeding occurring during treatment at MSH, was investigated immediately by repeating the coagulation profile (PT, APTT, and PF) as well as the platelet count. Routinely, heparin was temporarily discontinued while platelet concentrates (at least 10 units) were given pending the determination of the most likely cause of the bleeding. No attempt was made to neutralize the heparin in the circulation either before or after blood samples had been obtained for coagulation profile. Further management of the bleeding depended on the results of such determinations.

Abbreviations (cont'd):

ARA-C	cytosine arabinoside
AT-III	anti-thrombin III
CR	complete remission
DIC	disseminated intravascular coagulation
DNR	daunorubicin hydrochloride
FDP	fibrinogen degradation products
FVIIIC	Factor VIII coagulant activity
G.I.T.	gastrointestinal tract
G.U.T.	genito-urinary tract
PF	plasma fibrinogen
PLT TX	platelet transfusion
PRD	prednisolone
PT	prothrombin time
VCR	vincristine

Therapeutic leukapheresis was carried out prior to chemotherapy in patients 1 and 2 (Table 1). The procedure was done as part of a study evaluating the management of hyperleukocytotic leukaemic state with leukapheresis.

All four MSH patients (Patients 1–4, Table 1) received in continuous infusion cytosine arabinoside (ARA-C) 100 mg/m^2/24 h for 7 days, and daunorubicin 45 mg/m^2/t. i. d. In addition, each received a continuous intravenous infusion of heparin 12 000–15 000 units daily (approximately 7500 units/m^2/day). The dose was adjusted in accordance with changes in plasma fibrinogen levels or occurrence of haemorrhage. A total of 30–50 units of platelet concentrate per patient was given in the first week of management to the MSH patients either because of bleeding or prophylactically for thrombocytopenia of $\lesssim 20 \times 10^9$/l.

Patient 5 received a continuous infusion of ARA-C 100 mg/m^2/day for 7 days and doxorubicin 20 mg/m^2/t. i. d. He also received in continuous infusion heparin 12 000 units/24 h but received only 5 units of platelet concentrate when his platelet count had dropped to 20 000/mm^3. Patient 6 received only blood transfusion and died before definitive treatment could be started. Patient 7 received one dose of vincristine 0.5 mg/m^2, prednisolone 40 mg/m^2/day, and units of platelet concentrate.

Results

The characteristics of the patients are summarized in Table 1. There were 5 male and 2 female patients and their age ranged from 9 to 65 years. All presented with a bleeding diathesis of varying severity involving mainly the skin, the retina and the genito-urinary and gastrointestinal tracts.

The results of initial coagulation studies are shown in Table 2. APPT and PT were prolonged in all cases and plasma fibrinogen concentration was reduced to between 50 and 140 mg/dl. FVIIIC was estimated in two patients, and values of 60

Table 1

Patient characteristics on initial evaluation

Patient			Diagnosis	Site	Severity	WBC	Platelet
No.	age	sex		of bleeding		$\times 10^9$/l	
1	17	M	AMML	Retina	Moderate	288.0	100.0
2	65	M	AMML	Skin	Mild	122.0	81.5
3	52	M	APROL	Skin	Mild	2.2	8.0
4	33	F	APROL	Skin	Mild	7.8	23.0
5	41	M	APROL	G.U.T.	Severe	10.0	94.0
6	9	M	APROL	Skin	Mild	4.6	10.0
7	9	F	APROL	G.I.T.	Moderate	2.8	15.0

Patients 1–4 were treated in Mount Sinai Hospital, New York, USA, while 5–7 were treated in University College Hospital, Ibadan, Nigeria

and 70 per cent were obtained. These values probably represent relatively reduced levels since, according to Rosner et al. [17], FVIIIC is usually considerably elevated in acute leukaemia. Patients 5 and 7 had FDP values >320 μg/dl and >181 μg/dl, respectively. These results taken together with the clinical picture of severe haemostatic failure were, in our view, consistent with a diagnosis of disseminated intravascular coagulation (DIC) [8].

Table 2
Results of initial coagulation studies

Patient No.	PT* in s	PTT*	Fibrinogen in mg/dl	Factor VIIIC	FDP in μg/dl
1	19/11.4	117/48	50	ND	ND
2	15/12	79/49	100	ND	ND
3	14/12	60/48	125	ND	ND
4	17.2/14	56.4/48	75	ND	ND
5	21.1/14	41.1/32.2	80	ND	>320
6	26/14	48/30	100	70%	ND
7	23/14	41/30.1	140	60%	>181

ND not done
* Lower figures in each case were normal control values

Response to treatment

The changes in the fibrinogen level occurring in Patients 1, 2 and 4 during the course of treatment are shown in Fig. 1. Similar changes were observed in Patients 3 and 5. A prompt rise of the plasma fibrinogen was observed in all the 5 patients in whom serial values were obtained. Attempt in Patient 4 to reduce the heparin dose because of haemorrhage occurring within the first week of treatment was followed by a drop in the fibrinogen level. In the case of Patient 2, heparin had to be discontinued because of unexplained bleeding. His plasma fibrinogen concentration which up to that time had been on the increase, levelled off. On restarting heparin because of thrombophlebitis of a calf vein, again rapid increase of the fibrinogen level was observed.

Evidence of significant bleeding during heparin was observed in five of the patients. Some of these bleeding episodes occurred at early stages of treatment and might have been due to inadequate control of DIC (Patients 2 and 4, Table 3). In other cases, bleeding during heparinization was associated with marked thrombocytopenia, hence it was difficult to attribute the bleeding solely to the heparin. An uterine bleeding episode was observed in Patient 4 at a time when the plasma fibrinogen was higher than 200 mg/dl, the platelet count was above 50×10^9/l and APTT and PT were prolonged. This is the only bleeding episode believed to have been caused mainly by heparin.

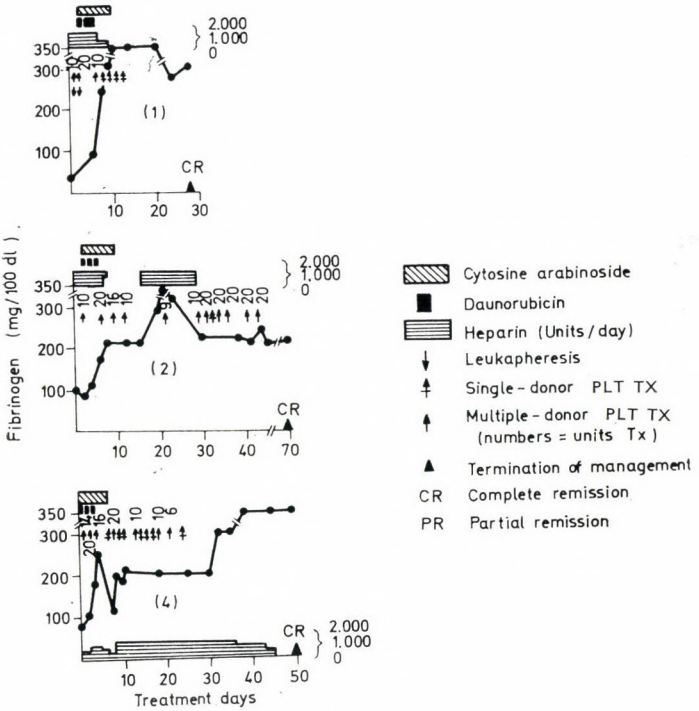

Fig. 1. Plasma fibrinogen level during management of Patients 1, 2, and 4, in relation to chemotherapy, heparinization and platelet transfusion

Table 3

Haemorrhagic complications during heparin treatment

Patient No.	Site	Severity	Presumed cause
1	Nose	Mild	Thrombocytopenia
2	Venopuncture	Mild	Active DIC?
3	G.U.T., Retina	Moderate	Severe thrombocytopenia
4	Uterus	Moderate	Heparin? overdosage
5	Venopuncture	Moderate	Active DIC?
6	Brain stem	Fatal	Thrombocytopenia

A clinical diagnosis of classical fatal brain stem haemorrhage was made in Patient 6. At the time of his death, which occurred on the 7th day of treatment, the patient showed a remarkable overall clinical improvement. Haematuria, the main clinical symptom, had ceased. Both APTT and PT had normalized and

plasma fibrinogen had risen from 80 mg/dl to 160 mg/dl. The patient received 5 units of platelets earlier in the day prophylactically for thrombocytopenia or 20×10^9/l.

Outcome of management

The results of treatment in all patients are shown in Table 4. Three complete remissions and 1 partial remission were recorded in the patients who received intensive chemotherapy, intensive platelet support and heparin, their survival times being 400+, 371, 516 and 368 days, respectively. The other 3 patients died early during treatment; their survival times were 11, 7, and 3 days, respectively. All the 3 had clinical evidence of cerebral haemorrhage, and in addition one had massive gastrointestinal haemorrhage terminally.

Table 4
Results of treatment

Patient No.	Treatment	Outcome	Survival, days
1	LEUK× 2, PLT TX+++ ARA-C + DNR, HP	CR	400+
2	LEUK× 3, PLT TX+++ ARA-C + DNR, HP	PR	371
3	ARA-C + DNR PLT TX+++, HP	CR	516
4	ARA-C + DNR PLT TX+++, HP	CR	368
5	ARA-C + DOX PLT TX+, HP	Cessation of symptoms: died*	11
6	Blood TX	Progressive disease: died*	7
7	VCR + PRD PLT TX+	Progressive disease: died**	3

CR complete remission; PR partial remission
PLT TX+++ 30–50 units of platelet concentrate in first week
PLT TX+ 2–5 units platelet concentrate in first week
LEUK leukapheresis
HP heparin: approx. 7500 units/m^2/24 h
Blood TX blood transfusion
* Fatal brain stem haemorrhage
** Fatal brain & G. I. haemorrhage

Discussion

The results of the coagulation tests obtained in the seven patients, who all presented with clinical evidence of haemostatic failure, were consistent with a diagnosis of disseminated intravascular coagulation complicating their primary disease of acute leukaemia. Although some coagulation tests that are believed to be more indicative of in vivo generation of thrombin, e.g. protamine sulphate paracoagulation test [13], fibrinopeptide A assay [15], and Factor Xa [19] were not done in any of our patients, the evidence in support of DIC in all of them was in agreement with the outlined criteria [8]. DIC which was first firmly associated with leukaemic promyelocyte by Hillstad [12], is attributed to a potent procoagulant activity present in the granules of the leukaemic promyelocyte [6]. This substance, apart from having a procoagulant activity similar to tissue thromboplastin, also has a fibrinolytic activity which could not be separated from the procoagulant activity [18]. The increased fibrinolytic activity of the substance was however observed to be less marked than its increased procoagulant activity [6]. The mechanism of DIC occurring in association with other granular subtypes of acute myelogenous leukaemia, e.g. acute myelomonocytic leukaemia, may have a similar basis.

The prognosis of APL was extremely poor until recently when the value of daunorubicin, heparin and platelet transfusion was recognized [1, 2, 4]. According to Gralnick and Tan [7] failure to control the disease was due to the lack of effective myelosuppressive agents. On the other hand, judging from the report of Bernard et al. [2] and Collins et al. [3] it appears that acute promyelocytic leukaemia is especially responsive to anthracyclene containing regimens. A substantial proportion of the cases reported by these authors experienced prolonged remissions. The most critical period during the process of remission induction in APL patients appears to be the early stage of treatment when death from haemorrhage is most frequent. Our observations reported here are in part similar to those of others [1–3, 7, 9]. Drapkin et al. [9] observed a statistically significant improvement in survival in patients who, apart from receiving intensive chemotherapy, were also given heparin to control bleeding. The 'favourable' outcome of the management of the bleeding diathesis in 4 of our patients prompts us to suggest that the minimum requirements of platelet transfusion should be in the range of 30–50 units, and of heparin about 12 000–15 000 units (7500 units/m^2) per 24 h in the first week following cytotoxic chemotherapy.

We attribute the satisfactory haemostatic control in the adequately managed patients to the joint effect of rapid decimation of the neoplastic cell population, protection of the coagulation system and effective platelet support. Clinical and laboratory evidence of deterioration in the status of the coagulation system was observed in two patients in whom heparin had prematurely been reduced or discontinued. These observations suggest that at least part of the haemostatic support provided by the combination of heparin and platelet transfusion was borne by the use of heparin.

Traces of heparin have been shown to accelerate the thrombin–antithrombin-III (AT-III) interaction. Thus, in the presence of heparin, AT-III rapidly neutralizes thrombin as well as other activated serine proteases [16]. Bleeding in the thrombocytopenic state, as was observed in some of our patients, requires platelet transfusion to arrest it. We therefore surmise that the favourable outcome of management in four of our patients was due to the effective use of heparin and platelet transfusion in addition to intensive chemotherapy.

In conclusion, we recommend that, at least, 30–50 units of platelets and 12 000–15 000 units (about 7500 units/m^2) per 24 h of heparin should be given during the first week following adequate antileukaemic chemotherapy in the management of leukaemia-associated DIC.

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Eosinophilia of Obscure Aetiology Study of Antigenic Properties

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A case with a high eosinophilic leukocyte count (100 000/μl) gradually decreasing to normal in the course of three weeks without any treatment is presented. The highly purified suspension of eosinophilic leukocytes (96 per cent of cells) separated from the peripheral blood following sedimentation with dextran and subsequent centrifugation on 1.148 g/ml density Verografin solution allowed to perform some assays of the presence of membrane antigens. In the complement-mediated cytotoxic reaction using anti T, anti Ia-like and anti AML xenosera we failed to find T, Ia-like antigens and leukaemic antigens on the purified eosinophilic leukocytes.

Key words: membrane antigens, separation of eosinophilic leukocytes

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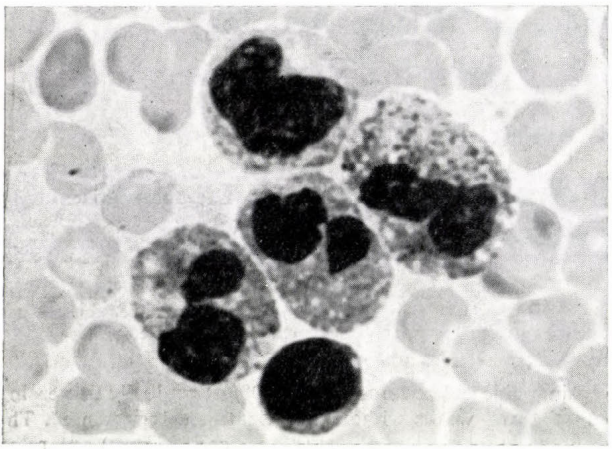
In the recognition of the pathogenesis and pathophysiology of some blood malignancies the immunological approaches to an asynchronic proliferation of pathological cellular clones using membrane differentiation markers are of considerable help [2]. In this paper we describe the unique case of a patient with high counts of eosinophilic leukocytes which normalized without treatment within a period of three weeks. The separated eosinophilic leukocytes were tested for some membrane differentiation antigens.

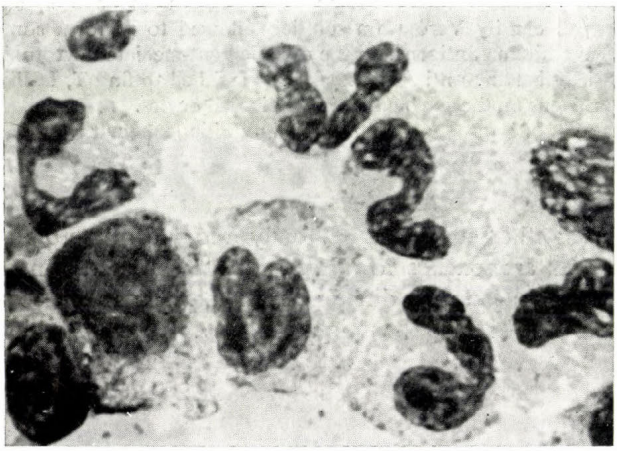
Report of a Case

A 26-year old man was admitted in July, 1977 for a rapid rise to 111 000/μl of leukocyte counts and 94 per cent eosinophilic leukocytes, febrile episodes and swelling of submandibular nodes. The history was negative except for the last two years when he had three episodes of bronchitis. On admission he had no complaints except for swollen (pea-sized) lymph nodes under the left mandible. The liver and spleen were of normal size, and the other findings were also negative.

Laboratory findings. Hb: 16.4 g/dl; Ht: 0.46 l/l; reticulocytes 10 per cent; leukocytes 57 600; thrombocytes 128 000; FW 23/32. The differential count is shown in Table 1, the smear in Fig. 1*a*. The sternal biopsy was cell rich, with 35.3 per cent eosinophilic leukocytes and a few atypical eosinophilic myelocytes (see Fig. 1*b*). No other atypical cells were found.



(*a*)



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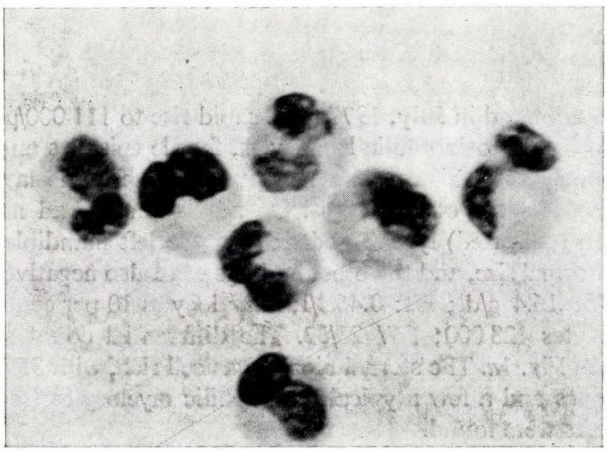

(*c*)

Fig. 1*a*. Eosinophilic leukocytes in peripheral blood of eosinophilic patient. Stained according to Pappenheim. Magnification × 600

Fig. 1*b*. Eosinophilic leukocytes in bone marrow of an eosinophilic patient. Stained according to Pappenheim. Cells magnified × 600

Fig. 1*c*. Eosinophilic leukocytes separated from peripheral blood on 1.145 g/ml density solution

Table 1

Cell counts obtained from the peripheral blood of a normal donor and of a patient with eosinophilia prior and after separation on Verografin density gradients

Differential	Normal donor			Patient		
	prior to separation	after separation		prior to separation	after separation	
		1.077 g/ml	1.148 g/ml		1.077 g/ml	1.148 g/ml
		density solution			density solution	
Neutrophil, segmented	45	1	75	3.5	2	1
Neutrophil bands	4			0.5	0.5	
Metamyelocytes						
Myelocytes						
Promyelocytes						
Myeloblasts						
Basophilic, segmented	1			0.5		
Eosinophilic, segmented	3		5	82	5.5	96
Eosinophilic bands				8	1.5	1
Eosinophilic metamyelocytes				0.5	0.5	
Eosinophilic myelocytes						
Monocytes	5	4	1	0.5		
Lymphocytes	42	95	19	4.5	90	2

Lung X-rays showed no focal change. The faeces contained no parasites, biochemical examinations gave normal findings and they failed to explain the eosinophilia. During hospitalization twice febrile episodes appeared with transitory rhinitis and conjunctivitis. Then the eosinophilia decreased gradually without any therapy and in three weeks the blood picture was fully normal (Fig. 2).

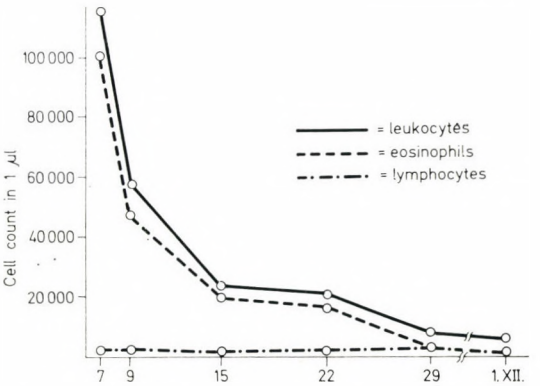

Fig. 2. Absolute leukocyte, eosinophilic leukocyte and lymphocyte count in peripheral blood of the patient. Eosinophilic leukocyte counts gradually decreased from the initial 100 000 µl to normal in 3 weeks without therapy. The differential count was normal

Eosinophilia of unknown aetiology was diagnosed associated perhaps with some allergic upper respiratory disturbance. The diagnosis of eosinophilic syndrome was not supported. Regular check-ups were negative, with a normal blood picture.

Methods

Cell separation. Eosinophilic leukocytes were separated on 1.145 g/ml density solutions according to Day [1] in our modification [3, 4] using as separation medium Verografin SPOFA (86.85 per cent N-methyl-D-glucamide salt and 13.15 per cent sodium salt of 3,5-diacetoamide-2,4,6-tri-iodobenzoic acid, see Fig. 1*c*).

Microcytotoxicity testing. The antigenic properties of eosinophilic leukocytes were tested in microcytotoxicity assays as described by Mittal [6] for HLA antigen tests, in our modification with anti-T, anti-Ia-like and anti-AML xenosera [5].

Results and Discussion

Table 1 shows the differential cell count prior and after separation on various density gradients, from the peripheral blood of a control person and from the eosinophilic patient. Evidently, the 1.077 g/ml separation solution concentrated mostly lymphocytes (90 per cent), with 1.148 g/ml density Verografin solution, 96 per cent eosinophilic leukocytes were separated. Following separation a normal donor shows eosinophilic leukocyte counts similar to those obtained prior to separation, without a significant repopulation of eosinophilic leukocytes.

Table 2

Cytotoxic reaction of anti-T, anti-Ia-like and anti-AML sera with eosinophilic leukocytes

Xenosera	Tested cells					
	T lympho-cytes*	B lympho-cytes*	T-line (Molt-3)	B-line (Raji, Daudi)	AML myelo-blast	Eosinoph. leukocytes
Anti-T (anti-T lymphocytes)	+**	−	+	−	−	−
Anti-Ia-like (anti-Daudi)	−	+	−	+	NT	−
Anti-AML	−	NT	−	NT	+	−

Note: * tested on enriched suspensions of T and B peripheral lymphocytes
** cytotoxic reactions + or − correspond to various counts (2–20) of tested cells. Eosinophilic leukocytes were tested twice with the above xenosera. As positive cytotoxic was regarded a reaction with over 30% of cells showing signs of cytotoxic damage due to antiserum diluted 1 : 2 or higher
AML—acute myeloid leukaemia. NT—not tested

Table 2 presents the cytotoxic reaction of anti-T, anti-Ia-like and anti-AML sera with various cell types. As it is seen, anti-T, anti-Ia-like and anti-AML sera failed to react with eosinophilic leukocytes in the direct cytotoxic test. We may therefore conclude that eosinophilic leukocytes isolated from the patient were lacking T, Ia-like and leukaemic AML xenoantigens, i.e. the determined phenotype was Ia-like (−), T (−), and M (−).

Perrillo and Fauci [7] demonstrated that, as to membrane markers, the population of eosinophilic leukocytes is less homogeneous than it has been assumed. They tested various membrane markers on the surface of isolated eosinophilic leukocytes of patients with eosinophilia and hypereosinophilic syndrome and failed to find a higher percentage of Fc receptors on the eosinophils of hypereosinophilic patients than on those of normal donors or patients with eosinophilia. The present observation has led us to assume that the eosinophilia in the reported patient was a plain eosinophilia the cause of which remains obscure.

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Stimulatory Effect of Blood Platelet Factor(s) on Glycolysis in Cell Cultures

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The addition of bovine blood platelet homogenate to confluent cultures of three established mouse cell lines (L-929, Balb c/3T3 and SC) brings about a rapid increase of lactic acid formation.

Homogenates prepared from quiescent cells previously treated with platelet breakdown products show an increased rate of glycolysis as compared with homogenates of nonstimulated cell cultures.

Activities of hexokinase, pyruvate kinase and lactate dehydrogenase remained unaltered but the activity of phosphofructokinase increased by about 60 per cent.

Transport of 2-deoxy-D-glucose into the cells was slightly stimulated.

It is concluded that the transport of glucose was not a limiting factor in confluent 3T3 cells. The findings suggest that the increase of phosphofructokinase activity may have accounted for the changes in glycolysis seen in intact cells and cell-free homogenates prepared from cultures exposed to some unknown factor(s) present in platelet homogenate.

Key words: blood platelets, fibroblasts, glycolysis-stimulating factor

Introduction

One of the functions of blood platelets seems to be the transport of platelet derived growth factor (PDGF) in the α granules and to release it at injured sites to initiate fibroblast growth and wound repair [1].

Prior to the onset of DNA synthesis stimulated by PDGF observed after 30 h [2, 3], it seems possible that this and/or other factors released during platelet aggregation induce a number of early 'events' which do not require *de novo* protein synthesis. Among these 'events' one of special importance is an increase of energy production in the activated cells.

Many studies have been performed to elucidate the mechanism of the stimulation of energy production (mainly glycolysis) by oncogenic viruses [4] and mitogens, e.g. epidermal growth factor or serum [5, 6]. Little is known about the stimulation of energy production in fibroblasts by factors released by blood platelets. Therefore it seemed worth while to study the effect of platelets on energy production in fibroblasts.

Materials and Methods

Chemicals

2-deoxy-D-1-[^{3}H] glucose was the product of Amershan, England. ATP, ADP, NAD, NADH, NADP, fructose-6-P, phosphoenol pyruvate (tricyclohexylamine salt), aldolase and the α-glycerophosphate dehydrogenase-triosephosphate isomerase mixture were purchased from Sigma Chemical Co. Glucose-6-phosphate dehydrogenase was obtained from the Boehringer Mannheim Corporation. LDH was obtained from POCH (Gliwice, Poland). Other reagents were analytical grade products.

Platelet homogenate preparation

Bovine blood was drawn into 9 : 1 vol 0.2 M trisodium citrate. The platelet-rich plasma was separated by centrifugation at $300 \times g$ at 20 °C for 55 min. The platelets were pelleted by centrifugation at $1000 \times g$ at the same temperature for 15 min and washed twice with 0.14 M NaCl + 1 mM EDTA.

The platelet suspension was cooled to 0 °C and ultrasonicated (4 bursts each at 20 kHz for 15 s). The concentration of protein in the homogenate was determined by the biuret micromethod according to Zamenhof [7].

Cell culture

Studies were performed on three mouse cell lines: L-929, Balb c/3T3 and SC. The cells were grown in Carrell culture flasks (surface of bottom, 25 cm^2) in 10 ml Eagle's minimal essential medium (MEM) with 15 per cent heat inactivated bovine serum. After four days the media from confluent cultures were removed and replaced with MEM without serum (10 ml/flask) supplemented with platelet homogenate in various concentrations.

Measurement of lactic acid production by intact cells

Lactic acid produced by the cells was measured in the medium by the procedure of Hohorst [8]. The cell layer was washed three times with ice-cold 0.14 M NaCl solution and dissolved in 2 ml of 0.2 N NaOH + 0.1 per cent sodium deoxycholate. Protein was determined by the method of Lowry et al. [9].

Measurement of lactic acid production in cell-free homogenates

Cultures of 3T3 cells after 5 h exposure to the platelet homogenate were washed three times with ice-cold 0.14 M NaCl solution, scraped off and suspended in 2 ml of medium containing 50 mM Tris-HCl pH 7.3; 5 mM K_2HPO_4; 40 mM nicotinamide; 5 mM $MgCl_2$; 45 mM KCl; 1 mM ATP; 0.5 mM NAD^+ and

sonicated (4 bursts each at 20 kHz for 15 s). Incubation was performed at 37 °C in 1.5 ml samples of homogenate supplemented with 30 μl of 0.5 M glucose to start the reaction. At the indicated points of time, 200 μl samples of homogenate were removed to measure lactic acid production.

Assay of glycolytic enzyme activities

The cell layer was rinsed three times with ice-cold 0.25 M sucrose, 1 mM EDTA and 1 mM β-mercaptoethanol pH 8.2, scraped off and homogenized in 2 ml of this buffer plus 3 mM ATP, using ultrasonication (4 bursts each at 20 kHz for 15 s at 0 °C). Aliquots were immediately assayed for hexokinase, phosphofructokinase, pyruvate kinase and lactate dehydrogenase activity. Hexokinase activity was determined according to Chou et al. [10], phosphofructokinase activity according to Mansour [11]. Pyruvate kinase activity was assayed according to recommendations of The International Committee for Standardization in Haematology [12]. Lactate dehydrogenase activity was determined according to Bergmayer [13].

2-deoxy-D-glucose transport assay

The cell layer was washed three times with buffer: 50 mM glycylglycine pH 7.4; 5 mM KCl; 2 mM $MgCl_2$; 4 mM Na_2HPO_4; 92 mM NaCl and incubated with 2.5 ml of this buffer at room temperature. The reaction was started by adding 6.25 μCi of [^{3}H]-2-deoxy-D-glucose along with unlabelled 2-deoxy-D-glucose to a final concentration of 10 mM. The reaction mixture was aspirated at the indicated time intervals and cells were dissolved in 2 ml of 0.2 N NaOH + 0.1 per cent sodium deoxycholate and radioactivity was counted. Phosphorylated sugar was precipitated as described by Kletzien and Perdue [14].

Free deoxyglucose was determined by counting the aliquot of the supernatant.

Results

It can be seen from Fig. 1 that the presence of platelet homogenate stimulates the quiescent cells to produce much more lactic acid than do control cells. Such an effect was found in all the investigated cell lines and may be observed in the presence of very low concentrations (0.01–0.02 mg/ml) of platelet protein. The increase in concentration of the platelet protein in culture medium up to 0.5 mg/ml brings about a further stimulation of lactate production while a subsequent increase of the protein concentration does not cause further stimulation.

As can be seen from Figs 2a, b, c, prolongation of incubation resulted in a continuous increase of lactate production in all cultures. The presence of platelet homogenate stimulated lactic acid production. Such a stimulatory effect was observed after one h of incubation.

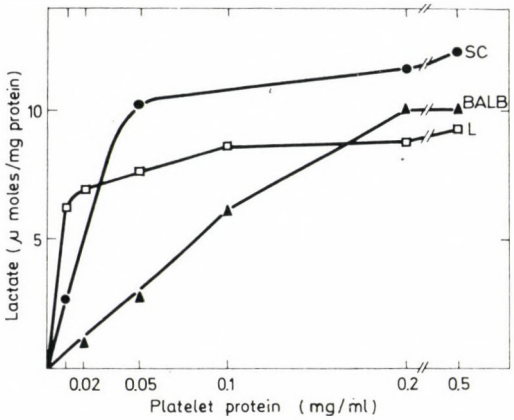

Fig. 1. Increase of lactate production induced by various concentrations of platelet homogenate. The amounts of lactate presented on the ordinate were calculated by subtracting the amounts of lactate produced by control cultures from those produced by cultures treated with platelet homogenate during 5 h incubation

Table 1

The rate of glycolysis in a cell-free system obtained from 3T3 cells treated with platelet homogenate

Incubation time, min	Lactic acid nmol/mg protein	
	control	+ platelet homogenate
0	166.71 ± 35.07	172.08 ± 34.64
30	170.30 ± 39.43	176.64 ± 13.48
60	213.84 ± 25.72	324.87 ± 27.04
90	295.03 ± 26.19	423.66 ± 22.91

Contact cultures of 3T3 cells were incubated in MEM with or without platelet homogenate (0.3 mg/ml) for 5 h. Then the cell-layer was washed, homogenized and incubated in the presence of added glucose, ATP, phosphate and NAD^+. At the indicated time the samples were withdrawn from the reaction mixture to measure the amount of lactate and protein. Further details see under Materials and Methods

The results presented in Table 1 show that the homogenates prepared from quiescent 3T3 cells previously treated with platelet breakdown products produce much more lactate than does the homogenate of control, non-treated cells.

As can be seen from the results summarized in Table 2, 5 h exposure of quiescent 3T3 cells to platelet homogenate caused a 60 per cent increase in total

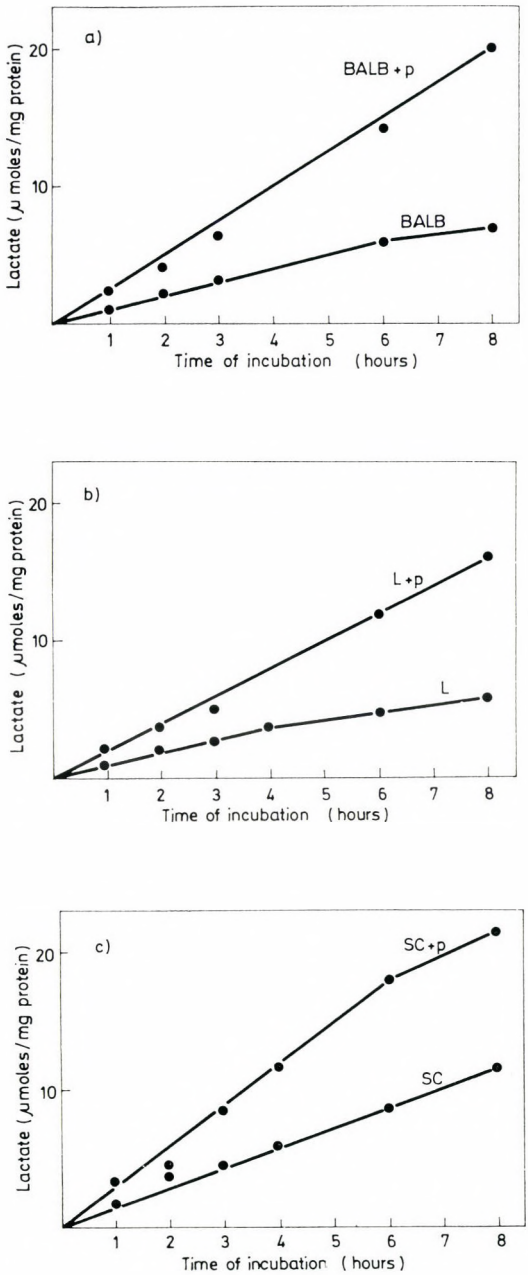

Fig. 2 *a*, *b*, *c*. Comparison of lactate production by cells treated with platelet homogenate and control cultures during various time of incubation (p — indicates the cultures treated with platelet homogenate)

Table 2

The effect of platelet homogenate on the activity of glycolytic enzymes in 3T3 cells

	Hexokinase (glucose-6-phosphate)	Phosphofructokinase (fructose-1,6-diphosphate)	Pyruvate kinase (phosphoenol pyruvate)	Lactate dehydrogenase (pyruvate)
	nmol/mg protein/min			
Control cells	32.27 ± 2.60	98.01 ± 5.99	592.95 ± 24.24	1463 ± 130.11
+ Platelet	34.86 ± 3.97	158.58 ± 5.84	584.48 ± 47.36	1320 ± 115.23

On the fourth day after seeding, the medium was replaced by fresh medium supplemented with platelet homogenate at a final concentration of 0.3 mg/ml. After five h incubation the medium was removed and enzyme activities were measured as described under Materials and Methods

phosphofructokinase activity. The activities of hexokinase, pyruvate kinase and lactate dehydrogenase remained unaltered.

We measured the total flux of 2-deoxy-D-glucose as the appearance of free sugar and 2-deoxy-D-glucose phosphate into intracellular pools of 3T3 cells. It can be seen from Fig. 3 that a 5 h exposure of the cells to platelet homogenate resulted in a 20 per cent stimulation of 2-deoxy-D-glucose uptake by 3T3 cells. The additionally transported sugar can be found in the cells both in free and phosphorylated form.

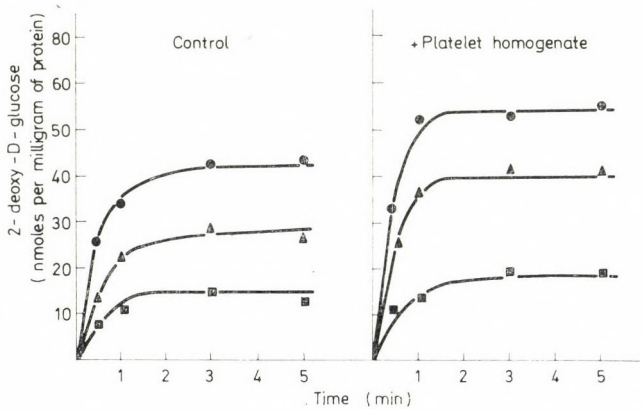

Fig. 3. Effect of platelet homogenate on uptake and phosphorylation of 2-deoxy-D-glucose. Confluent cultures of 3T3 cells treated and non-treated with platelet homogenate were incubated in the presence of unlabelled and [^{3}H] 2-deoxy-D-glucose. After incubation the amounts of total (● — ●), phosphorylated (■ — ■) and free (▲ — ▲) intracellular 2-deoxy-D-glucose were estimated. Further details see under Materials and Methods

Discussion

In formation of the haemostatic plug at wounded sites, a large number of platelets participate. Their aggregation and lysis cause a local increase in the concentration of various platelet factors. Each of them can interact with the surrounding cells for a long time. We therefore believe that a platelet homogenate added to connective tissue cell cultures *in vitro* may simulate the above described situation and such an experimental model may be appropriate to study the mechanism of effect of various platelet factors on cellular metabolism.

The results showed that blood platelets contain a factor which is able to stimulate glycolysis in quiescent cell cultures. The effect can be observed after 1 h of incubation, suggesting that the platelet factor acts quickly after its addition. Since glycolysis is the main source of energy in the studied cell, the platelet factor must stimulate the cells to increase its energy production.

What is the nature of the stimulating factor?

Bovine blood platelets are known to contain a factor(s) which stimulates fibroblasts to produce more protein, e.g. collagen [15], and is able to stimulate their proliferation [16]. Human platelets contain a well-characterized growth factor (PDGF) [2, 3] and probably some other still unknown factors [17]. Addition of growth-promoting factors, such as serum, insulin or epidermal growth factor rapidly increases the rate of lactic acid production by density inhibited cultures of 3T3 cells [5, 6]. It can be speculated that glycolysis is stimulated by PDGF though the results of preliminary experiments (not shown here) suggest that the glycolysis stimulating factor from bovine platelets has a much smaller molecular weight (below 2000) than the PDGF from human platelets.

The stimulation of glycolysis seen in intact cells treated with platelet breakdown products persists after cell homogenization. This suggests that stimulated cells increase their glycolytic capacity. The results of experiments on the activities of key glycolytic enzymes have confirmed this suggestion. We could observe a 60 per cent increase in the activity of phosphofructokinase. Such a change in the activity of this rate limiting enzyme might account for the enhanced glycolysis.

It has been suggested that the transport of glucose across the cell membrane is one of the main limiting factors in glycolysis [18]. However, incubation of 3T3 cells with platelet homogenate brings about only a 20 per cent increase in the uptake of 2-deoxy-D-glucose. Taking into account the great increase in glycolytic glucose degradation in quiescent 3T3 cells, the transport must exceed the cellular demand for glucose. The observed stimulation of lactate production in intact cells upon the addition of platelet homogenate appears to reflect an increase of glycolysis rather than of glucose transport.

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Fibrinolytic Activity of Leukocytes in Atherosclerotic Patients

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It was determined by microautography and the Astrup method that both the total number of leukocytes and the single leukocytes of patients with atherosclerosis have a lower fibrinolytic activity than the leukocytes of healthy persons.

Key words: atherosclerosis, fibrinolysis, leukocytes

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The fibrinolytic potential of tissues and cells in atherosclerosis has been a subject of great interest as it plays an important role in the pathogenesis [1] and in the thrombotic complications in atherosclerotic patients.

Some reduction in the fibrinolytic activity of blood plasma [1–3] and the vascular wall [4] has been noted in these patients. Information is scarce concerning the fibrinolytic activity of blood elements. The fibrinolytic activity of erythrocytes in atherosclerosis [5] and of thrombocytes [6] has been studied but little is known about the fibrinolytic properties of leukocytes [7].

Leukocytes reaching with the blood flow the organs and tissues carry information as they are markers of biochemical processes [8]. Complex enzyme composition in leukocytes and a great number of these cells in the organism suggest their active participation in the intimate mechanisms in atherosclerosis. Still, knowledge on the fibrinolytic properties of leukocytes in atherosclerosis is insufficient and this has stimulated us to undertake the present study.

Materials and Methods

We have examined 54 patients with atherosclerotic affection of the coronary vessels and manifest ischaemic heart disease, and 5 patients with obliterating endarteritis in their lower extremities diagnosed by thorough clinical and biochemical examinations. Biochemical studies included determination of total lipids and cholesterol by the Ilck method, triglycerides by the Karson method, phospholipids non-esterified fatty acids and total amount of beta- and prebetalipoproteins according to Burshtein. Fibrinolytic activity of leukocytes was determined by the fibrin standard plate method of Astrup and Mullertz [9] without modifications and

fibrinolysis microautography [10]. In the former method the lysis zones on heated and non-heated fibrin plates and plates with streptokinase were taken into consideration.

Thrombin and fibrinogen were received from the Kaunas plant of bacteriological preparations. Fibrinogen was dissolved in veronal buffer pH 7.8. The activity of thrombin was chosen so as to coagulate the standard plasma in 12–15 s. Fibrinogen and thrombin solutions were filtered. Streptokinase of 250 units/ml activity was supplied by the Leningrad Research Institute of vaccines and sera. As a control served leukocytes from healthy subjects and specimens with veronal buffer without leukocytes.

Leukocytes for the investigation of fibrinolytic activity were prepared according to the method of Germanov and Bobilev [11] with the modification that sedimentation was carried out at low temperatures to prevent the loss of enzyme activity and a sevenfold washing of leukocytes. The obtained leukocytes were used in human concentrations, $5.0–8.0 \cdot 10^9/l$. The final washing of leukocytes was carried out in buffer solution, which created optimal conditions for fibrinolysis. As a result of the washings, procoagulative and fibrinolytic substances were probably removed from the surface of leukocytes [12].

Destroyed leukocytes were prepared by twofold freezing and thawing and additional grinding. Destruction of cells was controlled under the microscope.

The method of fibrinolytic microautography used in the studies was based on the method introduced by Todd in 1959 and Kwaan and Astrup in 1967: 0.01 ml of thrombin and 0.02 ml of fibrinogen were placed on the edge of a slide together with 0.03 ml of leukocytes of the above-mentioned concentration, then the solutions of thrombin, fibrinogen and the studied specimen of leukocytes were quickly mixed on a slide. The length of the smear was 4 cm, its width 2.5 cm, and its thickness 0.06 cm. The slides were incubated in the humid chamber of the thermostat at 37 °C for 18 h and then fixed and stained by the Romanovsky–Giemsa method.

Fibrinolysis zones around the cells were determined with an eyepiece-micrometer according to the formula: $a = b - c$, where a is the area of fibrinolysis around the cell in mcm^2, b is the area of the leukocyte together with the fibrinolysis zone, and c is the area of the leukocyte, as determined by the formula: πR^2.

Results

In patients with atherosclerosis the mean amount of total blood lipids was 7.036 ± 0.316 g/l; the concentration of phospholipids 152.2 ± 8.3 mmol/l; mean cholesterol content was 5.7 ± 0.2 mmol/l; the triglyceride concentration $1.49 \pm \pm 0.07$ mmol/l; non-esterified fatty acid 0.623 ± 0.048 g/l; the total content of beta- and prebetalipoproteins was 5.616 ± 0.249 g/l.

The zone of fibrinolysis determined on heated fibrin plates was on the average 19.2 ± 1.7 mm^2, essentially lower than that of healthy subjects ($28.8 \pm \pm 3.2$) $p < 0.014$.

Fibrinolysis zones were broader on non-heated than on heated fibrin plates and the difference was 28.3 ± 2.5 mm^2, still much lower than for the healthy subjects (38.4 ± 4.5). The zones were broader on non-heated fibrin plates with streptokinase than on heated ones with streptokinase (15.5 ± 2.4 mm^2); and their value was 17.9 ± 2.2 mm^2, lower than those for healthy subjects (27.2 ± 3.6 mm^2 and 23.0 ± 3.0 mm^2, respectively).

Microautography revealed fibrinolysis zones in 10 per cent of polymorphonuclear leukocytes; they amounted to an average of 56.4 ± 4.9 mcm^2 while in healthy subjects to 86.4 ± 5.8 mcm^2. Zones of fibrinolysis were found around eosinophils and monocytes, too, only the lymphocytes turned out to be inert. Fibrinolytic microautography was either unable to detect insignificant zones of fibrinolysis or an additional activation of the cells is required.

The patients with atherosclerosis were divided into several groups according to Fredrickson's classification (1967). The lowest fibrinolytic activity was observed in type IIa, but the difference failed to reach significance (Table 1).

Discussion

The increase in lysis zones on non-heated fibrin plates in comparison with heated ones showed the presence of plasmin and plasminogen activator in the leukocytes of atherosclerotic patients, but the plasmin and activator activity was lower than in the healthy subjects ($p < 0.014$) [13]. Lysis zones of fibrin plates on the heated and non-heated plates with streptokinase were narrower in atherosclerotics

Table 1

Fibrinolytic activity of leukocytes in different types of hyperlipoproteinaemia in atherosclerotic patients

Investigated subjects	Statistical indices	Components of fibrinolytic activity of leukocytes			
		Plasmin	Plasminogen activator	Plasminogen proactivator	Plasminogen
Healthy persons	n	27	27	27	27
	M ± m	28.8 ± 3.2	38.4 ± 4.5	27.2 ± 3.6	23.0 ± 3.0
Patients with	n	9	9	9	9
hyperlipoprotein-	M ± m	13.4 ± 3.2	21.9 ± 6.6	15.4 ± 6.2	8.9 ± 1.9
aemia II a	P	<0.003	<0.05	>0.12	<0.05
Patients with	n	4	4	4	4
hyperlipoprotein-	M ± m	19.3 ± 4.2	31.0 ± 4.8	18.3 ± 7.6	12.5 ± 4.1
aemia II b	P	>0.07	>0.28	>0.32	<0.05
Patients with	n	7	7	6	7
hyperlipoprotein-	M ± m	19.3 ± 4.8	33.0 ± 9.9	12.9 ± 3.2	21.1 ± 10.3
aemia IV	P	>0.72	>0.62	<0.007	>0.62

than in healthy subjects which indicates that the proactivator and plasminogen values were also lower in patients with atherosclerosis than in healthy subjects. The data were obtained for destroyed leukocytes, i.e. for the total number of cells. In order to judge the activity of a leukocyte fibrinolysis microautography was applied. Leukocytes were found to be active fibrinolytically but activity was more often observed in polymorphonuclear leukocytes. The lymphocytes proved to be fibrinolytically inert or they should have been activated additionally.

Thus, it has been shown by two methods that both the total number of leukocytes and the single leukocyte cells of patients with atherosclerosis have a weaker fibrinolytic activity than the leukocytes of healthy subjects. This weakening may be brought about by the decrease of activators, as shown by us, or else an increase of inhibitors.

Leukocytes in atherosclerotic patients are probably less active in thrombolysis and in recanalization of thromboembolic vessels.

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In vitro Investigation of Rh Antibodies in Pregnant Women

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Seventeen Rh negative pregnant women who had Rhesus alloantibodies were investigated serologically. The antibody-mediated cellular cytotoxicity (ADCC) against target Rhesus positive human erythrocytes was determined. The investigation was repeated in the first, second and third trimester of pregnancy and after delivery. There was no correlation between ADCC and antibody titre.

The antibody titre and the cytotoxic index increased during pregnancy, but the cytotoxic index decreased after delivery. The cytotoxic index showed individual characteristics and was quite independent of the titre of anti-D.

There was no correlation between the heavy chain type of anti-D and the cytotoxic index.

Our data suggest that the individual variations during pregnancy of the supposed cytotoxic antibody acting together with anti-D play an important role in pregnancy and delivery.

Key words: cellular cytotoxicity, pregnant women, Rhesus alloantibodies

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No simple explanation of the mechanism by which the foetus escapes maternal immune destruction is yet available [1]. Masking of the foetal transplantation antigens by glycocalix [3] and blocking antibodies [5], as well as a decrease in maternal immune reactivity are accepted as important factors in this respect. Plum et al. [14] have shown that there is no evidence of a fundamental change in the production or distribution of the constituents of the maternal immune system, but the hormones may stimulate the monocyte-macrophage system.

The present study was undertaken to determine the effect of gestation on the lymphocyte dependent (K cell) cytotoxicity to human erythrocytes by Rhesus alloantibodies.

Materials and Methods

Rhesus alloantibodies were obtained from mothers sensitized during pregnancy. The anti-D serum used as a control was obtained from normal human donors immunized with Rh positive red blood cells. Specificity and titres of the

antibodies were determined by routine blood banking methods. The sera containing alloantibodies were inactivated at 56 °C for 30 min. Sera were diluted in Parker's medium supplemented with 10 per cent AB serum free of red cell antibodies.

Macrophage-depleted peripheral blood lymphocytes

Mononuclear leucocytes were obtained from heparinized venous blood by Ficoll-Uromiro density gradient centrifugation [2]. Contaminating monocytes and granulocytes were removed by iron carbonyl and a magnetic field. This was followed by using the adherence technique to deplete the mononuclear cells in Falcon Petri dishes in CO_2 atmosphere at 37 °C for 45 min. Adherence was repeated three times. Nonadherent cells were resuspended to provide 2×10^7 cells/ml in Parker's medium supplemented with 10 per cent AB serum. Contaminating monocyte macrophages were counted histochemically after esterase staining [8]. The monocyte contamination was less than 1 per cent.

Erythrocytes

Fresh human RBC of genotype R_1R_2 (CDe/cDE) were washed in saline and a portion of the red cells was treated by 1 per cent papain solution at room temperature for 10 min. The treated cells were then labelled with 100 μCi ^{51}Cr (LNK) at 37 °C; after 1 h incubation, the cells were washed three times and resuspended in Parker's medium. Target erythrocytes were adjusted to 1×10^6 cells/ml in Parker's medium supplemented with 10 per cent AB serum.

Cytotoxic assay

The assay was carried out in U plates. Each well contained 5×10^4 ^{51}Cr labelled target cells and effector lymphocytes at a 20 : 1 effector to target cell ratio. Of the sera of pregnant women containing anti-Rh antibodies 50 μl were measured into the wells, 50 μl of effector lymphocytes were added and they were made up to 200 μl total volume by Parker's medium with 10 per cent AB serum. The percentage of ^{51}Cr was calculated [9] and the results were expressed as cytotoxic index.

We investigated 17 Rh negative pregnant women who had anti-D antibodies. The investigation was repeated in the first, second and third trimester of pregnancy and after delivery.

Results

There was no correlation between antibody titre and ADCC in the serum of the pregnant women (Fig. 1). This finding supports our earlier supposition that together with anti-D, another cytotoxic antibody may exist [9].

In four cases, we found measurable anti-C titres. Three of these women also had high levels of ADCC. This finding supports our earlier observation that anti-C + D causes a higher ADCC than anti-D alone [9].

The anti-D titre increased during the pregnancy of 7 of the women; the cytotoxic index varied with each individual but in a different way. Generally, it was lowest in the first trimester and highest before delivery (Fig. 2). A very high cytotoxic index appeared in some cases in the second trimester. In at least two of these cases, births were before term. After delivery the cytotoxic index generally decreased (Table 1).

The heavy chain type anti-D produced in sensitized women was usually IgM, IgG-1. In 3 three cases, both IgG-1 and IgG-3 were found and in 2 cases, IgG-1 and IgG-4. In 1 case the anti-D was of the mixed heavy chain type. This pregnant woman

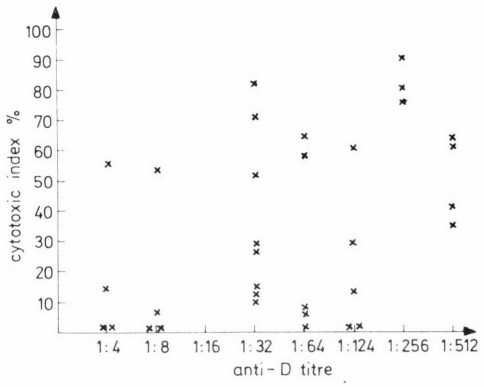

Fig. 1. Anti-D titre and cytotoxic index of pregnant women. No correlation is apparent between these characteristics

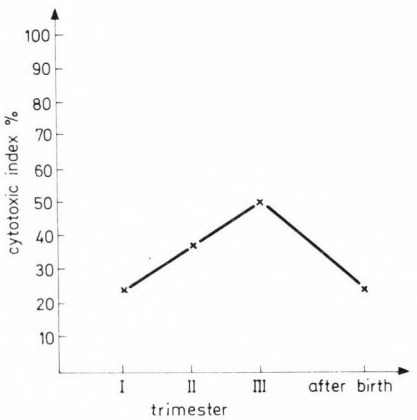

Fig. 2. Changes of cytotoxic index during pregnancy

Table

Anti-D titre and

Subjects	Anti-C titre*	I. Trimester		II. Trimester	
		Anti-D titre	Cytotoxic index	Anti-D titre	Cytotoxic index
H. L.	0			1 : 64	58.4
K. L.	1 : 8			1 : 32	81.4
K. B.	0	1 : 8	52.2	1 : 8	10.5
E. G.	0			1 : 4	14.8
K. K.	0	1 : 256	77.7	1 : 512	62.9
B. F.	0	1 : 64	10.2	1 : 64	8.2
E. E.	0			1 : 512	42.0
M. F.	1 : 4			1 : 8	−6.3
M. P.	0	1 : 8	1.7	1 : 8	0.0
M. J.	1 : 4			1 : 256	90.1
R. A.	0	1 : 128	13.4	1 : 128	60.4
T. K.	0	1 : 64	13.7	1 : 64	0.1
K. J.	0	1 : 4	0.0	1 : 4	0.4
M. J.	0				
A. J.	0			1 : 256	32.2
R. J.	1 : 4			1 : 128	55.6
R. L.	0				

* Anti-C titre did not change during pregnancy

had been plasmapheresed. In 4 cases, the anti-D was IgM. We did not find any correlation between the heavy chain type of anti-D and the cytotoxic index.

Figure 3 shows the changes of the cytotoxic index during pregnancy. The pregnant women could be divided into two groups, the first group with a high,

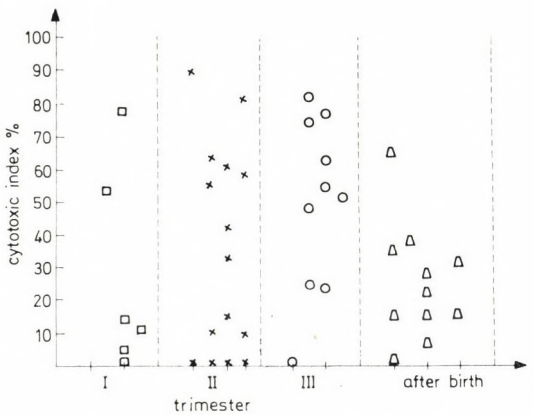

Fig. 3. Cytotoxic index formation during pregnancy

1

cytotoxic index, %				
III. Trimester		After delivery		Antibody type
Anti-D titre	Cytotoxic index	Anti-D titre	Cytotoxic index	
		1 : 128	7.4	IgM, IgG-1
1 : 128	47.5	1 : 128	27.6	IgM, IgG-1
1 : 64	22.7	1 : 64	13.9	IgM, IgG-1,2,3,4, IgA
		1 : 128	36.2	IgM, IgG-1,3
				IgM, IgG-1,3
		1 : 64	64.9	IgM, IgG-1
1 : 512	61.8	1 : 512	60.0	IgM, IgG-1
1 : 8	0	1 : 16	25.5	IgM
1 : 8	52.4	1 : 8	6.6	IgM, IgG-1
1 : 256	80.9			IgM, IgG-1,3
		1 : 128	33.5	IgM, IgG-1
		1 : 128	29.7	IgM, IgG-1
		1 : 4	−1.8	IgM
1 : 4	54.2	1 : 4	13.7	IgM
1 : 256	73.1	1 : 256	22,7	IgM, IgG-1,4
1 : 128	76.8	1 : 128	57.0	IgM, IgG-1,4
1 : 16	24.2	1 : 16	13.9	IgM

and the other with a low cytotoxic index. The high and low cytotoxic indexes were independent of the titre of anti-D.

The cytotoxic index of the immunized donors and the pregnant women both having anti-D were also compared (Table 2). In the donors, the cytotoxic index and the anti-D titre changed in parallel, but some of the pregnant women had a low anti-D titre and a high cytotoxic index while in others a high anti-D titre was associated with a zero or a very low cytotoxic index. The increase of anti-D titre did not run parallel with the increase of ADCC.

Discussion

Several human peripheral blood leukocyte populations have been shown to mediate ADCC. Depending on the conditions of the assay, cytotoxicity may be mediated by lymphocytes, monocytes, and neutrophils [6, 7, 10–13]. The rise in the leukocyte count during pregnancy is due to neutrophilia [4].

The present studies were undertaken to define the effect of lymphocyte dependent cytotoxicity to human erythrocytes with Rhesus alloantibodies during gestation. There was no correlation between antibody titre and ADCC. Antibodies eliciting ADCC might be different from the serologically detectable ones, as shown by our earlier studies.

Table 2

Antibody titre and ADCC in pregnant women and donors

Antibody titre	Cytotoxic index, %	
	donors	pregnant women
1 : 4	−0.88	14.8
	6.9	6.6
	11.4	52.2
	−0.5	0.0
	−3.9	0.4
1 : 8	1.8	52.2
		−6.3
		1.7
		0.0
		52.4
1 : 16	57.2	0.0
1 : 32	23.5	10.5
		27.6
		70.7
		13.7
1 : 64		70.2
		13.9
		0.0
		8.2
		64.9
1 : 128	51.9	7.43
	45.6	27.6
		36.2
		13.4
		60.4
		33.5
		0.1
1 : 256	42.7	77.7
	35.4	90.1
	21.8	80.9
	71.1	
1 : 512	28.8	62.9
	11.1	77.8
	80.8	42.0
	65.6	61.8

The cytotoxic index and the anti-D increased during pregnancy and decreased after birth, but the level of cytotoxic index was independent of the titre of anti-D. According to Plum et al. [14], the number of leukocytes significantly increases during the third trimester of gestation. This finding seems to support our observation that the cytotoxic index is highest before delivery. We believe that the anti-

bodies eliciting ADCC might be correlated more with the change of the status of pregnant women than with that of anti-D.

Rosenthal [15] observed a significant enhancement of the phagocytosis of immune complexes by normal peripheral blood polymorphonuclear leukocytes in the presence of pregnant serum. This observation suggests that sera of pregnant women contain a factor which may further stimulate the increased clearance of immune complexes. This quality and quantity of immune complexes might play a role in ADCC.

Our data suggest that the individual variations during pregnancy of the supposed cytotoxic antibody acting together with anti-D has an important role in pregnancy and delivery. We are conducting further studies to determine whether there is any connection between the cytotoxic index, the immune complexes, premature birth and foetal death.

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Book Reviews

A Colour Atlas of Haematological Cytology. By F. G. J. Hayhoe and R. J. Flemans. 2nd edition. Wolfe Medical Publications Ltd, London 1982. 240 pages, 750 colour photomicrographs.

The second edition of this Atlas is divided into five parts: 1. the red cells and their precursors; 2. granulocytes, monocytes and megakaryocytes; 3. lymphocytes, plasma cells and their derivatives and precursors; 4. miscellaneous cells from bone marrow and blood smears; and a new section 5. imprints of lymph nodes and spleen, cells from pleural, ascitic and cerebrospinal fluid. Normal and abnormal forms are illustrated. In the Appendix technical details of some staining and cytochemical methods are given.

Most of the photographs are from smears stained with the Leishman or May–Grünwald–Giemsa methods, but numerous smears ware stained for cytochemistry by methods used routinely in haematological diagnosis (Prussian blue reaction, Sudan black B staining, PAS reaction, techniques for peroxidase, acid and alkaline phosphatases, chloroacetate and butyrate esterases).

The intention of the authors was to present an atlas not a textbook. This aim seems to be fulfilled by this work. It contains 750 colour photomicrographs in comparison with the 349 of the first edition.

Each part of the Atlas is preceded by a brief introduction. The legends accompanying the photomicrographs do not give an exact morphological description of the cells illustrated, being mostly only the enumeration of the different cells in the given photo (e.g. 'a promyelocyte, two myelocytes, a metamyelocyte and a stab cell, of the neutrophil series') without mentioning the specific magnifications.

Some obvious errors are reprinted uncorrected from the first edition, e.g. the prescription of 250 mg benzidine in 10 ml solvent for the Graham–Knoll peroxidase technique.

The Atlas is printed on good quality paper and the colour rendering of the illustrations has been improved. The format of the book is more convenient than that of the first edition.

Despite these minor objections the book is warmly recommended to all who are interested in haematological cytology.

G. Lelkes

Roentgenologic Changes Observed in Blood System Diseases. By E. Z. Novikova. Medicina Publishing House, Moscow 1982. 256 pages, 66 figures

Professor Novikova, head of the Roentgenology Department of the Moscow Institute of Haematology, summarizes in this monograph her exceptional experience in the X-ray diagnosis of haematological diseases. Her monograph on Roentgen symptomatology of bone changes accompanying haematologic diseases appeared fifteen years ago, still serving today as a useful aid in clinical practice.

The new monograph is divided into five chapters. The first deals with thoracic cavity organs, the second with the gastrointestinal tract, the third with the spleen, the fourth

with lymphatics and the fifth with the osseous system and its changes of haematologic origin. The last chapter is, of course, only a shortened recapitulation of the 1967 monograph.

Professor Novikova has been following the patients of her institute for many decades and provides a comparative analysis of X-ray diagnostic observations with pathologic and histologic controls. Her conclusions, based on highly conscientious and careful studies, are supported by a strict follow-up of patients and X-ray diagnostic case records sometimes of several decades. In addition to the most frequent regular Roentgen-morphologic manifestations the book discusses numerous rarities the occurrence of which may be explained by the unusual number of examined patients.

The book is interesting and instructive for physicians dealing with haematologic diseases and a long-needed aid in the every-day practice of haematologic X-ray diagnostics.

J. Forrai

Abstracts

The formation of erythrocyte membrane proteins during erythropoietin-induced differentiation. B. D. Tong, E. Goldwasser (Department of Biochemistry, University of Chicago, Chicago, Ill. USA). *J. Biol. Chem. 256*, 12 666 (1981).

The effect of erythropoietin on the synthesis of the proteins characteristic of mature erythrocyte membranes was studied in cultures of bone marrow cells from adult, polycythemic rats. Stimulated synthesis of the major glycoprotein, glycophorin, was maximal at 30 h and fell to control level by 66 h. Stimulated synthesis of the major integral membrane protein, band 3, occurred at about 18 h, was maximal at 66 h, and fell to control level by 114 h. In contrast, stimulated hemoglobin synthesis did not start until after 24 h, was maximal at 96 h, and was at control level at 114 h. Erythropoietin had, in addition, a transient effect on the synthesis of some membrane proteins found in marrow cells but not in mature red cells.

G. Gárdos

Kinetic properties of the reconstituted glucose transporter from human erythrocytes. T. J. Wheeler, P. C. Hinkle (Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, N.Y., USA). *J. Biol. Chem. 256*, 8907 (1981).

The kinetic parameters of d-glucose transport in liposomes reconstituted with the purified glucose transporter were determined. Net uptake and efflux both had K_m values of 0.7 to 1.2 mM and V_{max} values of 1.6 μmol/mg of protein/min. Equilibrium exchange had a K_m of 35 mM and a V_{max} of 50 μmol/mg of protein/min. By separating the liposomes from unreconstituted protein using density centrifugation, the V_{max} of exchange was increased to 86 μmol/mg of protein/min, about 3 times that of the erythrocyte membrane. Trypsin which inhibits erythrocyte glucose transport only from the cytoplasmic side, inhibited reconstituted transport activity about 40% when added externally. With internal treatment as well, the inhibition was about 80%. This suggests that the reconstituted transporter is oriented about equally in both directions. Antibody prepared against the purified transporter inhibits transport to a maximum of about 50%, also indicating a scrambled orientation. External trypsin treatment decreased the K_m for uptake and increased the K_m for efflux, consistent with asymmetric kinetic parameters for the two faces of the transporter. However, the calculated K_m values are lower than those reported for erythrocytes. Phloretin and diethylstilbestrol inhibit the reconstituted transporter. However, they bind to liposomes, producing anomalous results under some experimental conditions. When this binding is taken into account, phloretin inhibits completely and symmetrically. The binding accounts for the apparent asymmetric effects of phloretin reported by others. The inhibitory effects of mercuric ions are consistent with action at two classes of binding sites. Treatment with trypsin increases the sensitivity to Hg^{2+}, indicating that the more sensitive site is on the external face of the transporter.

G. Gárdos

Membrane-bound ATP fuels the Na/K pump. Studies on membrane-bound glycolytic enzymes on inside-out vesicles from human red cell membranes. R. W. Mercer, P. B. Dunham (Department of Biology, Syracuse University, Syracuse, N.Y., USA). *J. Gen. Physiol. 78*, 547 (1981).

ATP stimulates Na transport into inside-out vesicles (IOVs) made from human red cell membranes; strophanthidin inhibits the ATP-stimulated transport. The substrates for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) (glycolytic enzymes bound to the cytoplasmic surface of the red cell membrane) also stimulate Na transport into IOVs without added ATP. The elution of GAPDH from the membranes prevents the stimulation by the substrates, but not by exogenous ATP. Hexokinase plus glucose (agents that promote breakdown of ATP) prevent stimulation of Na transport by exogenous ATP but not by the substrates for GAPDH and PGK. [^{32}P]orthophosphate is incorporated into a membrane-bound organic phosphate compound shown chromatographically to be ATP. The level of membrane-bound ATP is decreased when Na is added, and this decrease is inhibited by strophanthidin. When further synthesis of [^{32}P]ATP is blocked by the addition of unlabeled orthophosphate, all of the membrane-bound [^{32}P]ATP is dissipated by the addition of Na. From these observations it was concluded that membrane-bound glycolytic enzymes synthesize ATP and deposit it in a membrane-associated compartment from which it is used by the Na/K pump.

G. Gárdos

Membrane alterations in cellular aging: Susceptibility of phospholipids in density (age)-separated human erythrocytes to phospholipase A_2. S. D. Shukla, D. J. Hanahan (Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas, USA). *Arch. Biochem. Biophys. 214*, 335 (1982).

Human erythrocytes were separated into four density (age) groups representing the top 10% (young), bottom 10% (old), and two middle fractions of 40% each (intermediary ages). When these erythrocytes of different age groups were treated with the low levels of a purified basic phospholipase A_2 from *Agkistrodon halys blomhofii*, under conditions where little or no hemolysis occurred, the optimum extent of phosphatidylcholine (PC) hydrolysis in all age groups was the same, but interestingly, the rate of its hydrolysis was two to three times faster in the older cells compared to younger erythrocytes. On the other hand, hydrolysis of phosphatidylethanolamine (PE) of younger erythrocytes by the phospholipase A_2 was negligible under the particular experimental conditions. However, in erythrocytes of older age groups, both the rate and extent of PE hydrolysis by the enzyme increased in a distinctive fashion. Concomitant with the above pattern of PC and PE hydrolysis, the shape changes in the erythrocytes also were different; whereas all older erythrocytes became echinocytic only two-thirds of the younger erythrocytes showed a similar shape change. These observations firmly establish that during *in vivo* aging of normal erythrocytes in circulation significant changes in the structural organization of membrane phospholipids take place. Importance of this phenomenon in membrane phospholipid asymmetry studies and in the elimination of senescent cells also is discussed.

G. Gárdos

Localization of 2',3'-cyclic nucleotide 3'-phosphodiesterase in human erythrocyte membranes. C. E. Dreiling (Division of Biochemistry, School of Medicine, University of Nevada, Reno, Nevada, USA). *Biochim. Biophys. Acta 649*, 587 (1981).

The location of 2',3'-cyclic nucleotide 3'-phosphodiesterase in human erythrocyte membranes was determined. This was accomplished by comparing the enzyme's accessibility with that of glyceraldehyde-3-phosphate dehydrogenase (cytoplasmic surface marker) and acetylcholinesterase (external marker) in sealed and unsealed ghosts and normal and inverted membrane vesicles. The results showed that 2',3'-cyclic nucleotide 3'-phosphodiesterase, like glyceraldehyde-3-

phosphate dehydrogenase, meets several criteria for an inner (cytoplasmic) membrane location: (1) the enzyme was accessible to substrate in unsealed ghosts and inside-out vesicles but not in sealed or right-side-out vesicles, (2) latent activity in sealed ghosts could be exposed with detergent (Triton X-100), (3) activity in unsealed ghosts was gradually sequestered during resealing and could be re-exposed with detergent, and (4) the enzyme was susceptible to trypsin proteolysis only in unsealed ghosts. These results demonstrate that the active site of 2',3'-cyclic nucleotide 3'-phosphodiesterase faces the cytoplasm of erythrocytes and that the enzyme may not span the lipid bilayer of the membrane. The localization of the phosphodiesterase on the inner membrane surface of erythrocytes suggests that the similar enzyme of myelin may be embedded within the major dense line of the compact lamellae.

Ilma Szász

Control of the erythrocyte membrane shape: Recovery from the effect of crenating agents. E. Alhanaty, M. P. Sheetz (Physiology Department, University of Connecticut Health Center, Farmington, Conn. USA). *J. Cell Biol. 91*, 884 (1981).

Intact erythrocytes become immediately crenated upon addition of 2,4-dinitrophenol (DNP) or pyrenebutyric acid (PBA). However, when cells are incubated at 37 °C in the presence of the crenating agents with glucose, they gradually (4–8 h) recover the normal biconcave disc form. The recovery process does not reflect a gradual inactivation of DNP or PBA since fresh cells are equally crenated by the supernatant from the recovered cells. Further, after recovery and removal of the crenating agents, cells are found to be desensitized to the readdition of DNP as well as to the addition of PBA, but they are more sensitive to cupping by chlorpromazine. This alteration in the cell membrane responsiveness was reversible upon further incubation in the absence of DNP. Recovery is dependent upon cellular metabolic state since an energy source is needed and incubation with guanosine but not adenosine will accelerate conversion to the disc shape. It is suggested that the conversion of cells from crenated to disc shape in the presence of the crenators, represents an alteration or rearrangement of membrane components rather than a redistribution of the crenators within the membrane. This shape recovery process may be important for erythrocyte shape preservation as well as shape control in other cells.

Ilma Szász

Transport of neutral amino acids by human erythrocytes. E. A. Al-Saleh, K. P. Wheeler (School of Biological Sciences, University of Sussex, Brighton, UK). *Biochim. Biophys. Acta 684*, 157 (1982).

The transport of several neutral amino acids by human erythrocytes *in vitro* was studied. The measurements made included steady-state distributions, kinetics of initial rates of uptake, effects of monovalent cations and anions, general mutual inhibitory interactions, kinetics of inhibitions, effluxes, ability to produce accelerative exchange diffusion, and the inhibitory action of the thiol reagent N-ethylmaleimide. The results are interpreted as showing that the human erythrocyte membrane possesses several distinct transport systems for these amino acids, including one Na^+-dependent system and one dependent on both Na^+ and a suitable anion, that are qualitatively similar to those systems previously described in pigeon erythrocytes and mammalian reticulocytes. Quantitatively, however, the systems differ among the different kinds of red cell and a major difference lies in their abilities to produce accelerative exchange diffusion.

Ilma Szász

The association of human erythrocyte catalase with the cell membrane. I. Aviram, N. Shaklai (Department of Biochemistry, The George S. Wise Center of Life Sciences and Department of Chemical Pathology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel). *Arch. Biochem. Biophys. 212*, 329 (1981).

A specific interaction of human erythrocyte catalase with the inner surface of the

red cell membrane was demonstrated. The dependency of catalase affinity on pH and ionic strength implies that the interaction is dominated by electrostatic forces. Scatchard analysis of the binding at pH 6.0 and 5 mM Mes buffer reveals a single class of approximately 10^6 binding sites/ghost with an association constant of 2.5×10^7 M^{-1}. The membrane-bound catalase retains its enzymatic activity. Competition binding studies of catalase and other proteins known to associate with the membrane inner surface were carried out. It was found that the binding of catalase is inhibited by aldolase, glyceraldehyde-3-phosphate dehydrogenase as well as by hemoglobin. The advantage of membrane-bound catalase in protection of the cell membrane against peroxidative damages is discussed.

Ilma Szász

Anti-transferrin receptor monoclonal antibody and toxin-antibody conjugates affect growth of human tumour cells. I. S. Trowbridge, D. L. Domingo (Department of Cancer Biology, The Salk Institute for Biological Studies, San Diego, CA, USA). *Nature 294*, 171 (1981).

The development of the monoclonal antibody technique has led to renewed interest in whether the growth of tumour cells may be specifically inhibited by antibodies or antibody-toxin conjugates directed against antigenic determinants selectively expressed on tumour cells. Recently, monoclonal antibodies were obtained against the transferrin receptor of human cells, which is thought to have an essential role in transport of Fe across the cell membrane and which is selectively expressed on proliferating cells *in vivo* and *in vitro*. In some cases, transferrin receptors can be used as a marker to distinguish between tumour cells and normal tissue. Here it is shown that human tumour cells are specifically killed *in vitro* by anti-transferrin receptor antibody covalently coupled to ricin or diphtheria toxic subunits. In experiments designed to test the effectiveness of these antibody-toxin conjugates *in vivo*, it was found that anti-transferrin receptor antibody alone inhibits the growth of human melanoma cells in nude mice. *A. Egyed*

Transferrin and iron uptake by human lymphoblastoid and K-562 cells. H. M. Schulman, A. Wilczynska, P. Ponka (Lady Davis Institute for Medical Research of the Sir Mortimer B. Davis Jewish General Hospital, Montreal, Quebec, Canada). *Biochem. Biophys. Res. Comm. 100*, 1523 (1981).

Two human lymphoblastoid cell lines and K-562 cells were found to take up radioiodinated transferrin and transferrin-bound iron in amounts comparable to reticulocytes. These cell lines were also shown to possess transferrin receptors whose numbers and affinity for transferrin were similar to those of reticulocytes. However, unlike reticulocytes, in which at least 90% of the iron taken up is incorporated into heme, in the lymphoblastoid and K-562 cells only around 10% of the incorporated iron is found in heme. In addition, in contrast to the hemoglobin synthesizing cells, excess heme does not inhibit the removal of iron from transferrin by the lymphoblastoid and K-562 cells, suggesting that only during erythroid differentiation do cells acquire a specific mechanism for removing iron from transferrin which is subject to feedback inhibition by heme.

A. Egyed

Asymmetric metabolism of phosphatidylethanolamine in the human red cell membrane. G. V. Marinetti, K. Cattieu (Department of Biochemistry, University of Rochester Medical Center, Rochester, N.Y., USA). *J. Biol. Chem. 257*, 245 (1982).

The incorporation of labelled fatty acids into phosphatidylethanolamine (PE) on the two sides of the human red cell membrane was studied by use of the vectorial probe trinitrobenzene sulfonate (TNBS). A small population of PE molecules on the outer surface of the membrane has a 4-fold higher turnover rate than the remaining PE molecules. This effect is greatest with palmitic acid, less with linoleic and linolenic acids, and not seen with stearic acid. By use of the hydrophobic penetrating probe fluorodinitrobenzene (FDNB), a second larger population of PE and phosphatidylserine (PS) molecules

is found which reacts with FDNB and has a higher specific activity than the PE and PS molecules which do not react. With human polymorphonuclear cells, the labelled PE molecules inside the cell have a higher specific activity than the PE molecules located on the outer cell surface. These results suggest that there are heterogeneous populations of PE and PS on both halves of the red cell membrane which show different metabolic turnover rates of their fatty acids.

B. Sarkadi

The role of calmodulin in the regulation of (Mg^{2+} + Ca^{2+})-ATPase activity in erythrocyte membranes of cystic fibrosis patients and controls. S. Katz, D. L. Emery (Division of Pharmacology, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, B.C., Canada). *Cell Calcium 2*, 545 (1981).

(Mg^{2+} + Ca^{2+})-ATPase activity has been found to be significantly reduced in EDTA-washed erythrocyte membrane preparations from cystic fibrosis patients compared to aged-matched controls. Calmodulin was found to be present in erythrocytes from cystic fibrosis patients and characterized similarly to calmodulin isolated from control preparations. Calmodulin from control erythrocyte preparations stimulated the (Mg^{2+} + Ca^{2+})-ATPase activity of EDTA-washed erythrocyte membranes derived from cystic fibrosis patients to the same extent as those membranes derived from controls. Similarly, calmodulin obtained from erythrocytes of cystic fibrosis patients stimulated the (Mg^{2+} + Ca^{2+})-ATPase activity of control and cystic fibrosis erythrocyte membrane preparations to a similar extent. These results indicate that this decrease in (Mg^{2+} + + Ca^{2+})-ATPase activity in erythrocytes from cystic fibrosis patients is not due to an alteration in the regulatory function of calmodulin.

B. Sarkadi

Metabolism of phosphoinositides in the rat erythrocyte membrane. A reappraisal of the effect of magnesium on the ^{32}P incorporation into polyphosphoinositides. P. Marche, S. Koutouzov, P. Meyer (Inserm U7/CNRS La 318 Research Unit, Department of Nephrology, Hôpital Necker, Paris, France). *Biochim. Biophys. Acta 710*, 332 (1982).

The metabolism of phosphoinositides was investigated in the red blood cell membrane of the rat by measuring ^{32}P-incorporation into phospholipids after incubation of membranes with [γ-^{32}P]ATP in a medium containing magnesium. A new chromatographic procedure has been developed which facilitates the separation of triphosphoinositide, diphosphoinositide and phosphatidylinositol from the phospholipids present in lipid extracts of incubated 'ghost' membranes. Under the experimental conditions only two phospholipids, diphosphoinositide and triphosphoinositide, were ^{32}P-labelled. Furthermore, the results indicate that either di- or triphosphoinositide could be labelled preferentially, depending upon the magnesium concentration of the incubation medium. This clarifies some apparent discrepancies reported in the literature between the ^{32}P labelling of polyphosphoinositides observed in intact erythrocytes and that observed with 'ghost' membranes. In addition, the enzymatic pathways involved in the phosphoinositide metabolism are discussed.

B. Sarkadi

Erythrocyte-ghost Ca^{2+}-stimulated Mg^{2+}-dependent adenosine triphosphatase in Duchenne muscular dystrophy. M. J. Dunn, A. H. M. Burghes, V. Dubowitz (Jerry Lewis Muscle Research Centre, Department of Pediatrics and Neonatal Medicine, Hammersmith Hospital, London, UK). *Biochem. J. 201*, 445 (1982).

The Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activities (Ca^{2+}-ATPase) of erythrocyte-ghost membranes from patients with Duchenne muscular dystrophy (DMD) and carriers of DMD were compared with activities of normal controls. The Ca^{2+}-ATPase activity of DMD-patient ghost preparations was found to follow the same pattern of activation by Ca^{2+} as the control mem-

branes. However, the Ca^{2+}-ATPase activity in DMD and some DMD-carrier preparations was substantially elevated compared with controls. To characterize further the elevated Ca^{2+}-ATPase activity found in DMD-patient ghost membrane preparations, kinetic parameters were estimated using both fine adjustment and weighting methods to analyze the experimental data. It was established that in both DMD and DMD-carrier preparations the increase in Ca^{2+}-ATPase activity was reflected by a significant increase in V_{max} rather than by any change in K_m. The response of the membrane Ca^{2+}-ATPase activity to changes in temperature was also investigated. In all preparations a break in the Arrhenius plot occurred at 20 °C, and in DMD and DMD-carrier preparations an elevated Ca^{2+}-ATPase activity was detected at all temperatures. Above 20 °C the activation energy for all types of preparation was the same, whereas below this temperature there appeared to be an elevated activation in DMD and DMD-carrier preparations compared with normal controls. The concept that a generalized alteration in the physicochemical nature of the membrane lipid domain may be responsible for the many abnormal membrane properties reported in DMD is discussed.

B. Sarkadi

News Items

The IXth Congress of the International Society on Thrombosis and Haemostasis

will take place in

Stockholm (Sweden), 3–8 July, 1983

Main topics:

Coagulation
Blood-surface interactions
Platelets
Arachidonic acid metabolism and its inhibition
Natural and synthetic anticoagulants
Thromboembolism
Atherosclerosis
Haemorrhagic disorders
Immunology

Further information:

IXth ISTH Congress, Stockholm Convention Bureau AB, Jakobs Torg 3, S-111 52 Stockholm, Sweden. Telex 115 56, Telegrams: Congressus

*

The 7th Meeting of the European-African Division of the International Society of Haematology

will take place in

Barcelona (Spain), September 4–9, 1983

President: Prof. C. Rozman

Enquiries: Secretariat, 7th Meeting ISH, OTAC, S. A., Congress Organization, Sepúlveda 45–47, Barcelona-15, Spain

*

The 20th Congress of the International Society of Haematology

will take place in

Buenos Aires (Argentina), September 1–7, 1984

President: Prof. L. J. Bergna

Enquiries: XX Congress of the International Society of Haematology, Congress Secretariat, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina. Tel.: 83–0702

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Academic Year 1983 – 1984

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Applications must be submitted before 1st March, 1983, and awards will be announced by the Trustees in June.

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The original and *two* additional copies of the manuscript should be submitted. They should be typed double-spaced on one side of a good quality paper with proper margins. The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which the work has been carried out; (4) name and full postal address of the author to whom communications regarding the manuscript should be directed; (5) a short title not to exceed 40 characters (including space) to be used as a running head. The second page should contain an abstract of 50—100 words, summarizing the reasons for the study, the methods used, the results, and the major conclusions. This page should also contain 4—8 key words placed in alphabetical order. Original papers should not exceed 15 printed pages including tables, figures, and references. Case reports should not be more than four, technical notes and Letters to the Editor not more than two printed pages in length. In the manuscripts the approximate location of tables and figures should be indicated in the margin. The manuscript of original papers should be divided into summary, introduction, materials and methods, results, discussion, acknowledgements and references. Review articles should also be appropriately divided. SI units should be used in the manuscript, except that, for the time being, litre (l) may be used as a unit of volume.

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Contents

Index: 26861

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Cryopreservation of Blood Cells and Other Tissues: Current Status*

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(Received August 6, 1982; accepted August 24, 1982)

Freezing Injury

It is now well established that freezing injury results either from excessive loss of cell water or from the formation of intracellular ice. The loss of cell water occurs when freezing is slow and ice forms in the extracellular solution. The freezing out of water concentrates the extracellular solution, increasing its osmotic pressure and water leaves the cell in response to the osmotic gradient. How the loss of cell water actually causes injury is still debated. It is probable that the resulting reduction in cell volume places stresses on the membrane which lead to membrane damage. Intracellular ice generally develops when the freezing rate is rapid and there has been insufficient time for water to leave the cell. Very low temperatures are reached while the intracellular solution is still dilute and intracellular ice forms. Alternatively, membrane rupture can result from the stresses of extracellular freezing permitting ice to enter the cell from the outside. It is presumed that intracellular ice is mechanically destructive to intracellular structures.

Cryoprotection

The simplest approach to the prevention of freezing injury is to increase the solute concentration. Any solute at high concentration will reduce both the freezing point and the amount of ice formed at any subfreezing temperature. To be effective with cells, the solute added must be non-toxic and must easily enter the cell. Only a few compounds fulfil these criteria. Most commonly used are glycerol, dimethyl sulfoxide (DMSO) and some of the other glycols. For simple anti-freeze cryoprotection, concentrations of glycerol or DMSO of the order of 25 per cent to 35 per cent are necessary. Such concentrations can reduce the amount of ice formed to such an extent that injurious cell dehydration never occurs. Storage can be at any temperature low enough to prevent biochemical reactions from proceed-

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Summing-up lecture held at the International Congress ISH–ISBT, Budapest, August, 1982.

ing at a significant rate, usually $-60\,^{\circ}C$ or lower. This is the cryopreservation method used for human red cells.

Alternatively, the so called 'kinetic' approach can be used. If freezing is relatively rapid, there may not be sufficient time for all of the freezable water to leave the cells in response to the osmotic gradient created by extracellular ice. It is thus possible to avoid dehydration injury by accelerating the cooling rate. However, if too much water remains in the cell, intracellular ice will form. The presence of a cryoprotectant can reduce both the amount of extracellular ice formed and the chance that intracellular ice will develop. Since success depends on leaving some freezable water inside the cell, storage must be at a very low temperature, generally below $-120\,^{\circ}C$, to prevent dehydration of the cell from going to completion during storage. This is the type of procedure used to cryopreserve platelets using 5 per cent DMSO with freezing at $1\,^{\circ}C$/min. Figure 1 illustrates schematically the influence of cooling rate on cell injury.

The introduction and removal of cryoprotectants is a major obstacle in many applications. Since these agents, particularly the glycols, penetrate cells rather slowly, large osmotic gradients may be transiently created and can do serious damage to cells. Most of the expense of frozen red cells results from procedures necessary for the removal of glycerol. As later paragraphs indicate, there are substantial differences between cells in their tolerance to osmotic stresses that significantly affect their response to cryopreservation.

Red Cells

The cryopreservation of red cells, whether by the high [1, 2] or low [3–5] glycerol method, is now an established clinical procedure and no longer considered experimental. The primary virtues of frozen red cells are: (i) long-term preservation, (ii) the nearly complete elimination of leukocytes and (iii) the washing out of virtually all plasma. The appropriate uses for this product are for long-term storage of rare types or for autotransfusion, for patients unusually susceptible to nonhemolytic, febrile reactions where conventional leukocyte-poor red cells are inadequate, or where alloimmunization against white cell and platelet antigens is to be avoided at all costs. Although there is some evidence that the risk of post transfusion hepatitis may be reduced with frozen cells [6] modern methods of donor testing and the elimination of paid donors has minimized the magnitude of this risk.

In the American Red Cross Blood Services, the use of frozen cells rose steadily from the time that they were introduced into the system in 1972, reaching a level of nearly 100 000 units/year in 1978. Since that time the rate of use has fallen by almost half (Fig. 2). We believe this reduction in use to be the result of three developments. First, the diminution of hepatitis B as a transfusion problem and lack of evidence that frozen cells are an effective solution. Second, whereas in 1978, 50 per cent of frozen cells were used by dialysis centers both to avoid allo-

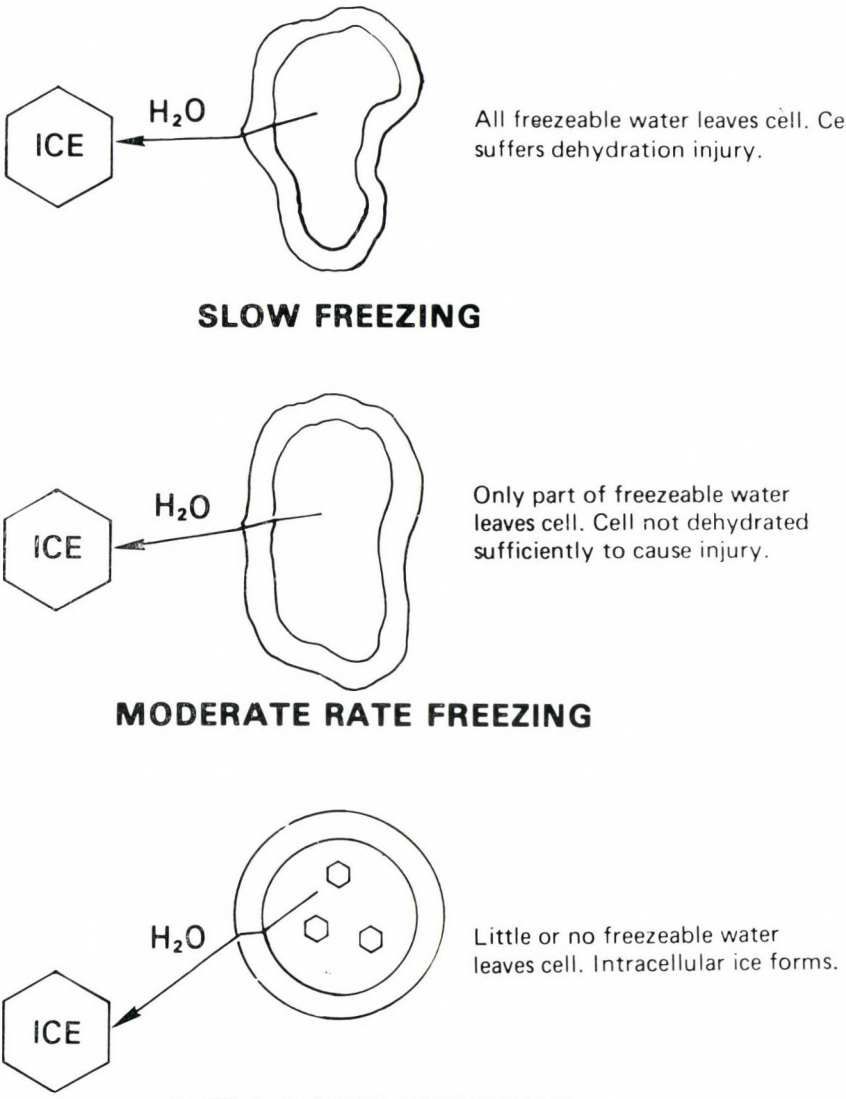

Fig. 1. Schema of the effects of varying the cooling rate on the nature of freezing injury. During slow freezing (top panel) the cell interior remains in near equilibrium with the external solution. At any temperature, all water that is freezable at that temperature is lost by the cell. Injury is the result of excessive cell dehydration and volume reduction. During very rapid cooling (bottom panel) insufficient time is available for water to leave the cell. At around −40 °C homogeneous nucleation occurs and intracellular ice develops, also a generally lethal event. In the center panel, an intermediate situation exists and it is possible that there may be insufficient dehydration to cause injury, yet sufficient concentration of cell contents to prevent the development of lethal intracellular ice [36]

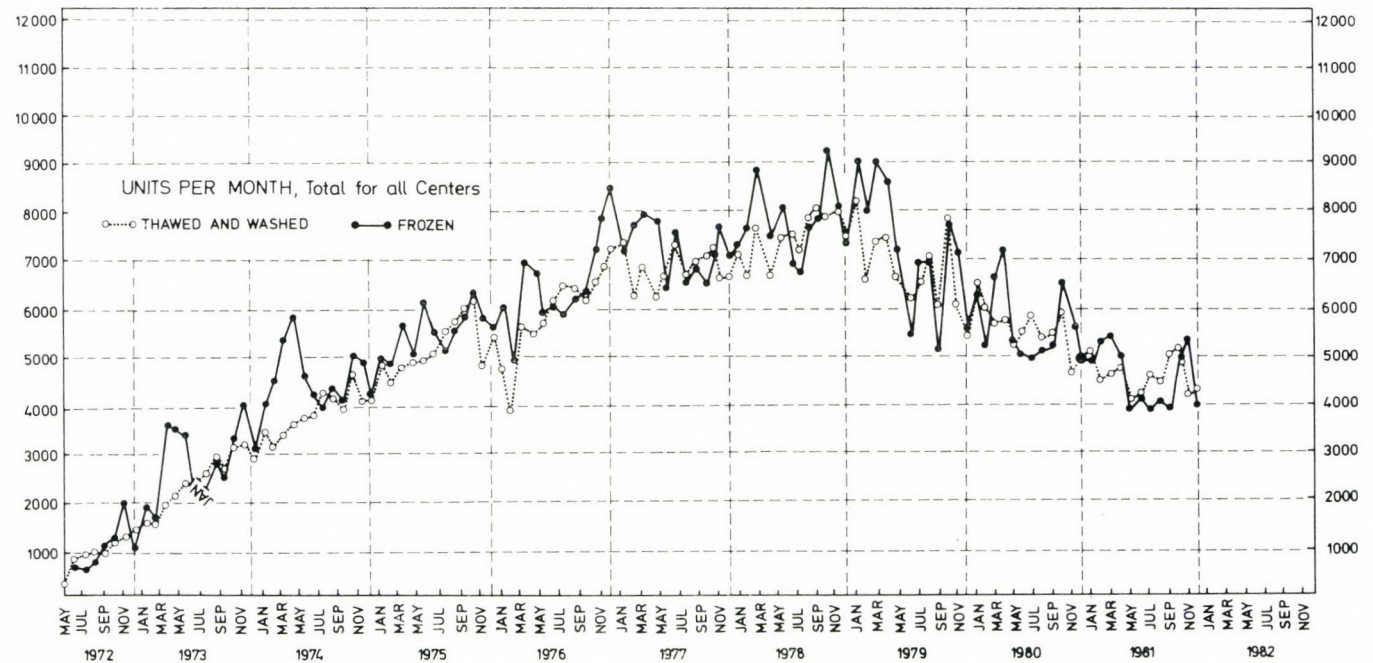

Fig. 2. Monthly production of Red Cells Frozen and Red Cells Deglycerolized by the AmericanRed Cross Blood Services. During 1972 to 1978 additional Blood Centers were joining the program, accounting for much of the increase in use. Changes in the practices of dialysis centers and the substitution of Red Cells Washed account for the subsequent reduction in use

immunization and minimize hepatitis transmission, evidence that transfusions of unfrozen blood improve the survival of transplanted kidneys has led to changes in this practice. Third, the development of improved techniques for the removal of white cells has provided less expensive ways to avoid febrile reactions in sensitized patients.

Recent advances in red cell freezing have been limited to minor modifications such as freezing cells at high hematocrit to reduce storage volume and to simplifying deglycerolizing, and attempts to store red cells at temperatures higher than $-60\,^{\circ}C$. Two presentations at the recent International Congress have addressed this latter question. Klos and colleagues [7] stored red cells at $-30\,^{\circ}C$ with glycerol concentrations ranging from 5.6 to 8.6 M with or without the addition of adenine, adenosine or inosine. They found that ATP, 2,3 DPG and cell potassium fell to 80–85 per cent of control after six months and to 60–70 per cent after 12 months. Our experience [8] was similar although we subsequently found a progressive increase in post thaw hemolysis after six months and frequently a loss of between 30 and 40 per cent of the cells during the first 30 min post transfusion. We would agree with these investigators that storage at $-30\,^{\circ}C$ might be feasible for periods of up to six months but have some reservations whether the advantages of the higher temperature would justify the relatively short storage time and the reduced cell viability.

Beaujean and colleagues [9] compared storage at -80 and $-20\,^{\circ}C$ using the conventional high glycerol procedure. They observed a decrease of both function and survival after 2 months at $-20\,^{\circ}C$. This is also consistent with our experience which led us to the conclusion that both the addition of adenine and the reduction of storage temperature to $-30\,^{\circ}C$ would be necessary to obtain a useful duration of storage.

New developments in red cell freezing in the foreseeable future will probably be limited to new techniques for introducing and removing glycerol with the goal of reducing processing costs. Such techniques as dialysis or the use of microporous filters are being explored. It is doubtful, however, that costs can be reduced sufficiently to make frozen red cells competitive with refrigerated cells for routine use.

Platelets

This author has published a review of the recent literature on platelet cryopreservation [10] and for a more detailed review of the subject reference should be made to that article. In general, with only rare exceptions, little has changed since the report in 1967 by Lundberg et al. [11] of clinical results using a DMSO procedure that included a post thaw wash step. DMSO concentrations between 4 and 6 per cent have been the rule with a cooling rate ranging from 1 to $5\,^{\circ}C$/min. In general, the numerical loss of platelets from freezing and thawing is 20 to 30 per cent and the functional integrity of the remaining cells ranges from 30 to 50 per cent of control values. This generality is not inconsistent with studies

reported at the present Congress. Velden and colleagues [12] compared freezing with 5 per cent and 10 per cent DMSO. With 5 per cent DMSO the numerical recovery averaged 86 per cent and the assay of function by the hypotonic shock reaction averaged 57 per cent of control. With 10 per cent DMSO, the values were 82 and 47 per cent. Itoh and colleagues [13] using both 5 and 10 per cent DMSO reported a numerical recovery of 85 per cent. The *in vivo* survival of the remaining cells was 67 per cent of control. Zaroulis and Leiderman [14], also using 5 per cent DMSO, reported an 81 per cent numerical recovery following automated processing in the IBM 2991 Blood Cell Processor. No functional assays were reported. Hackensellner et al. [15] reported an improvement in *in vitro* function after cryopreservation when the anti-oxidants seleno-methionine, tocopherol or dithioerythritol were included in the preservation solution.

There have in the past been promising reports of the effectiveness of glycerol as a cryoprotectant for platelets. This compound has the advantage of clinical acceptability and the procedures reported have generally not included a washing step to reduce the cryoprotectant concentration prior to transfusion. However, following the initial report in 1976 by Dayian and Rowe [16], attempts to duplicate the procedure have met with mixed results. The two reports presented at this Congress are no exception to that pattern. Körbling and colleagues [17] reported a numerical recovery of 76 per cent with an *in vivo* increment equal to 50 per cent of fresh cells. On the other hand Velden et al. [12], comparing platelets frozen in glycerol with those frozen in DMSO, reported a 70 per cent numerical recovery of the glycerol frozen platelets but a functional recovery measured by the hypotonic shock reaction of zero.

It appears safe to say that there is still room for improvement in the cryopreservation of platelets. A possible explanation for the relative refractoriness of platelets to various freezing procedures was suggested by the report at the Congress by Meryman and colleagues [18]. These investigators showed that the human platelet has a very limited tolerance to anisotonic conditions. Platelet function, assessed by aggregation, by hypotonic shock response and by morphological scoring, is progressively reduced as the osmolality of the suspending solution departs from isotonicity in either the hypo- or the hypertonic direction. Unlike red cells, which are unaffected by osmolalities as high as 1500 mOsm, platelets have lost 50 per cent of their functional capacity at 800 mOsm. Since the introduction and removal of cryoprotectants involves transient hypo- and hypertonic gradients and since freezing imposes a substantial hypertonic stress, it is not surprising to find these cells more difficult to freeze than is true of cells with more extensive osmotic tolerance. Some of the problems with glycerol as a cryoprotectant may also be attributable to this factor. Since the diffusion of glycerol across the platelet membrane is relatively slow compared to DMSO, osmotic gradients developed during the introduction and removal of the cryoprotectant may be greater and more prolonged.

There is little question but that cryopreservation of platelets is of clinical importance. It is the only way to maintain cells for autologous use. For that pur-

pose, even the present DMSO procedure is generally considered clinically useful. However, if the effective functional recovery could be increased from the present 20–30 per cent to a value approaching that of a fresh preparation, cryopreservation might well find considerable use for short term preservation of HLA matched pheresed platelets to provide donor convenience and perhaps even for the stockpiling of HLA typed platelets for future matched recipients. It is unlikely that frozen platelets could become cost-effective for the storage of random unit concentrates.

Granulocytes

A notable aspect of the literature on granulocyte cryopreservation is the extreme variation in results by investigators using comparable procedures and the general inability of other investigators to confirm encouraging reports. The literature through 1980 has been concisely summarized in tabular form by Bank [19]. A major reason for many of the variable results is undoubtedly the different sensitivity of different assays. In their presentation at the Congress, Meryman and colleagues [18] showed that when assays such as the fluorescein diacetate and ethidium bromide assays, which primarily demonstrate plasma membrane integrity, are used, granulocytes are apparently tolerant to hypertonic exposures as high as 1200 mOsm. However, when assayed by chemotaxis and bacterial killing, exposure to osmolalities in excess of 600 mOsm results in an instantaneous loss of function. Electronmicroscopic studies have shown extensive intracellular damage at osmolalities of 750 mOsm and above [20].

For this reason, although the fluorescent dye assays are useful in demonstrating the presence of injury to granulocytes, they can be very misleading as indicators of survival and function. Many of the earlier reports of successful cryopreservation were based on fluorescent dye assays and cell survival could not be confirmed when the more sensitive functional assays were used. The use of animal models other than primates is probably equally risky. The experience of Cohen and Gardner [21] who developed an excellent cryopreservation technique for dog platelets which was unsuccessfully applied to human platelets, is illustrative of the species differences that can influence the tolerance of cells to cryopreservation.

The variability in reported results is not, however, solely due to differences in assay and processing procedures. A recent report by Hill et al. [22] describes the same variability between presumably identical samples processed simultaneously.

It is apparent that the granulocyte is sensitive to a number of the environmental parameters that are altered during freezing. Takahashi et al. [23] have reported 4 factors that can be responsible for cell damage. First, there is limited tolerance to the hypertonic stress created by freezing. Second, injury can result from exposure to temperatures below $-5\ ^{\circ}C$ even in the absence of freezing. The rate at which this injury develops can be reduced by the presence of dimethylsul-

foxide but not by glycerol. Third, cells are injured by being cooled at velocities much above 1 °C/min [24]. Fourth, the intolerance of granulocytes to hypotonic stresses can result in injury during the post-thaw dilution stage. It is apparent from past experience that successful cryopreservation of granulocytes probably cannot be achieved by empirical manipulation of cryoprotectants and cooling rates but will require a methodical study of the response of these sensitive cells to the various changes in environment that accompany a cryopreservation procedure.

There still remains some question regarding the clinical usefulness of granulocyte transfusions in the treatment of sepsis and, therefore, regarding the importance of granulocyte cryopreservation. One important research application would be the investigation of the clinical effectiveness of transfusions of much larger doses than can be collected by a single pheresis procedure, a question that could best be answered by freezing successive collections from the same donor.

Monocytes, Lymphocytes and Stem Cells

These three cell types are grouped together because of the relative ease with which they can be frozen. As shown in the Congress presentation by Meryman et al. [18] these three cells have an extended tolerance to osmotic stress.

Successful monocyte cryopreservation has been reported by others [25, 26] and, in our experience, we can routinely obtain in excess of 95 per cent functional recovery of monocytes as assayed by chemotaxis and bacterial killing [27]. In view of the marked stability of these cells it would be interesting to know whether monocyte transfusions might be effective in the treatment of sepsis at lower doses than is apparently true of granulocytes.

Lymphocyte cryopreservation is well established in the preparation of panels for HLA testing. It has been suggested that differences might exist between lymphocytes and stem cells in their sensitivity to freezing, and that this might be utilized as a way of reducing T-lymphocyte contamination of bone marrow transplants. Our [18] comparison of the osmotic tolerance of lymphocytes and stem (CFU-c) cells demonstrated essentially identical osmotic tolerance, implying that this would not be an effective way of discriminating between the two cell types. Fedotenkov et al. [28], however, presented a report at the Congress to the effect that a glycerol freezing technique had been developed that decreased the activity of lymphocytes in the transplant and avoided the problem of graft-vs-host disease as well as of interaction within a mixed donor transplant.

Empirically designed techniques for the cryopreservation of bone marrow have been in clinical use for several years. The basic procedure, which consists of diluting the marrow sample with an equal volume of 20 per cent DMSO and freezing at 1 °C/min, is crude by comparison to the care that must be taken with many other cell types. Again, this is undoubtedly due to the remarkable tolerance of these cells to osmotic stress.

There are some indications that the present methods of stem cell cryopreservation may not result in maximum recovery of viable cells following storage [29–31]. One interesting approach to improving viability was the suggestion to the Congress by Matthes and Hackensellner [32] that a reduction in oxidative damage during cryopreservation could be achieved by the use of antioxidants to minimize lipid peroxidation and oxidation of sulfhydryl groups.

The uses of cryopreserved stem cells are at the moment limited to autologous transplants, either to permit more aggressive chemotherapy and irradiation for the treatment of solid tumors or for use in the treatment of leukemia. A recent report that leukemic cells in murine marrow could be selectively removed, rendering the marrow non-leukemic for the subsequent transplant [33], suggests that this latter application may become more important in the future.

Other Cells and Tissues: Future Developments

Although the focus of the Congress is clearly on blood transfusion and hematology, it is not inappropriate to comment briefly on certain new areas of endeavor involving cryopreservation in which blood bankers may become increasingly involved. The storage of human tissues other than blood cells has been conducted largely by single purpose banks collecting skin or corneas, by individual surgeons collecting such tissues as bone and dura for their own use, and a very small number of true tissue banks collecting and distributing a variety of tissues. However, the organizational structure and many of the facilities of a regional blood center are precisely those required for tissue banking and blood banks are showing an increasing awareness of this potential area of service.

With the exception of skin, corneas and kidneys, the tissues commonly transplanted at the present time include bone, cartilage, nerve, artery, dura, fascia, and heart valves. These are preserved by freeze drying and are nonviable. They provide either structural support or a matrix for the invasion and substitution by host cells. For reasons not fully understood, freeze dried tissue does not evoke an immune response and typing and matching are unnecessary. Skin is generally preserved by freezing and is viable at the time of transplant. Its primary use is in burn patients to provide a temporary cover. The transplant will be rejected after 2–4 weeks by which time the patient has stabilized and grafts from unburned areas can be applied.

Corneal transplants may be either lamellar grafts, consisting of only the outer surface of the cornea, or penetrating grafts which include also the endothelial layer on the inner surface. Lamellar grafts include no living cells and present no serious problems in preservation. Long term preservation of penetrating grafts requires cryopreservation of the endothelial layer, a goal that has not been satisfactorily achieved as yet. Since the endothelium is responsible for controlling hydration of the cornea, its integrity is of paramount importance.

As advances in understanding in immunology lead to better control of immune rejection, there will be an increasing need for cryopreservation techniques for organized tissues and organs. Recent studies of the cryopreservation of kidneys indicate that the presence of ice may well be incompatible with subsequent function of the organ. In cell suspensions, the presence of extracellular ice is physically innocuous, its only effect being to increase the solute concentration of the extracellular solution. However, in an organized tissue, extracellular ice can still disrupt tissue architecture, rupture intercellular connections and can be particularly damaging when it develops in the vascular network.

Recent studies have therefore been directed at ways by which low temperature preservation might be achieved without the development of ice. One particularly novel approach, vitrification, shows considerable promise and will be equally applicable to tissues and cell suspensions. The creation of a vitreous (glassy) state at low temperature may well provide an alternative approach to long term preservation for a variety of biological materials [34, 35]. In order to present the theory of vitrification in understandable fashion a few introductory comments regarding the crystallization and growth of ice are necessary.

First, the development of an ice crystal must be initiated by a nucleation process. This generally requires the presence of a physical nucleating site, called a heterogeneous nucleus, which is thought to be a region on a macromolecule where

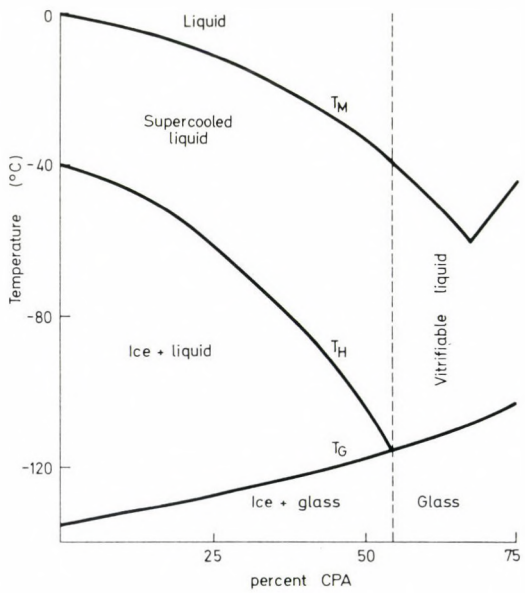

Fig. 3. A schematic phase diagram for a typical cryoprotectant (CPA). T_M = melting point, T_H = homogeneous nucleation temperature, T_G = glass transformation temperature. At CPA concentrations higher than indicated by the dashed line, vitrification can be achieved

a molecular arrangement similar to ice can act as a template. The size of the nucleus will determine the temperature at which it will support ice; the smaller the nucleus, the lower the temperature must be for it to become critical. For this reason, an aqueous solution will rarely freeze at or near its melting point but must be cooled until the largest nucleus present can support crystal growth. Figure 3 is a schematic phase diagram of a typical cryoprotective agent. Like any other solute, as the concentration increases, the melting point of this solution will be reduced. The curve T_M illustrates this relationship but it can be assumed that the spontaneous freezing point will be at some temperature below T_M and will vary from sample to sample depending on the size of the largest heterogeneous nucleus present. However, as the temperature is lowered, the size of the nucleus necessary to support crystal formation is progressively reduced until random aggregations of water molecules are sufficient to act as nuclei. This temperature, referred to as the homogeneous nucleation temperature, T_H, is also reduced as the solute concentration increases. In the absence of heterogeneous nuclei, water can be supercooled to approximately $-40\,^{\circ}C$ and solutions can be supercooled further depending on their concentration.

A final landmark illustrated in Figure 3 is the glass transformation temperature T_G. This is the temperature below which the solution is so viscous that ice growth is impossible. For a relatively dilute aqueous solution, it is impossible to reach T_G without nucleation and crystallization. However, it is apparent from Figure 3 that if the cryoprotectant concentration were sufficiently high, T_H and T_G would intersect so that it would be possible to cool to below the glass transformation temperature without the formation of ice, as illustrated by the vertical dotted line.

Although vitrification at high solute concentrations can easily be achieved in solutions, these concentrations are far too high for use with living tissues. However, in addition to reducing freezing temperature by the addition of solutes, it is also possible to reduce it by the application of hydrostatic pressure. For example, at approximately 30 000 lb/sq.in, the melting point of pure water is reduced to $-22\,^{\circ}C$. T_H is similarily reduced but T_G is relatively uneffected. If anything, it rises slightly. The principle being employed to vitrify biological material is to superimpose hydrostatic pressure on cryoprotection in the hope that the concentration of cryoprotectant required can be reduced to a feasible level.

Attempts to put this theory into practice reveal that there are several associated sources of damage to be dealt with. Pressure alone, in the absence of temperature reduction can cause injury, depressing the ability of cells to maintain the normal sodium–potassium ratio. However, DMSO, for reasons not as yet understood, can substantially increase the pressures tolerated by cells. The presence of cryoprotectants was found to stimulate the development of a destructive high pressure form of ice, Ice III, at a higher temperature and pressure than expected. However, the polymers polyvinylpyrrolidone or hydroxyethyl starch could forestall the development of Ice III. It was found that toxicity from high concentrations of cryoprotectants, particularly DMSO, could be minimized by the addition

of acetamide. It was also found that various mixtures of cryoprotectants such as glycerol, ethylene glycol and DMSO could increase or decrease the probability of vitrification at the same total cryoprotectant concentration. It appears probable that further exploration of other cryoprotectants and mixtures of cryoprotectants can further reduce the total concentrations necessary to achieve vitrification. Once vitrification has been achieved and the temperature has fallen below T_G, the pressure can be released without devitrification for storage in liquid nitrogen.

The problems associated with thawing large masses of tissue, such as intact kidneys, are substantially relieved by vitrification. In a frozen cell suspension, where 50 per cent of the liquid volume may have been converted to ice, the heat of fusion that must be supplied to convert ice at 0 °C to water at the same temperature will require nearly the same amount of heat as would be necessary to warm the frozen sample from −100 °C to 0 °C. Furthermore, when very rapid thawing by means of microwave heating is attempted, the poor energy absorption by ice and the good absorption by water create an uncontrollable situation where melted portions may boil before frozen portions are melted. In a vitrified sample, no ice exists and no latent heat need be supplied. Initial calculations indicate that microwave heating will provide an effective and controllable way to warm a vitrified specimen so rapidly that devitrification will not occur and it will be unnecessary to reapply hydrostatic pressure while warming the specimen.

Although we expect that further advances in cryobiology will improve the recovery of cell suspensions following conventional cryopreservation and will be extended to cells that are currently refractory to frozen storage, vitrification promises to provide an approach to the cryopreservation of complex tissues and organs where conventional techniques have been unsuccessful and may be inapplicable.

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Thalassemia and Abnormal Hemoglobins*

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At the Congress we were presented with new findings on the heterogeneity of thalassemia, bone marrow transplantation for treatment of thalassemia, and prospects for gene therapy. Several new abnormal hemoglobins were described, and other abnormal hemoglobins were reported for the first time in several countries. We also heard about progress made in development of antisickling compounds.

Thalassemia

At the first Stratton Lecture, Professor David Weatherall discussed the heterogeneity of thalassemia at the molecular and clinical levels. He focused on α thalassemia and described five deletion defects which can affect one or both of the duplicated α-globin loci, and three nondeletion defects – Hb Constant Spring, an intron deletion, and Hb Quong Sze, a very unstable mutation. Hb-H disease is usually caused by the malfunctioning of three of the four α-globin genes due either to deletion or nondeletion defects. Both Professor Weatherall at the Stratton Lecture and Professor Antonio Cao at the Thalassemia Symposium noted that patients with nondeletion Hb-H disease tend to be more anemic and have a higher percentage of Hb-H in their hemolysate than patients with deletion form of the disease. The extreme heterogeneity of α thalassemia was illustrated by a case reported by Dr. Dimitri Loukopoulos and Professor Phaedon Fessas at a poster session. The combination of a deletion and a severe nondeletion defect produced hydrops fetalis. Professor Weatherall also described a syndrome of mental retardation associated with extensive DNA deletion affecting the α-globin loci. As similar deletions were not found in the parents, this raises the possibility of a new mutation in the germ line.

Professor Weatherall also discusses the modifying effects of α thalassemia on the clinical course of homozygous β thalassemia. Homozygous patients with thalassemia intermedia coinherit the α thalassemia genes more often than trans-

*Summing-up lecture held at the International Congress ISH–ISBT, Budapest, August, 1982.

fusion-dependent patients. The differences in frequency of α thalassemia between these two types of β thalassemia homozygotes are more pronounced in Greek Cypriots than in Sardinians, probably because β^+ thalassemia is common in Cypriots and β^0 in Sardinians.

At the Thalassemia Symposium Professor Cao discussed other factors that may contribute to the clinical heterogeneity of β thalassemia. He emphasized that in addition to α thalassemia and heterocellular HPFH, there are other unknown modifying factors. He described families in which two siblings had the identical genotype and yet one was transfusion dependent while the other had thalassemia intermedia. It is important to continue to define these modifying factors for genetic counseling purposes.

At a plenary session Dr. Stuart Orkin reviewed the extreme polymorphism of β thalassemia at the molecular level. Already some 20 lesions are known to produce β thalassemia. These have been defined by restriction mapping, DNA polymorphism, gene cloning, DNA sequencing, and the use of surrogate systems to analyze the defect in mRNA transcription or processing. The lesions include several deletions which vary from small ones involving a portion of the β-globin gene, to large ones up to 60 kb long which include $^G\gamma$-, $^A\gamma$-, δ-, and β-globin genes. Two promoter abnormalities due to single nucleotide mutations affect the RNA polymerase binding site and result in β^+ thalassemia. Many examples of intervening sequence mutations have been described. Some lead to completely abnormal splicing of β-globin mRNA and result in β^0 thalassemia; others lead to partial splicing abnormality and result in β^+ thalassemia. He noted also that the low level of Hb E production is also due to a partial splicing abnormality of the β^E-globin mRNA. There are several examples of early termination of β-globin chains due either to nonsense mutations or to frameshifts as a result of the deletion or addition of one, two or four nucleotides. Not discussed, but also a cause of thalassemia are missense mutations which produce extremely unstable abnormal globin chains. Examples are Hb Indianapolis affecting the β chain, and Hb Quong Sze, affecting the α-globin chain. No doubt many other abnormalities will be found, rivaling those in the abnormal hemoglobins.

Dr. Edward Benz at the Thalassemia Symposium reemphasized the heterogeneity of the molecular lesions in β thalassemia. He also described a sensitive method for assaying abnormal spliced mRNAs in reticulocytes and detecting the heterogeneity of thalassemia in patients with β thalassemia.

A highlight of the meeting was Dr. Donnell Thomas' report of two successful bone marrow transplants in homozygous β thalassemia presented at the second Stratton Lecture and at the Thalassemia Symposium. One was performed six months ago, and the other seven weeks ago. He emphasized that at this early stage it is important to select suitable patients. The patients must be young and receiving minimal or preferably no transfusion, and the donors must be histocompatible. We keenly await the progress reports on this work.

The future prospects of gene therapy were discussed by me (Y. W. Kan) at a plenary session, and by Dr. Francesco Baralle at the Thalassemia Symposium.

The availability of normal globin genes through recombinant DNA technology makes this a distinct possibility. The two primary requirements are the efficient insertion of the normal gene into bone marrow cells and the adequate expression of the inserted genes. While both of these conditions have not yet been fulfilled the various strategies designed to achieve these goals were discussed.

Abnormal Hemoglobins

Several new abnormal hemoglobins were described at the poster session. Among them were Hb Le Lamentin by Dr. M. Sellaye in Professor Rosa's group, and four unstable hemoglobins discovered by Dr. T. Nakatsuj in Professor Miwa's group during a survey of 10 000 blood samples: Hb Miyashiro (β 23 Val–Gly), Hb Tottori (α 59 Gly–Val), Hb Yokohama (β 37 Leu–Pro), and Hb Saitama (β 117 His–Pro).

A number of abnormal hemoglobins were described for the first time in several countries. These included Hb H in Hungary described by Dr. M. Horányi from Professor Hollán's group, and the combination of Hb O^{Arab} with β thalassemia in Turkey by Professor M. Aksoy. Time does not allow me to enumerate all the others, but it was interesting to see new reports of abnormal hemoglobins from the USSR, Cuba and Viet Nam.

Many reports addressed the clinical syndromes associated with sickle cell anemia. Dr. Martin Steinberg presented a progress report on the natural history of sickle cell disease which is being compiled in the United States. Drs Dominique Labie and Ronald Nagel co-chaired a symposium on antisickling compounds. A number of different agents were described including thiol reagents by Dr. Yves Beuzard, cytiedil by Dr. E. P. Orringer and Dr. L. P. Berkowitz, and ticlopidine by Dr. H. Wajcman's group. A multicenter trial of cytiedil has been started, and preliminary results indicate the drug may shorten the duration of crisis.

An interesting report was given by Drs Karel de Ceulaer and Graham Sergeant at the poster session. The drug Depo-provera (medroxyprogesterone acetate) given to female sickle cell anemia patients for contraception was found to increase their hemoglobin levels, fetal hemoglobin percentages, and red blood cell survival rates, while decreasing the number of irreversibly sickled cells and the frequency of crises. We keenly await further confirmation of these results.

Finally, not reported at this meeting but worth mentioning is a new method of prenatal diagnosis of sickle cell anemia by amniocentesis. It was previously reported that the restriction enzyme *Dde* I could be used to directly detect the sickle mutation. A new enzyme, *Mst* II, has now been found to be much more useful and was reported simultaneously by Judy Chang and Y. W. Kan, Stuart Orkin's group, and John Wilson's group. This test is extremely sensitive and amniotic fluid cell culture is no longer necessary. The DNA can be directly extracted from cells obtained from 15 ml of amniotic fluid. *Mst* II cleaves the β-globin gene at a position corresponding to amino acid number 5, 6 and 7. The mutation of GAG (Glu) to

GGG (Val) abolishes this recognition site. Hence, using *Mst* II, a larger DNA fragment (1.35 kb) is produced instead of the normal two fragments (1.15 and 0.2 kb).

This was an extremely interesting meeting for people in the field of hemoglobin and thalassemia. Professor Susan Hollán and the organizing committee are to be gratulated for the excellent Congress.

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Platelets and Atherogenesis*

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The longevity of the various animal species appears to be more or less fixed, and each ultimately dies of some species-specific disease at a typical age provided no catastrophe intervenes. Thus, dogs may live for about 15 years, whereas man can enjoy about six times this life span. Moreover, each species has characteristic disorders which appear to provide the basis for the terminal event.

In man occlusive arterial disease is the most common of these, but it is uncertain how much prolongation of life would result from its elimination. Nevertheless, sufficiently large numbers of younger individuals succumb to or are disabled by arterial thrombosis of various organs to warrant major research efforts in this area. Atherosclerosis is universally found, and typically occurs even in childhood [1]. It is probable that its clinical manifestations represent a combination of progressive augmentation of lesions initiated at an early age, together with an ongoing establishment of newly afflicted arterial sites. Although the process of atherogenesis may be accelerated by many metabolic disorders, the following discussion will be confined to that disease for which there is no obvious explanation of dramatic risk factor: this is the variety most commonly encountered in Western societies.

Transformation of a Normal to a Thrombosed Artery

The lumenal diameter of normal cerebral or coronary arteries can be measured in millimeters, and a single de-endothelialization of vessels with such diameters in experimental animals is followed by deposits of thrombotic material that produces trivial compromise of the lumen and no evidence of reduced flow [2]. Further, even the intimal hyperplasia that follows such a vascular insult is insufficient to produce clinically significant arterial narrowing [3]. In many of these experiments, massive removal of endothelium was accomplished by intralumenal passage of inflated balloon catheters, a manipulation designed to yield maximal

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effects. Even when multiple injury was produced by repeated balloon de-endothelialization, the augmented intimal hyperplasia, sometimes accompanied by lipid deposits, was still insufficient to produce occlusive atherosclerosis [4]. These data imply that occlusive atherosclerosis is the consequence of prolonged and perhaps sustained or repeated stimuli; these stimuli presumably act over many years, and may have different effects at different periods of life. In any event, the high shear and rapid flow conditions that prevail in more normal arteries do not allow occlu-

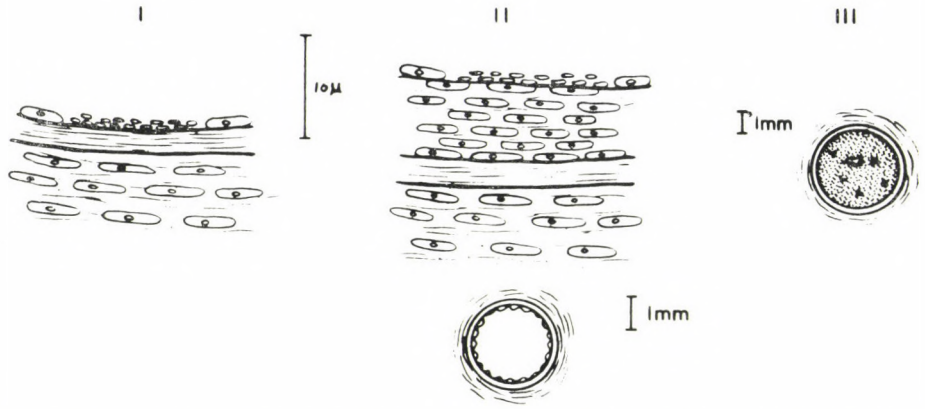

Fig. 1. Diagrammatic evolution of an arterial occlusion. I: De-endothelialization leads to a thin platelet layer on exposed subendothelium. II. After a single insult, there is a hyperplastic smooth muscle neointima of little consequence in lumenal narrowing. III. After a long history of repeated or continuous adverse stimuli, the lumen is so compromised that a final occlusive thrombus is possible

sive thrombotic buildup; such potential deposits are scoured away. For occlusion to occur, there must be sufficient compromise of flow so that a thrombotic mass can both form and sustain itself: the atherosclerotic lesion must be virtually occlusive itself, as illustrated in Figure 1.

At each stage of atherogenesis, platelets participate in a particular way, reflecting the versatility of these cell fragments. Thus, they release mitogenic materials into the vessel wall to initiate smooth muscle migration and proliferation; they facilitate the terminal occlusive event by undertaking their familiar adhesion and aggregation reactions.

The Precipitating Events

Entrance of mitogenic material into the deeper layers of the arterial wall implies loss of the endothelial barrier which normally prevents this process. Although the atherogenic process may be readily initiated in experimental animals by procedures which elicit massive endothelial detachment, there is no evidence

that human disease is commonly the consequence of such events. More subtle alterations of endothelial integrity are probably operating in such high risk disorders as hypertension and diabetes, and in the usual aging process itself. In fact, one needs no extensive review of the literature to find reports of infectious, dietary, or hypersensitivity bases for endothelial damage. Probably the one requirement of the lesion is that it allows adhesion of platelets to the subendothelium, and that these platelets are sufficiently perturbed to release their mitogenic material.

For this event to occur, the interaction of platelets with the exposed subendothelial connective tissue must be adequate to elicit a platelet release reaction, thereby discharging mitogen from the alpha granules [5]. Moreover, the quantity of mitogen must be adequate to exceed the threshold required by a responding smooth muscle cell, and it must be assumed that much of the platelet-released contents is rendered locally unavailable by diffusion and convection away from the reaction site. Thus, exposed subendothelium must have geometric and chemical properties that will achieve platelet events of mitogenic caliber. Note that the geometry of the lesion will influence such factors as the rate of platelet accumulation and residence time, and the dispersion of any released material [6]. Moreover, the state of the platelets themselves, and the properties of the blood in which they are carried represent additional variables that will determine the response to any arterial insult. At present, the spectacular biochemical advances of recent years have not been matched by corresponding physiological application even in experimental animals, not to mention human disease.

In young rabbits, de-endothelialization of the aorta by passage of a balloon catheter is immediately followed by the onset of platelet adhesion to the exposed subendothelium (Fig. 2), a reaction that is rapidly extended and soon accompanied by the platelet release reaction. Although presently the subject of speculation only, the subendothelium that human platelets 'see' must be a complex of intercellular matrix components, which may differ with the source and severity of the lesion, the past history of the site, and age, sex, and other genetic and environmental influence. Candidates for platelet-reactive matrix components include collagens of various types, proteoglycans, fibronectin, and matrix-bound von Willebrand factor [7] as those promoting platelet accumulation, and possibly heparan sulfate as a substance that is inhibitory [8]. Additionally, production of prostacyclin by smooth muscle cells at sites of de-endothelialization may reduce local thrombogenic potential [9].

Intimal Hyperplasia and Atherogenesis

When smooth muscle cells of the arterial media are subjected to a sufficient mitogenic stimulus, they migrate lumenally across the internal elastic lamina, and they proliferate. In experimental animals, there is evidence that the proliferative response comes first [10]. Our data in rabbits and rats indicate that this hyperplastic reaction, although brisk initially, rapidly subsides; incorporation of tritiated

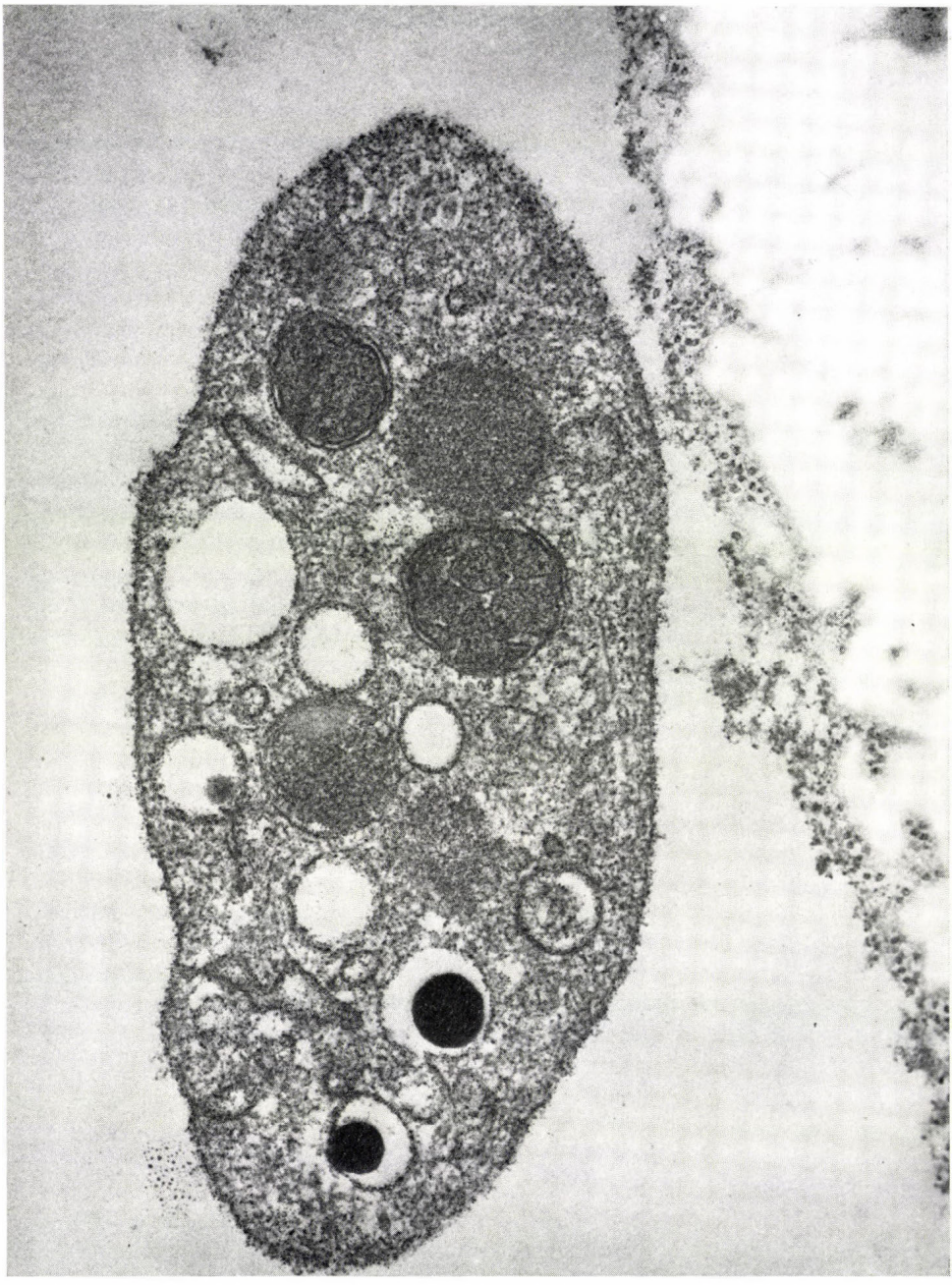

Fig. 2. Initial adhesion of a platelet to the subintima of a rabbit aorta de-endothelialized by passage of a balloon catheter within seconds of fixation. About 1 % of the surface is so covered at this time, and the attachment of the platelet appears to be quite precarious. Electron micrograph, original magnification × 40 000

thymidine into smooth muscle cells reaches a peak in the first week after trauma, but returns to normal within a month despite incomplete restoration of the endothelial cell cover [11]. As a consequence, the degree of intimal hyperplasia that follows a single de-endothelialization episode even of massive degree, is of little consequence in narrowing the lumen of a major artery.

If long-term events in human disease are a reflection of animal data, findings on the effects of certain manipulations are instructive. We have subjected rabbits to second aortic balloon de-endothelialization procedures after a one month period of healing from the first insult, and healing is again allowed to proceed for an additional month. As shown in Figure 3 a new internal elastic lamina often marks the extent of the initial hyperplastic response, and additional intimal growth of almost equal degree occurs. These observations suggest that repeated vascular insult may be the mechanism whereby intimal hyperplasia becomes sufficiently massive to be occlusive. It had been shown previously that continued arterial insult in rabbits led to atherosclerotic lesions even in the absence of hypercholesterolemia, and that de-endothelialization greatly augmented the atherogenesis induced by dietary means [12, 13]. Our own data emphasize the requirement that even the hyperplasia itself depends upon renewed stimulation. In fact, some rabbits develop typical atherosclerotic lesions without cholesterol feeding if allowed prolonged healing (up to 2 years) [3], but these aortae show no more intimal thickening than those from animals with fibrous plaque formation alone.

The total of events leading from a normal, patent artery to one with occlusion sufficient to yield tissue damage are thus multiple and complex. They are influenced and modified by such factors as the intrinsic properties of the vessel at risk, and its history of exposure to mechanical, metabolic, and exogenous noxious stimuli. Additional variables may affect the blood hemostatic components. Moreover, if an atherosclerotic lesion is sufficient to produce a thrombotic event, this may be productive of clinically detectable disease only if the affected organ has insufficient collateral circulation, and if this organ is intolerant of the infarct mass produced.

Prophylaxis and Management

Theoretically, beneficial influence upon atherogenesis and its consequences may be applied at each of its phases.

Prevention of initial endothelial damage would appear to at least be partially possible by certain known measures including control of hypertension, rapid and effective management of acute infections, and perhaps certain diet manipulations. A pharmacological approach, whereby vascular endothelium is rendered less susceptible to cytological damage or less liable to detachment is hypothetically possible, and there are data in rabbits which suggest that sulfinpyrazone protects the endothelium from certain types of viral insult [14]. The long-term consequences of such intervention are completely unknown at present, and it would be expected

Fig. 3. Light micrograph of a rabbit aorta subjected to 2 episodes of balloon de-endothelialization one month apart. L — Lumen; M — Media; IEL-1 — initial internal elastic lamina; IEL-2 — internal elastic lamina formed at presumed sites of re-endothelialization, and probably representing the origin of the second hyperplastic response

that any agent of adequate 'protective' potency may well have additional and undesirable effects on other tissues and organs.

Manipulation of the platelet–vessel wall interaction is a second potential prophylactic measure, and several bases for such an approach are available. That these may have at least a limited effect was demonstrated by Friedman and associates [15] who prevented intimal hyperplasia in ballooned rabbits by sustained and profound thrombocytopenia. Although such data favor the view that the atherogenic response is manipulable in its early stages, obviously such drastic measures are not feasible. If thrombocytopenia is impractical, platelets might be modified so that their smooth muscle mitogenic activity is removed or made unavailable. Thus, Tiell et al. [16] achieved similar inhibition in rats by hypophysectomy, but the animals were able to recover spontaneously their ability to form intimal hyperplasia, after about a month. A possible mechanism for Tiell's data was a transient depletion of a pituitary-derived mitogen.

Alternatively, the platelet–vessel wall interaction is susceptible to modification by several means. These include manipulation of the platelets themselves, and numerous possibilities are suggested by the vastly increased understanding that has emerged in the past few years concerning platelet function. These are well beyond the scope of the present discussion, but will be touched upon briefly below and in another context. Similarly, administration of prostacyclin or augmentation of its intravascular level from endogenous sources, or development of a safe and effective analog represent vasoprotective approaches that are favored by certain investigators. An additional possibility is that the thrombogenic extracellular components of the vessel wall could be beneficially modified, also at present a speculation only. Many of these considerations are discussed in greater depth in a recently published collection of detailed reviews [17].

Atherosclerotic lesions grow at sites of predilection [18], and presumably new sites are recruited with the passage of time. It is not known whether those initially formed are the pathogenetic culprits because of their prolonged residence, or whether more recently evolved plaques are most dangerous. The latter could well have different composition and properties, since aging processes in the entire organism could be operating. In any event, the magnitude of these lesions is a function of cells, connective tissue, and debris. There is evidence in experimental animals that the debris including cholesterol and calcium deposits may be removable [19], but the fibrous material is irreversibly laid down [20] even though the smooth muscle cells responsible for its formation may depopulate. It would thus appear that the rate of atherogenesis as it evolves is a promising target for application of already established information, as well as future research.

Finally, the atherosclerotic lesion develops to the point at which a clinically significant event is immanent or has already occurred, presaging a more ominous outlook. There has been considerable controversy concerning the nature of this final 'coup de grâce,' particularly in the coronary circulation, and the possibility of platelet embolization downstream as a pathogenetic or contributing factor, together with arterial occlusion and spasm [21]. Two subjects are to be considered:

the subsequent management of the ominous patient, and possible measures at the time of an acute thrombotic episode. Furthermore, there has been a spate of recent reports [22–24] concerning large and small clinical trials of various agents and surgical interventions so that the following remarks represent a personal and limited overview of this complex topic.

A patient may be considered 'ominous' when clinical judgment predicts the development of a major thrombotic episode in the near future. Such events as transient ischemic attacks or certain varieties of angina pectoris are familiar examples. General medical measures are sometimes effective in halting their progress, and these include effective treatment of hypertension, weight loss in the obese, restriction of tobacco use, and the like. If the symptoms persist, surgical bypass procedures, or medical measures are available. Aspirin is perhaps the most commonly used antithrombotic drug in the treatment of arterial disease, and a major question has concerned the dosage schedule that is most effective. Many of the clinical trials have arbitrarily employed about one gram per day, based upon the concept that this dose achieves maximal depression of platelet function in all patients [25]. However, aspirin also inhibits prostacyclin production by cultured endothelial cells, thereby inhibiting their presumed antithrombotic potential [26]. Furthermore, if aspirin is given in a single dose of 40 mg, it has been reported that maximal inhibition of platelet function is achieved [27], and 20 mg/day gives a similar sustained effect [28]. Additional data supporting low dose regimens derives from evidence that such prolong the bleeding time, whereas massive doses shorten it again. On the other hand, it appears that progressively increasing the dose of aspirin correspondingly increases depression of platelet aggregation by collagen, suggesting the possibility of increasing clinical effects with increasing doses. Unfortunately, there are no clinical data available which demonstrate convincingly (or otherwise) that there is an ideal aspirin dosage range for arterial thrombotic disease.

The studies attempting to demonstrate the clinical efficacy of aspirin in coronary or cerebral arterial disease have often given conflicting or disappointing results [24]. At present there appears to be no possibility of resolving this dilemma in view of the small differences anticipated and the corresponding need for an enormous number of patients to be followed for prolonged periods of time. Add to this the need to determine the appropriate dosage schedule, and wisdom would dictate reservation of resources until more promising agents or measures come along.

If the aspirin data are disappointingly unclear, those with the two closest competitors, dipyridamole, sulinpyrazone, and combination therapy with these agents are even more so, as is the case with several newly introduced drugs. This topic has been well reviewed in several recent publications, as noted above, which tend perhaps to give a rosier view than that of the present essay.

When the patient presents with an acute coronary occlusion or cerebral vascular accident, the therapeutic approach is obviously quite different. Since recent data are available primarily for the cardiac problems, only these will be considered.

An approach that has received recent widespread attention is that of attempting to restore patency to the occluded coronary artery before the ischemic consequences to the myocardium have become irreversible. One pharmacological orientation is that of eliciting a fibrinolytic state in an attempt to disperse the thrombotic deposit. Streptokinase has been improved as a drug to reduce its undesirable side effects, and is the most widely used thrombolytic agent; urokinase has also been employed, but it is considerably more expensive and less available. Data are accumulating which suggest that thrombolysis is indeed achieved following systemic [28] or direct intracoronary [29] administration. These promising results notwithstanding, the evidence is less convincing that reduced mortality accompanies the anatomical improvement, and the objective of the lytic state is to render the patient available for subsequent coronary bypass surgery. It is of interest that blood clotted *in vitro* is resistant to lysis by streptokinase added as soon as an hour after clotting has occurred [30], suggesting that an *in vivo* streptokinase effect may transcend simple clot lysis. In any case, the results of current studies will be watched with interest, and it is hoped that euphoria does not prevail.

Even more recent is the introduction of prostacyclin as a therapeutic agent. Its rationale is based upon the concept that coronary occlusion is largely precipitated by the formation of a platelet thrombus in the responsible artery, as noted above. It is additionally possible that myocardial damage results from platelet emboli originating at the thrombotic site, which come to lodge in smaller vessels downstream. If these platelet aggregates are dispersible, disaggregation potentially has a therapeutic effect. Our own data in a small series of patients with myocardial infarct seen within a few hours of onset suggest that the intravenous administration of prostacyclin in doses sufficient to produce mild systemic symptoms is ineffective in re-establishing coronary flow [31]. There have been preliminary reports on the use of prostacyclin given directly into the coronary circulation [32, 33], and these suggest that the drug does indeed re-establish patency when so used, although long-term effects are not as yet reported. Combined prostacyclin and thrombolytic agents represent an attractive possibility for future intracoronary therapy.

Conclusions

The past several years have been characterized by many insights and exciting new developments in arterial biology and vessel behavior during pathological alterations. It is these developments that have produced the limited therapeutic advances now coming to fruition. Empirical use of various techniques is sometimes followed by dramatic therapeutic benefit; more often it is wasteful and even harmful. Vessel biology should prove to be no exception to these concepts. The likelihood of continuing rapid progress is now favored by the dramatic improvement in technology and information transmission. Thus, scientists the World over, in free communication, can solve problems as never before.

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Hereditary Disorders of Erythrocytes Associated with Enzyme Deficiencies*

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The mature human erythrocyte is a disadvantaged cell. Lacking a nucleus, mitochondria, the ability to synthesize proteins, heme, and lipids, a complete cytochrome system, and an intact tricarboxylic acid cycle, it can neither divide nor carry out many of the commonly accepted biochemical reactions with which nucleated cells are endowed. During its maturation from a nucleated precursor through the stage of being a reticulocyte, it progressively loses its biosynthetic abilities and the requisite machinery. The activities of many enzymes decrease, and then decline sharply as the cell changes from a reticulocyte to a mature, circulating erythrocyte. Many, but not all, enzymatic activities continue to decay throughout the life of the cell, changes that may play critically important roles in the ultimate death and destruction of the erythrocyte.

Despite its serious metabolic handicap, the human erythrocyte must perform a number of duties required to discharge its *raison d'être*, the transport of oxygen to the tissues and carbon dioxide to the lungs (Fig. 1). Fortunately, the reversible binding of oxygen to hemoglobin and the transport of carbon dioxide do not require the expenditure of energy.

The human erythrocyte, however, is metabolically active. It is a flexible, biconcave, discoid container, limited by a plasma membrane composed of lipids and specific proteins arranged in what appear to be specific structural relationships. The erythrocyte carries out a number of reactions, related directly or indirectly to the generation and utilization of the high-energy compound, adenosine triphosphate (ATP) to withstand the buffeting that it encounters during its 120 day, 300-mile journey through the circulation. For its modest metabolic needs, the mature erythrocyte is left dependent upon the anaerobic Embden–Meyerhof glycolytic pathway and its associated shunts (Fig. 2). This least efficient route for the metabolism of the only substrate normally available to the erythrocyte, glucose, is essential for the cell to survive. This sugar is metabolized by the cell primarily through the direct anaerobic glycolytic pathway which has the potential for generating four molecules of ATP for every two molecules utilized by hexokinase and phos-

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FUNCTIONS OF THE ERYTHROCYTE

Transport O_2 to tissues, CO_2 to lungs
Maintain intracellular organization, structure, shape
Maintain intracellular K^+ and Na^+ concentrations
Maintain lipids, proteins of membrane
Maintain hemoglobin in functional state
Maintain intracellular concentration of GSH
Maintain intracellular concentrations of cofactors

Fig. 1. The functions of the mature circulating human erythrocyte

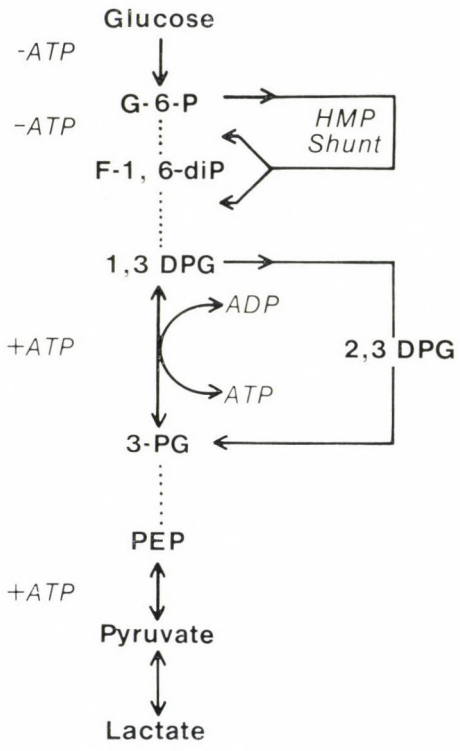

Fig. 2. Abbreviated anaerobic glycolytic pathway and its associated shunts in human erythrocytes. Dotted lines indicate omitted glycolytic intermediates. ATP = adenosine triphosphate. HMP shunt = hexose monophosphate shunt. G-6-P = glucose-6-phosphate. F-1,6-diP = fructose-1,6-diphosphate. 1,3-DPG = 1,3-diphosphoglycerate. 2,3-DPG = 2,3-diphosphoglycerate and its shunt. ADP = adenosine diphosphate. 3-PG = 3-phosphoglycerate. PEP = phosphoenolpyruvate

phofructokinase activity. The final product of this glycolytic pathway is pyruvate or lactate, both of which can diffuse out of the cell and be metabolized by other tissues. The high-energy ATP that is generated by phoshoglycerate kinase and pyruvate kinase from each of the two three-carbon fragments arising from the cleavage of fructose-1,6-diphosphate is utilized for many of the erythrocyte's needs. These include maintaining the intracellular organization, structure, and shape of the cell, maintaining the intracellular concentrations of cations, especially sodium, potassium and calcium, against electrochemical gradients, preserving the lipids and proteins of the erythrocyte membrane, and maintaining the intracellular concentrations of nucleotides and cofactors. The observation that only 20 per cent to at most 50 per cent of the ATP-generating capacity of the erythrocyte can be accounted for by the known needs of this cell emphasizes the importance of the glycolytic regulatory processes inherent in the enzymatic reactions of the Embden–Meyerhof pathway. These regulatory processes are complex, even though simpler than in other cells. The Rapoport–Luebering 2,3-diphosphoglycerate (2,3-DPG) shunt permits the generation of 2,3-DPG, an important regulator of oxygen binding by hemoglobin. This shunt may also serve as an 'energy clutch' that bypasses the first ATP generating reaction, phosphoglycerate kinase.

It is not surprising, therefore, that aberrations in the activities of enzymes in the Embden–Meyerhof pathway are associated with hemolytic disorders. Dr. S. Miwa reviews the numerous hereditary deficiencies of this pathway's enzymes. All of these deficiencies are inherited as autosomal codominant characteristics, requiring the homozygous or doubly heterozygous state for manifest pathology, except phosphoglycerate kinase deficiency which is a sex-linked disorder.

Normally, some 10 to 15 per cent of the glucose phosphorylated by hexokinase flows through the hexose monophosphate (HMP) shunt pathway. The first two reactions of this pathway are the only ones available to the mature erythrocyte for the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH generated appears to function primarily in the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) and the reduction of mixed disulfides of hemoglobin with GSH. The percentage of glucose flowing through the HMP is normally directly related to the rate of oxidation of GSH. When the erythrocyte is exposed to 'oxidant' stress, more glucose-6-phosphate is diverted into the HMP. The hereditary deficiency of glucose-6-phosphate dehydrogenase (G-6-PD) activity is the most common known enzymatic deficiency of human erythrocytes. The gene controlling this enzyme's synthesis resides on the X-chromosome. Professor G. Jacobasch reviews the fascinating story of deficiencies in hexose monophosphate shunt enzymes and their biochemical and clinical consequences.

With the virtual exhaustion of additional glycolytic and HMP enzyme deficiencies for biomedical scientists to discover, attention has turned to hereditary defects in enzymes not directly involved in the metabolism of glucose. Adenine nucleotides are essential cofactors for glycolysis. Their maintenance in the erythrocyte and elsewhere is dependent upon both glycolytic activity and enzymes that

can function in their salvage. Thus, marked deficiency in the activity of adenylate kinase, which catalyzes the reversible conversion of two molecules of adenosine diphosphate (ADP) into one of adenosine monophosphate (AMP) and one of ATP, results in a hemolytic disorder similar to what is seen with pyruvate kinase and other anaerobic glycolytic enzyme deficiencies. In contrast, excess activity of adenosine deaminase, which converts adenosine into inosine and ammonia, is associated with significantly reduced erythrocyte ATP levels and a hemolytic syndrome, while deficiency is accompanied by immunoincompetence. More remote from the glycolytic pathways are the degradative enzymes of nucleotide metabolism. Severe, genetically determined deficiency of erythrocyte pyrimidine-5'-nucleotidase is associated with a moderately severe hemolytic disorder and the accumulation of much cytidine and some uridine in the erythrocytes. Dr. D. E. Paglia considers the disorders of the non-glycolytic pathways that may result in premature destruction of the circulating erythrocyte.

Finally, maintaining hemoglobin in a functional state so that it can bind oxygen reversibly is an important, albeit secondary, function of the erythrocyte. Protection of the heme iron of hemoglobin against oxidation to useless methemoglobin may be provided by reactions utilizing GSH and other processes. Once formed, methemoglobin is readily reduced to hemoglobin by a methemoglobin reductase system that has been shown to involve cytochrome b_5 reductase. Normally, the reduced nicotinamide adenine dinucleotide (NADH) generated at the glyceraldehyde-3-phosphate dehydrogenase step in glycolysis is used to reduce pyruvate to lactate. When methemoglobin accumulates, NADH is utilized by cytochrome b_5 reductase to reduce it to hemoglobin. Deficiency of cytochrome b_5 reductase is not associated with hemolysis, but rather congenital cyanosis that may, in its most extreme form, be accompanied by a severe, generalized neurological disorder. The final discussion, therefore, will deal with enzymopenic hereditary methemoglobinemia.

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Hereditary Disorders of Enzymes in the Embden Meyerhof Pathway of Glycolysis*

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Recent advances in hereditary disorders of red cell enzymes in the Embden–Meyerhof pathway and the Rapoport–Luebering cycle are discussed with a stress on pyruvate kinase deficiency. Broad genetic heterogeneity exists in all the known erythroenzymopathies. The primary structure of normal human red cell phosphoglycerate kinase has been determined recently and single amino acid substitutions of four mutant phosphoglycerate kinases have been clarified. These studies allowed an analysis of the structure–function relationships at the molecular level more precisely than has been possible previously. It is the consensus of the investigators working in this field that the pathogenesis in three-quarters of the congenital nonspherocytic hemolytic anemia patients remains unknown even after adequate red cell enzyme studies and isopropanol test. This means that broad studies have to be carried out in this field.

Key words: Embden–Meyerhof pathway, enzymopathies, erythroenzymopathies, hereditary hemolytic anemia, pyruvate kinase deficiency, red cell enzymes

Introduction

Since the discovery of pyruvate kinase (PK) deficiency in 1961 by Valentine et al. [1], a total of seven hereditary red cell enzymopathies associated with chronic nonspherocytic hemolytic anemia have been described in the Embden–Meyerhof glycolytic pathway. These are deficiencies of hexokinase, glucosephosphate isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, phosphoglycerate kinase and PK [2, 3]. In addition, deficiency of diphosphoglycerate mutase, an enzyme in the Rapoport–Luebering cycle which generates an important glycolytic intermediate, 2,3-diphosphoglycerate (2,3DPG), has been reported in association with either chronic hemolytic anemia or polycythemia [4, 5]. The most common enzymopathies of the Embden–Meyerhof pathway associated with chronic hemolytic anemia is PK deficiency, followed by glucosephosphate isomerase deficiency and then phosphofructokinase deficiency (Table 1).

The deficiency of red cell lactate dehydrogenase (LDH), i.e. a deficiency of the B-subunit of LDH described initially by Kitamura et al. [6], is not associated with hemolysis, in spite of definite deviations from normal of some of the glyco-

* Lecture held at the International Congress ISH–ISBT, Budapest, August, 1982

Table 1

Enzymes of the Embden–Meyerhof pathway and the Rapoport–Luebering cycle which may cause hereditary nonspherocytic hemolytic anemia

Enzyme	Number of reported cases
Hexokinase	12
Glucosephosphate isomerase	39
Phosphofructokinase	21
Aldolase	3
Triosephosphate isomerase	15
Phosphoglycerate kinase	11
Pyruvate kinase	more than 300
Disphosphoglycerate mutase*	3

*One out of three did not show hemolysis and showed polycythemia

lytic intermediates [7]. In this connection it is worth mentioning that recently a deficiency of the A-subunit of LDH was found in four members of a Japanese family by Kanno et al. [8]. The patient with A-subunit deficiency complained of exertional pigmenturia and easy fatigability. An abortive increase in blood lactate and a marked increase in blood pyruvate were observed immediately after ischemic forearm exercise, accompanied by myoglobinuria. The patients had no hemolytic anemia.

Pyruvate Kinase (PK) Deficiency

More than 300 cases of this deficiency have been reported. The clinical severity varies considerably among the patients. Red cell morphology is that of congenital nonspherocytic hemolytic anemia. Echinocytes may be seen occasionally before splenectomy but they usually increase in number and may become conspicuous after splenectomy. High reticulocyte counts of above 40 per cent are often observed after splenectomy. Thus, echinocytosis and a high reticulocyte count after splenectomy are the characteristics of red cell enzymopathies of the Embden–Meyerhof pathway associated with hemolytic anemia.

Most cases of PK deficiency are caused by the production of mutant enzymes with abnormal characteristics due to structural gene mutation. Standardization of the methods for characterization of PK variants has recently been achieved through the collaborative efforts of a working group of the International Committee for Standardization in Haematology [9]. Thirty to fourty ml of patient's blood is usually required to perform PK variant characterization. The major problem of characterizing PK variants is its being an autosomal recessive disorder, as compared with G6PD deficiency, which is sex-linked. Hence, many PK deficiency cases

are heterozygous for two different mutant genes. In such cases, although it is important to see the characteristics of the mixture of two mutant PKs, it is almost impossible to characterize two different PK variants in one patient.

At least eight PK variants have been described up to now (Table 2). Biochemical characteristics of PK variants that seem most markedly to influence the clinical severity include (i) thermal instability; (ii) decreased affinity to phosphoenolpyruvate (PEP); (iii) decreased sensitivity to allosteric activation by fructose-1,6-diphosphate (FDP); (iv) increased sensitivity to feedback inhibition by ATP; and (v) a marked decrease in PK activity [10, 11]. In this context it is interesting that recently Paglia and Valentine [12] have observed that the red cell PK from a patient with PK deficiency showed a $K_{0.5S}$ for ADP five times greater than normal. $K_{0.5S}$ for PEP in this mutant was normal, as was its response to FDP activation.

There are three electrophoretically, immunologically and kinetically distinct PK isozymes in human tissues. These are called M_1, M_2 and L. Kahn et al. [13] showed that L-type PK seemed to be synthesized in the erythroid precursors initially as an L'_4 enzyme, which then, as a result of partial proteolysis, was converted into the $L_2L'_2$ isozyme. The change of L'_4 into L_4 is associated with marked kinetic changes, which improve the regulatory properties of the enzyme. Marie et al. [14] found, however, a mutant PK which is unable to undergo normal proteolytic maturation.

We have initially shown that few cases of PK deficiency had M_2-type PK in their mature red cells and called them classical PK deficiency [15]. The same situation occurs in the PK-deficient basenji dog [16]. The M_2-type is regarded as a prototype of PK isozyme. Recently, Max-Audit et al. [17] reported cases which showed increased red cell PK activity and ATP levels, decreased 2,3DPG concentration and the presence of M_2-type PK in the red cells. This is probably the same disorder that was reported by Zürcher et al. in 1965 [18].

Red cells, leukocytes and platelets are derived from the same multipotential stem cell. There must be a conversion from M_2-type to L-type PK during maturation of the erythroid series. We have recently investigated this problem by the fluorescent antibody technique [19]. The fluorescence of M_2-type PK was strongest in the proerythroblast stage, and then steeply declined. In contrast, the fluorescence of L-type PK was stronger in basophilic or polychromatic erythroblasts than in proerythroblasts. It appears that the conversion from M_2-type to L-type PK isozymes probably occurs during erythroid maturation just like the switch from fetal to adult hemoglobin. Compensatory M_2-type PK production is probably going on in the erythroblasts in classical PK deficiency. The PK hyperactivity reported by Max-Audit et al. might be regarded as a disorder similar to the hereditary persistence of fetal hemoglobin.

The mechanisms of hemolysis in PK-deficient red cells, especially in reticulocytes, appear to be that proposed by Mentzer et al. [20]. PK-deficient reticulocytes depend upon mitochondrial oxidative phosphorylation to maintain an adequate ATP level. In the spleen, stasis of red cells occurs, which is more marked in reticulocytes owing to their considerable adhesive tendency. In the spleen, PO_2

Table 2

Characteristics of eight pyruvate kinase variants determined by the ICSH methods [10, 11]

	Tokyo	Nagasaki	Sapporo	Maebashi	Itabashi	Fukushima	Aizu	Fresno
Activity %	156	136	147	39	51	92	63	16
$K_{0.5S}$(PEP)	high	high	high	high	high	high	high	low
Hill coefficient	low	normal	normal	normal	low	slightly high	normal	normal
$K_{0.5S}$(ADP)	normal	low	low	normal	normal	normal	normal	normal
Nucleotide specificity	slightly abnormal	normal	abnormal	normal	abnormal	abnormal	abnormal	normal
ATP inhibition	normal	normal	strong*	normal	strong*	strong*	strong*	weak**
F-1,6-DP (FDP) activation	normal	normal	normal	weak**	normal	weak**	weak**	strong*
Thermostability	labile	labile	normal	labile	labile	labile	normal	normal
pH curve	acidic	acidic	normal	normal (narrow)	normal (narrow)	normal	normal	slow
Electrophoretic mobility	slow	slow	fast	normal	slow	slow	normal	normal
Hemoglobin g/dl	8.0	8.6	9.1	10.1	7.8	9.8	8.6	11.7
Reticulocytes %	35.9	64.0	5.0	9.2	16.5	70.0	16.7	–
Splenectomy	+	+	–	–	–	+	–	+
Bilirubin, indirect mg/dl	7.0	2.0	2.2	8.2	2.2	2.3	12.4	–

* stronger than normal inhibition or activation
** weak activation (more FDP required for activation) or inhibition (more ATP required for inhibition)

is low and oxidative phosphorylation in the red cells is greatly impaired, resulting in a decreased ATP-level. ATP depletion alters the red cell membrane, resulting in potassium loss and dehydration. Echinocytes appear and as the deformability of these cells is reduced, finally the cells are phagocyted by macrophages of the spleen. There is probably a considerable degree of heterogeneity among the red cells in the severity of enzyme deficiency, and those that have the most residual activity have the greatest chance of survival in the circulation.

Hexokinase (HK) Deficiency

Although HK deficiency is a rare disorder, a clear genetic heterogeneity exists among the characteristics of HKs of the patients' red cells [21].

Glucosephosphate Isomerase (GPI) Deficiency

Almost 40 cases of GPI deficiency have been reported. Most mutant GPIs of the patients associated with chronic hemolytic anemia showed unstable characteristics [22]. However, recently Zanella et al. [23] have observed a stable variant associated with severe hemolytic anemia, mental retardation and muscular hypotonia. In this case, the question remains as to why the red cells do not survive normally.

Phosphofructokinase (PFK) Deficiency

Up to now about 21 cases of this deficiency have been reported. Vora et al. [24] have recently demonstrated that the normal red cell PFK consisted of a heterogeneous mixture of five tetrameric isozymes. In the light of these findings, they investigated the molecular basis of PFK deficiency in a case displaying myopathy and hemolytic anemia. The PFK from the patient's red cells consisted exclusively of the L_4 isozyme, and there was a complete absence of the other four. Reviewing the literature, Vora et al. concluded that the most common form of PFK deficiency (Tarui's disease) originates from the exclusive deficiency of the M-type subunit of PFK, although other types such as due to unstable M-type subunit or absence or the extreme instability of L-type subunit exist.

Aldolase Deficiency

After the initial report of Beutler et al. in 1974 [25], there has been no mention of this condition and our two cases recently found in a Japanese family appear to be the second one [26]. The red cell aldolase in our cases was heat labile and in addition had a high $K_{0.5S}$ for FDP. These findings strongly suggest that our cases

were caused by a structural gene mutation. This is in contrast with the case described by Beutler et al. because their patient did not show any enzymatic characteristics different from those of the normal red cell aldolase. Furthermore, the present cases did not show mental or growth retardation nor did they show dysmorphic features, while Beutler's case included these abnormalities.

Triosephosphate Isomerase (TPI) Deficiency

Only about fifteen cases have been reported. Recent observations of three TPI deficiency cases showed that in all of them the deficiency seemed to result from a marked instability of the enzyme [27, 28] rather than a reduced catalytic activity.

Phosphoglycerate Kinase (PGK) Deficiency

So far, about nine unrelated families with PGK deficiency associated with chronic hemolytic anemia have been reported.

Recently, Huang et al. [29, 30] have succeeded in determining the primary structure of the normal red cell PGK molecule. Human PGK is composed of 417 amino acids. The amino acid substitution of four PGK variants has been clarified [31–33]. In order to determine the amino acid substitution, 500 ml of patient blood was required except for PGK Tokyo, for which only 18 ml of blood was needed, as the authors developed an ingenious method. They separated lymphocytes from the blood and incubated them with EB-virus, cultured the transformed lymphoblastoid cells, and then the purified mutant PGK from these cells.

The tertiary structure of horse PGK was determined by Banks et al. [34] based on X-ray crystallographic studies, and they found that only 14 amino acids were different. Therefore, the three-dimensional structure of human PGK must be almost identical to that of the horse enzyme, and this fact gave the impetus to determine the exact structural abnormalities of PGK variants. For example, the structural abnormality of PGK Uppsala is a single amino acid substitution from arginine to proline at the 206th position. This substitution is expected to induce a dislocation of the ADP binding site, resulting in a markedly disadvantageous mutant enzyme and severe clinical symptoms. These studies from Yoshida's laboratory represent some of the most important recent progress in the field of red cell enzymopathies.

Diphosphoglycerate Mutase (DPGM) Deficiency

Two different phenotypes of DPGM deficiency have been reported. Total deficiency of the enzyme was observed by Rosa et al. in 1978 [4]. The patient showed a moderate degree of polycythemia but no sign of hemolysis. In contrast, Schröter in 1965 [5] reported on severe hemolytic anemia in a neonate.

Concluding Remarks

It is the consensus of the investigators working in this field that in the three-quarters of the congenital nonspherocytic hemolytic anemia patients the pathogenesis remains unknown even after adequate red cell enzyme studies, and in spite of isopropanol tests for unstable hemoglobin being done [35, 36]. Therefore, it is necessary to perform more detailed enzyme assays using both conventional and low substrate systems as well as to study the red cell glycolytic intermediates and adenine nucleotides so as to avoid overlooking enzyme anomalies. Efforts to discover new enzyme anomalies should also be made.

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Hereditary Disorders of Erythrocyte Enzymes in Non-Glycolytic Metabolic Pathways*

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A number of non-glycolytic metabolic abnormalities may occur in erythrocytes without significantly altering cell function or life span. They include deficiencies of adenine or hypoxanthine-guanine phosphoribosyltransferases, adenosine deaminase, nucleoside phosphorylase, and hyperactivity of ribosephosphate pyrophosphokinase. Three principal enzyme defects are causally associated with hemolytic anemia: hyperactive adenosine deaminase and deficiencies of adenylate kinase and pyrimidine nucleotidase. These produce hemolytic syndromes of variable severity ranging from mild or subclinical in the adenosine deaminase defect to severe in adenylate kinase deficiency. Pyrimidine nucleotidase deficiency is much more common and is associated with intermediate degrees of anemia. Acquired nucleotidase deficiency may occur secondary to lead toxicity and produces a syndrome virtually identical to the hereditary deficiency states.

Key words: adenosine deaminase, adenylate kinase, erythroenzymopathies, pyrimidine nucleotidase

Compared to nucleated cell types, the mature human erythrocyte is a relatively simple system. Yet this very simplicity imposes unique metabolic restrictions within which the cell must function to survive. For example, its inability beyond the reticulocyte stage to synthesize most complex molecules means that its complement of enzymes and other crucial constituents must serve for the full life span of the cell. These components, however, are subject to progressive losses and at some point, one or another probably limits the flow rate of essential metabolic processes, and the cell can no longer generate energy sufficient for survival.

There are basically two reservoirs of energy within the mature red cell. One is a pool of energy contained in reduced glutathione and pyridine cofactors which serves to counter ambient oxidative stresses on various cell components. The other consists of pool of high energy phosphates, most importantly the adenine nucleotides.

The terminal high energy phosphate of ATP provides the fuel to pump cations against concentration gradients, to maintain the plasticity and functional status of the cell membrane, and to drive important reactions in glutathione synthesis, glucose catabolism, and nucleotide preservation. Both energy reservoirs are de-

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pendent upon glycolysis for maintenance of adequate levels. Degradation of glucose to lactate, either anaerobically through the Embden–Meyerhof pathway or oxidatively through the pentose phosphate shunt, provides essentially the only means for mature erythrocytes to generate the energy necessary for normal survival. This dependence is emphasized dramatically by the deleterious effects of the numerous glycolytic enzymopathies that have now been defined. These are a heterogeneous group, yet despite the diversity of their mutant gene products, they have in common an association with premature hemolysis, so a causal pathogenetic role seems an inescapable conclusion [1].

In addition to glycolytic enzymopathies, certain purine and pyrimidine disorders are known to affect erythrocyte metabolism. Subsets within this group include disturbances that occur without apparent effects on cell longevity, others that may be associated with megaloblastic features, and finally, those that result in shortened cell life spans and hemolytic anemia. This discussion will largely be confined to the latter group for two reasons. First, they are becoming increasingly important in the differential diagnosis of hemolytic conditions, and second, their study has made fundamental contributions to the understanding of processes that are crucial to normal erythrocyte function and viability. The conditions we will review briefly are the hemolytic syndromes associated with hyperactive adenosine deaminase and with deficiencies of adenylate kinase and pyrimidine nucleotidase.

Much of our current knowledge of nucleotide metabolism in red cells evolved from blood-banking studies [2]. When blood was stored at 4 °C in ACD, it was noted to have a progressive loss of high energy phosphates and a limited post-transfusion survival. It was soon discovered that certain nucleosides such as inosine and adenosine could counter the phosphate losses and simultaneously improve post-transfusion viability. Under certain conditions adenosine was considerably more effective than inosine, so these effects could not be explained solely on the basis of ribosephosphate contributions for further degradation via the Embden–Meyerhof pathway.

Direct phosphorylation of adenosine to AMP, mediated by adenosine kinase, was demonstrated *in vitro* [3], and a number of investigators suggested that this might be an important mechanism for maintenance of the adenine nucleotide pool, since an active adenylate kinase is also present in red cells.

A rare experiment of Nature provided strong support for this hypothesis in the form of a family afflicted with a compensated nonspherocytic hemolytic anemia [4–6]. An intrinsic cell defect was confirmed by cross transfusion, and it was found to be transmitted through three generations as a genetic dominant. Affected cells had a decreased concentration of ATP and unprecedented elevations in activity of adenosine deaminase. The concentration of total adenine nucleotides within affected erythrocytes was actually about one-third of the expected level, since total adenine nucleotides are present in higher concentration in young cell populations.

When erythrocyte adenosine deaminase was assayed, it was found to be elevated from 45 to 70 times the mean levels found in either normal subjects or in

subjects with pronounced reticulocytosis. Extensive studies of the enzyme purified from affected erythrocytes showed that the enzyme protein itself was normal by all conventional biochemical criteria. Very similar findings were subsequently observed in a Japanese family with hyperactive adenosine deaminase [7]. The basic abnormality thus appeared to result from overproduction of structurally normal adenosine deaminase, presumably through an alteration of genetic control mechanisms for switching off the synthesis of this particular enzyme protein in erythroid precursors. It is pertinent that the overproduction appeared to be confined to erythroid elements, since granulocytes, lymphocytes and cultured skin fibroblasts did not share the abnormality. If the increased activity were due to some structural alteration, as apparently may occur with hyperactive ribosephosphate pyrophosphokinase, then in addition to increased specific activity, other tissues with the same isozyme would be expected to share the anomaly.

The mechanism by which pronounced hyperactivity of an enzyme (as opposed to deficiency) could account for severe reductions in the adenine nucleotide pool, may be evident in the reactions shown in Figure 1. Adenosine is present in the plasma in low concentration and is available to erythrocytes by diffusion and by facilitated transport. The direction of its flow within the erythrocyte is influenced by the relative activities and substrate affinities of adenosine kinase and deaminase. Under certain conditions adenosine may be preferentially deaminated because this enzyme is normally much more active than the kinase and is also in close physical association with the membrane components responsible for adenosine transport. At very low adenosine concentrations, however, phosphorylation may predominate, because the kinase has a much greater substrate affinity relative to the deaminase. Hyperactive adenosine deaminase could then account for reduced concentrations of cellular ATP, if the adenosine kinase pathway was normally necessary for replenishing and maintaining the adenine nucleotide pool by generating AMP.

Deaminase activity in the affected cells in this kindred was increased almost two orders of magnitude, perhaps even more in subpopulations, and could compete very effectively for ambiently available adenosine, despite the greater substrate affinity of the kinase. Deprived of the normal kinase-mediated salvage pathway then, the defective cells might not be able to compensate for random nucleotide losses and low concentrations of adenine nucleotides would result.

In support of this hypothesis, the converse situation has been observed in some cases of severe immunodeficiency disease associated with absent adenosine deaminase activity. These cases may exhibit markedly increased concentrations of cellular adenine nucleotides including deoxyribose forms. The absence of deaminase activity apparently allows an inordinate amount of adenosine to be converted to AMP, and the erythrocyte nucleotide pool is consequently expanded. The biochemical abnormalities in these two distinct genetic anomalies strongly suggest that the availability of adenosine and a balanced competition for it between adenosine kinase and deaminase are necessary for the normal maintenance of the adenine nucleotide pool in mature erythrocytes.

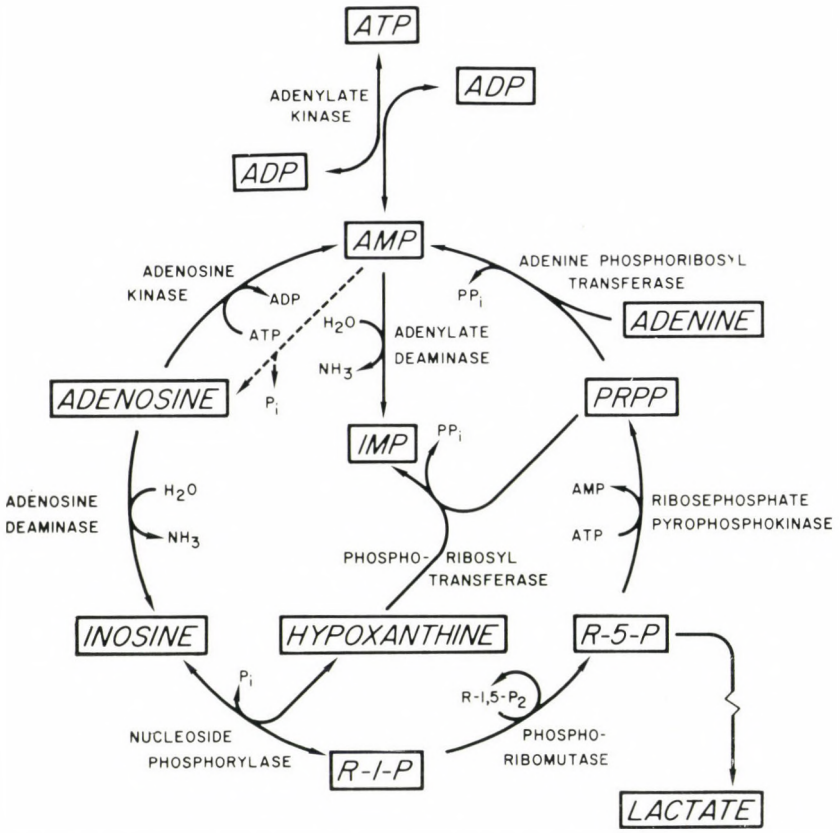

Fig. 1. Pathways of adenine nucleotide degradation and salvage in mature human erythrocytes. PRPP = phosphoribosyl pyrophosphate; R-5-P = ribose-5-phosphate; R-1-P = ribose-1-phosphate; R-1,5-P_2 = ribose-1,5-diphosphate; PP_i = inorganic pyrophosphate; P_i = inorganic phosphate; IMP = inosine monophosphate

Additional support for this hypothesis is provided by certain other 'experiments of Nature'. A few cases of adenylate kinase deficiency have been reported in which chronic hemolytic anemia was considerably more severe than that associated with adenosine deaminase hyperactivity [8–10]. A number of relatives had partial deficiencies consistent with heterozygosity for the defect, whereas those with frank anemia had adenylate kinase activities in the region of 5–10 per cent or less of normal means. Even though one family was complicated by coexistent G6PD deficiency, it seemed clear that adenylate kinase was the primary molecular lesion in both kindreds.

The sequence of events through which defective adenylate kinase could induce premature hemolysis is uncertain, but one might well speculate that this enzyme is critical to the same nucleotide salvage pathway discussed above. Since

AMP formed from plasma adenosine by adenosine kinase cannot be phosphorylated to ADP (and eventually to ATP) without adenylate kinase, it could not become an effective component of the adenine nucleotide pool in the absence of this enzyme.

AMP is in a special position of jeopardy, since its deamination either before or after dephosphorylation yields inosine monophosphate or diffusible inosine, and mechanisms do not exist in mature erythrocytes to retrieve the purine moiety from these compounds. This effect could be especially deleterious to older cells where salvage mechanisms are probably of greater relative importance as glycolysis diminishes with age. Therefore, ineffective use of this salvage pathway may be the primary pathogenetic mechanism of premature hemolysis both in instances of adenylate kinase deficiency and adenosine deaminase hyperactivity.

The most common enzyme defect within the category of nucleotide anomalies in red cells is that of pyrimidine nucleotidase deficiency [11]. This enzyme mediates the hydrolytic dephosphorylation of either uridine or cytidine monophosphates. Similar nucleotidases are widely distributed throughout nature as a heterogeneous group of isozymes. Virtually all react with both purine and pyrimidine substrates but with variable affinities. The cytosol nucleotidase in erythrocytes, however, has evolved in a highly specific direction so that its catalytic capability is sharply limited to pyrimidine substrates [12, 13]. Teleologically, such substrate specificity would seem almost mandatory in erythrocytes, since a nucleotidase also capable of dephosphorylating adenosine monophosphate would impose a constant drain on the adenine nucleotide pool that would require compensation by other salvage pathways if the red cells were to survive normally.

This nucleotidase probably functions only during reticulocyte maturation, serving to dephosphorylate the pyrimidine products of RNA degradation without jeopardizing the purine components. Nucleotidase deficiency, therefore, results in accumulation of pyrimidines which cannot diffuse from the cells as long as they remain phosphorylated. Impaired degradation of reticulocyte RNA is reflected in basophilic stippling, the particles of which are composed of ribonucleoprotein. This provides the most distinctive hematologic finding in this disease, pronounced stippling in the Wright's stained peripheral smears, but diagnosis ultimately requires demonstration of decreased nucleotidase activities, generally to about 5 per cent of that observed in comparably young cell populations and/or the detection of intracellular accumulation of various pyrimidine compounds, which normally are not present in erythrocytes in detectable amounts.

Chromatographic techniques are required to identify specific pyrimidines, but a simple determination of the ultraviolet absorption spectrum can readily detect their presence and establish the diagnosis, since pyrimidines accumulate in no other known erythrocyte condition. These spectra are principally a characteristic of the purine or pyrimidine base, and not the attached ribose or phosphates.

In Figure 2, the absorption spectrum of ADP in pure solution is compared with spectra of uridine and cytidine phosphates. These may then be compared with acid extracts of blood from normal subjects, from those with reticulocytosis

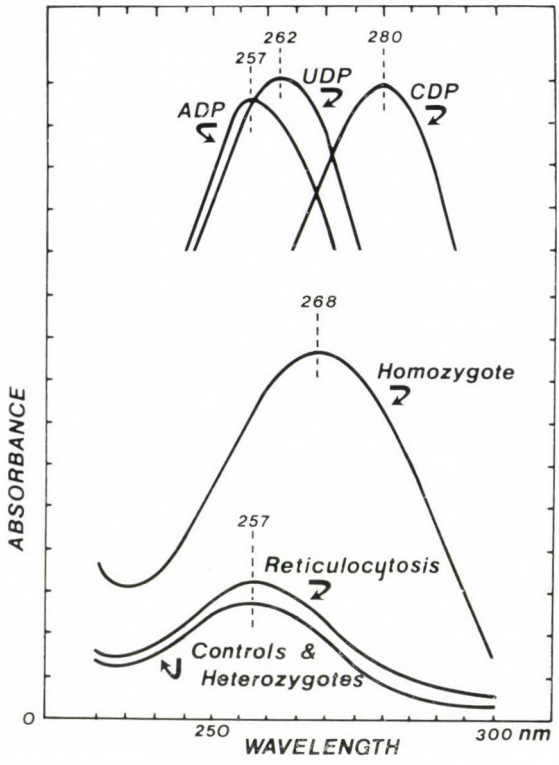

Fig. 2. Ultraviolet absorption spectra of equimolar concentrations of adenine, uridine and cytidine diphosphates compared to spectra of acid extracts of equicellular blood samples from subjects with homozygous or heterozygous pyrimidine nucleotidase deficiency, with reticulocytosis from other causes, and with no hematologic abnormality

due to other causes and from subjects with partial nucleotidase deficiency, who never exhibit pyrimidine accumulation. By contrast, in subjects with severe hereditary or acquired nucleotidase deficiency, the presence of significant intracellular concentrations of pyrimidine compounds produces shifts in the spectra from the usual 257 nm peak often up to 265–270 nm. These curves are normalized to comparable cell quantities, so the relative magnitudes demonstrate also that young cells have increased complements of adenine nucleotides compared to normal samples and that total nucleotides are markedly increased in homozygous deficiency states, generally ranging from 4–6 times normal mean concentrations. Since these nucleotides are often as much as 80 per cent pyrimidine compounds, the adenine nucleotide pool is actually diminished on an absolute scale, and this may be at least partially responsible for premature hemolysis.

Two epiphenomena have been consistent concominants in this disorder. Erythrocyte glutathione concentrations are approximately doubled, and ribose-

phosphate pyrophosphokinase activities are decreased to about one-fourth expected values. There are as yet no definite explanations for the etiologies of pathophysiologic significance of these epiphenomena, but it is quite possible that the elevated concentrations of glutathione may result from CTP inhibition of the normal membrane transport system that expels oxidized glutathione from the erythrocyte [14].

Deficiencies of pyrimidine nucleotidase may occur on both a hereditary or acquired basis, transmitted as an autosomal recessive trait or secondary to lead toxicity. The remarkable sensitivity of this enzyme to inactivation by lead and certain other heavy metals, and the common feature of basophilic stippling led tc elucidation of its role in the pathogenesis of lead-induced hemolytic anemia. Concentrations of lead that totally obliterate nucleotidase activity have minimal effects on most other erythrocyte enzymes, although they may have perceptible effects on heme biosynthesis. Humans exposed to chronic low-level overburden of industrial lead insufficient to cause anemia or basophilic stippling may still exhibit significant depressions of nucleotidase activity with otherwise normal erythrocyte enzyme profiles [15]. When blood lead levels approach 200 μg/dl packed cells, pyrimidine nucleotidase activity is depressed to levels comparable to those found in homozygous deficiency states. Basophilic stippling then becomes apparent and pyrimidine nucleotides begin to accumulate to detectable levels within the erythrocytes [16–20].

The three conditions reviewed, hyperactivity of adenosine deaminase and deficiencies of adenylate kinase and of pyrimidine nucleotidase, all induce hemolytic anemia of variable severity. The first two are very rare, with only two kindreds described in each instance. But these indicate that the anemia of hyperactive adenosine deaminase may be mild enough to escape clinical detection, whereas that of adenylate kinase deficiency can be life-threatening. Pyrimidine nucleotidase deficiency is one of the more common erythroenzymopathies and is associated with hemolytic anemia of intermediate severity, infrequently necessitating transfusions. Acquired deficiencies of nucleotidase activity result from lead overburden which, if sufficiently severe, almost completely recapitulate the syndrome associated with homozygous deficiency state. Splenectomy has not been demonstrably effective, and therapy is restricted to supportive measures.

Studies of cellular perturbations resulting from these defects have provided a broader understanding of normal erythrocyte metabolism, as well as the pathophysiology of underlying hemolytic mechanisms.

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Enzymopenic Hereditary Methemoglobinemia*

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The normal erythrocyte is well endowed with a system to convert useless methemoglobin to functional hemoglobin. The major mechanism for this reductive capacity resides in the soluble cytochrome b_5/NADH cytochrome b_5 reductase of the cytosol which presumably arise from the microsomal proteins of the endoplasmic reticulum through proteolytic cleavage of the proteins' hydrophobic tails during the maturation of nucleated erythrocyte precursors.

NADH cytochrome b_5 reductase is coded for by a gene on the human chromosome 22. Inheritance of a pair of abnormal alleles which specify an enzyme with decreased activity or stability occurs only rarely, but leads to enzymopenic hereditary methemoglobinemia.

Type I, uncomplicated, benign methemoglobinemia is attributed to mutation in paired alleles that affect primarily the catalytic capacity, stability, or solubilization of the polar, soluble segment of the enzyme. It does not appear to affect significantly the well being or life expectancy of the homozygous subject. The cosmetic affliction or the minimal symptoms can rather easily be controlled with methylene blue, ascorbic acid, or riboflavin. The heterozygote is entirely asymptomatic, but may have an increased tendency to develop methemoglobinemia on exposure to methemoglobin-inducing drugs or chemicals.

Type II, severe lethal methemoglobinemia is a generalized disorder in which the NADH cytochrome b_5 reductase is apparently defective in all tissues. It is thought to result from either gene deletion or mutation in paired alleles that determine the function, stability, or attachment to the endoplasmic reticulum of the entire enzyme, both the polar and the hydrophobic segments. As in Type I, the heterozygote is asymptomatic, and the homozygote's methemoglobinemia is readily controlled. The generalized disorder including the neurologic dysfunction, however, is not amenable to treatment at this time. Prenatal diagnosis by examination of amniotic fluid cells is both feasible and useful.

Key words: methemoglobinemia, erythrocytes; (NADH) cytochrome b_5 reductase, (NADH) methemoglobin reductase, cytochrome b_5 reductase deficiency

Introduction

Methemoglobin is an oxidation product of hemoglobin in which the sixth coordination position of ferric heme is bound to water, imparting to blood a brown color. It has a characteristic absorption spectrum, useful for its identification. Because of the loss of electrons from the heme irons and the positive charges made available, methemoglobin can no longer bind oxygen reversibly. It can, however,

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readily form complexes with anionic ligands that affect its physicochemical properties. Methemoglobin, as an isolated alteration, is not a drastic change, and can be reversed by the erythrocyte's metabolic processes. The relatively constant concentration of less than one percent methemoglobin in normal human blood may be regarded as the expression of an equilibrium between the slow, spontaneous formation of methemoglobin, counteracted by protective processes, and the rate of its reduction to hemoglobin. Methemoglobinemia, therefore, occurs when the rate of oxidation is increased sufficiently to overwhelm the protective and reductive capacities of the cells (acute, toxic methemoglobinemia), when an alteration in the structure of the globin moiety stabilizes methemoglobin and renders it resistant to reduction (hemoglobin M disorders), or when there is a significant deficiency in the methemoglobin-reducing ability of the erythrocytes [1, 2].

The present review will focus on the last of these mechanisms for the occurrence of methemoglobinemia. Suffice it to note that the spontaneous formation of methemoglobin, auto-oxidation, occurs slowly *in vitro* and *in vivo*. The molecular events of auto-oxidation are still poorly understood. The alpha chains of hemoglobin auto-oxidize more rapidly than beta chains. Various drugs, chemicals, or their metabolites accelerate the formation of methemoglobin one hundred to one thousand fold. Protection against oxidation of the heme moieties is provided by their lying within hydrophobic crevices formed of proximal and distal histidines and amino acids with nonpolar side chains that tend to shield the heme iron from excessive exposure to free radicals or hydrogen peroxide. These oxidants are normally scavenged by superoxide dismutase or glutathione peroxidase. Catalase can also destroy hydrogen peroxide when the latter is present at a high concentration. Amino acid substitutions in and around the heme pocket directly affect the hemoglobin bond in the hemoglobin M's. New, very stable bonds are formed between the substituted amino acid and the heme iron so that the latter is maintained in the ferric state, cannot bind oxygen reversibly, and resists the normal reductive processes of the erythrocytes.

Reduction of Methemoglobin to Hemoglobin

From the pioneering investigative work of Gibson [3] and Scott [4], persuasive evidence has accumulated that the normal human erythrocyte contains a methemoglobin-reducing system dependent upon the generation of reduced nicotinamide adenine dinucleotide (NADH or DPNH). The NADH-dependent methemoglobin reductase system, variously named diaphorase, NADH-methemoglobin reductase, NADH-dehydrogenase, NADH-methemoglobin-ferrocyanide reductase, and NADH-cytochrome b_5 reductase, is considered to be the most important one for the conversion of any methemoglobin that is formed, either by auto-oxidation or by chemicals, to functional, oxygen-carrying hemoglobin. When normal human erythrocytes, treated with sufficient nitrite to convert most of the hemoglobin to methemoglobin, are washed thoroughly and incubated with a substrate that can be metabolized by the cells to generate NADH, reduction of methemo-

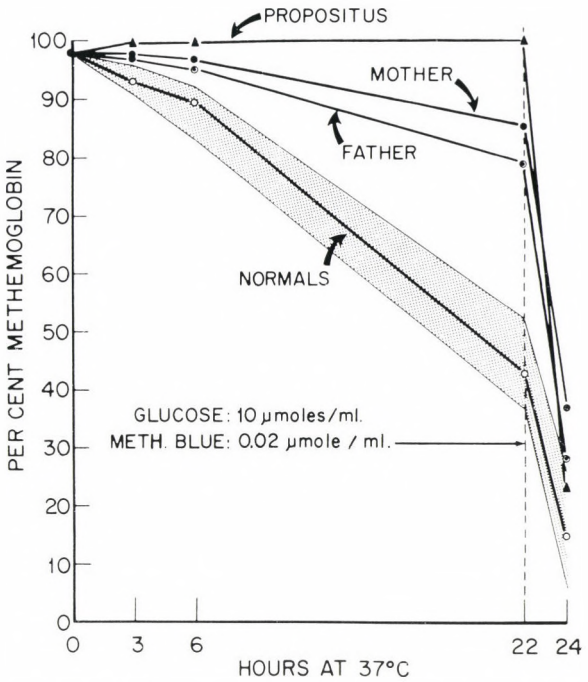

Fig. 1. Reduction of methemoglobin by nitrite-treated, washed human erythrocytes incubated with glucose as substrate, before and after addition of methylene blue. The propositus was a child homozygous for NADH-methemoglobin reductase deficiency. (Reproduced with permission from the *Amer. J. Med.* [5])

globin occurs [5] (Fig. 1). Under identical conditions, erythrocytes from patients with hereditary methemoglobinemia due to cytochrome b_5 reductase deficiency reduce methemoglobin very slowly, if at all. Erythrocytes from heterozygotes with intermediate levels of this enzymatic activity demonstrate an intermediate rate of methemoglobin reduction.

The anaerobic glycolytic pathway of mature human erythrocytes includes two NAD/NADH (DPN/DPNH)-linked enzymatic reactions [6] (Fig. 2). Glyceraldehyde-3-phosphate dehydrogenase reduces NAD to NADH, and lactate dehydrogenase uses NADH to form lactate from pyruvate. During steady-state glycolysis, there is no net accumulation of NADH because the equilibria of these reactions are in opposite directions. When nitrite-treated normal human erythrocytes are incubated with glucose, pyruvate accumulates in a nearly stoichiometric amount, consistent with the diversion of NADH to the enzymatic reduction of methemoglobin. Under similar conditions, little if any pyruvate accumulates in the cells of patients with cytochrome b_5 reductase deficiency.

The dramatic catalytic effect of methylene blue on the reduction of methemoglobin in normal erythrocytes, as well as in those deficient in cytochrome b_5

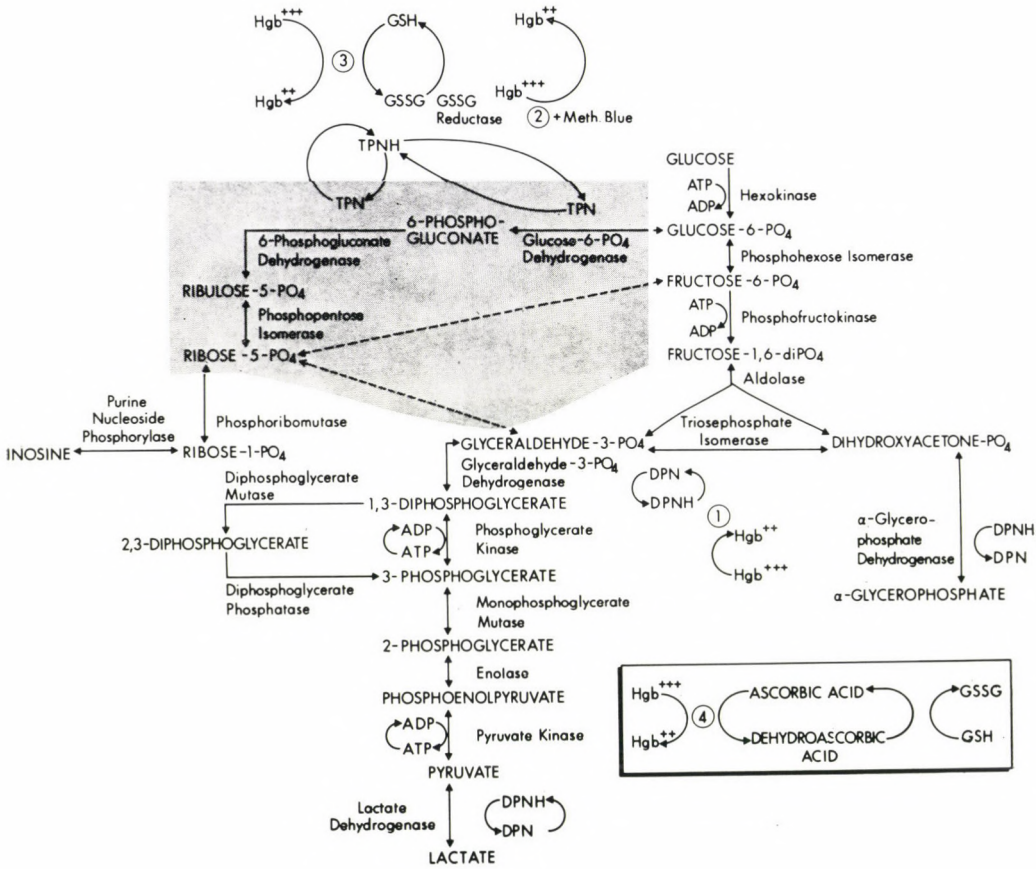

Fig. 2. Pathways by which glucose and purine nucleosides are metabolized by human erythrocytes, and the manner in which their metabolism is related to the reduction of methemoglobin (Hgb^{+++}) to hemoglobin (Hgb^{++}). Reactions enclosed in the shaded area comprise the hexose monophosphate shunt. Interrupted lines indicate omission of several reactions. 1 = NADH-(DPNH-) methemoglobin reductase (cytochrome b_5 reductase) system. 2 = NADPH-(TPNH-) methemoglobin reductase system with methylene blue (Meth. Blue). 3 = Nonenzymatic reduction of methemoglobin by reduced glutathione (GSH). 4 = Nonenzymatic reduction of methemoglobin by ascorbic acid. (Reproduced with permission from the *Amer. J. Med.* [6])

reductase activity, supports the thesis that a second pathway exists for the reduction of methemoglobin (Fig. 1). NADPH-methemoglobin reductase (NADPH-dehydrogenase) rapidly reduces methylene blue to leukomethylene blue which, in turn, spontaneously reduces methemoglobin with the regeneration of methylene blue. Since neither patients with glucose-6-phosphate dehydrogenase deficiency nor patients with NADPH-dehydrogenase deficiency have methemoglobinemia, and because of other biochemical data, it is believed that little if any NADPH is normally used for the reduction of methemoglobin. NADPH-methemoglobin

reductase appears to be a dormant reserve system that only becomes active in the presence of an artificial electron carrier, such as methylene blue or riboflavin [7].

Ascorbic acid and reduced glutathione can reduce methemoglobin directly, but slowly. The dehydroascorbate and oxidized glutathione that result are reduced to the original compounds by specific reductases. It is highly unlikely that either ascorbate or reduced glutathione plays an important role in the reduction of methemoglobin under physiologic conditions. Tetrahydropterin can also reduce methemoglobin *in vitro*, and may be regenerated through glutathione-induced reduction of the dihydro form [8].

The currently available data indicate that the four pathways capable of reducing methemoglobin to hemoglobin provide the erythrocyte with a great excess in reducing power [4]. At least 95 per cent of the capacity of normal human erythrocytes to reduce methemoglobin can be accounted for by the NADH-cytochrome b_5 reductase system.

The Methemoglobin Reductase: Cytochrome b_5 Reductase

Despite all of the data supporting the thesis that the NADH-dependent methemoglobin reductase system was the most important for human erythrocytes, the normal endogenous electron transport material eluded discovery for many years. Hemolysates of human erythrocytes, even when fortified with all of the necessary cofactors and substrates, failed to undergo reduction of methemoglobin, except at very high hemoglobin concentrations, in the absence of artificial electron carriers. Petragnani, Nogueira, and Raw [9] demonstrated in 1959 that cytochrome b_5 and cytochrome b_5 reductase, prepared from pig liver, rapidly reduced horse methemoglobin with NADH, and that methylene blue could not substitute for the cytochrome. They postulated that the erythrocyte methemoglobin reductase might be a similar multi-enzymatic system, but lacked one fully active component. In 1971, Hultquist and Passon [10] finally detected cytochrome b_5 and a NADH-diaphorase (cytochrome b_5 reductase) in hemolysates of human erythrocytes which together catalyzed the reduction of methemoglobin. These observations, confirmed by Kaplan [11], Sugita [12], Schwartz [2], and others, led to the thesis that reduced cytochrome b_5 was the physiologic electron carrier between the enzyme, NADH-cytochrome b_5 reductase, NADH, and methemoglobin. The sequence of reactions involved was visualized as shown in Fig. 3 [1]. The overall model of the kinetics of methemoglobin reduction that has emerged from the various studies would account for (i) the observed rate of methemoglobin reduction (1 mM/h) in nitrite-treated normal erythrocytes; (ii) the inability of the normal system to reduce very rapidly accumulations of methemoglobin resulting from exposure to toxic agents; (iii) the inability of erythrocytes deficient in cytochrome b_5 reductase activity to reduce even physiologic amounts of methemoglobin; and (iv) the predisposition of infants and subjects heterozygous for cytochrome b_5 deficiency to develop toxic methemoglobinemia.

$$NADH + (FAD)\ Reductase \rightarrow (FAD)\ Reductase{:}NADH \rightarrow (FADH_2)\ Reductase{:}NAD^+$$
$$(FADH_2)\ Reductase{:}NAD^+ + 2Fe^{3+}Cyt\ b_5 \rightarrow (FAD)\ Reductase + NAD^+ + 2Fe^{2+}Cyt\ b_5$$
$$Fe^{2+}Cyt\ b_5 + Fe^{3+}Hgb \rightarrow Fe^{2+}Cyt\ b_5{:}Fe^{3+}Hgb \rightarrow Fe^{3+}Cyt\ b_5 + Fe^{2+}Hgb$$

Fig. 3. Proposed scheme for the transfer of electrons from NADH to cytochrome b_5 reductase, by the reduced reductase to ferricytochrome b_5, and from the resulting ferrocytochrome b_5 to methemoglobin. (FAD) Reductase = flavin-adenine dinucleotide-containing cytochrome b_5 reductase. ($FADH_2$) Reductase = reduced reductase. Fe^{3+} Cyt b_5 = ferricytochrome b_5. Fe^{2+}Cyt b_5 = ferrocytochrome b_5. Fe^{3+} Hgb = methemoglobin. Fe^{2+} Hgb = hemoglobin. (Reproduced with permission from *Clin. Haemat.* [1])

Kaplan and his associates [13] have provided evidence that the lysine residues, beta 66 (E 10) and beta 95 (FG 2), located near the heme crevice in the beta chains, together with the lysine residues alpha 56 (E 5) and alpha 60 (E 9) of the alpha chains comprise the main part of the reduced cytochrome b_5 binding region. They postulated that interactions between complementary charged domains around the heme groups brought the heme moieties of the interacting proteins close together, thereby facilitating the transfer of electrons.

The properties and characteristics of the various reduced pyridine nucleotide-dependent methemoglobin reductases reported to exist in normal human erythrocytes and in the erythrocytes of subjects with hereditary methemoglobinemia not due to an abnormal hemoglobin have been reviewed extensively elsewhere [2]. Disagreements about reduced pyridine nucleotide specificity, flavin content, and requirement for an artifical electron carrier persist, but the reported differences may well be due to variations in experimental conditions, or arise from protein modifications induced by the isolation procedures. Current evidence favors the proposition that the methemoglobin-reducing activities of normal human erythrocytes, whether characterized as NADH dehydrogenase I [14], NADH methemoglobin-ferrocyanide reductase [15], or NADH cytochrome b_5 reductase [16], are different biochemical manifestations of a single enzyme protein which is separate and distinct from the NADPH-linked methemoglobin reducing ability. The latter almost invariably requires the intervention of a dye capable of serving as an electron carrier.

The cytochrome b_5 and cytochrome b_5 reductase of mature mammalian erythrocytes probably originate from the endoplasmic reticulum of nucleated erythrocyte precursors where they perform entirely different functions. Nucleated mouse erythrocyte precursors have endoplasmic reticulum and contain these two proteins. The concentrations of these latter proteins decrease as the cell matures, but small amounts of the water-soluble forms persist in the cytoplasm even after the organelles have disappeared [17]. Comparisons of the structures of cytochrome b_5 and cytochrome b_5 reductase obtained from mature erythrocytes and liver microsomes suggest that the proteins in the erythrocytes have arisen by proteolytic cleavage of the parent molecules in the endoplasmic reticulum. The striking similarities in amino acid composition and molecular weight of human erythro-

cyte cytochrome b_5 reductase and the lysosome-digested bovine microsomal enzyme support the hypothesis that the erythrocyte enzyme represents the polar protein segment of an amphipathic parent molecule originally bound to the endoplasmic reticulum of nucleated precursor cells [18]. Additional support for this thesis is derived from immunologic studies which indicate antigenic similarity between microsomal and soluble forms of cytochrome b_5 and of cytochrome b_5 reductase [19–21].

Evidence has been provided that the human erythrocyte cytochrome b_5 reductase is not only soluble, but also tightly bound to the inner face of the membrane [22]. The erythrocyte membrane enzyme is antigenically similar to the soluble enzyme, and the two forms are determined by the same gene. It is, therefore, postulated that the erythrocyte membrane cytochrome b_5 reductase is the primary gene product, and that post-translational partial proteolysis gives rise to the soluble form of the enzyme which serves as the methemoglobin reductase.

Enzymopenic Hereditary Methemoglobinemia: Clinical Manifestations

Hereditary methemoglobinemia due to an enzymatic defect has probably been described for more than a century. Congenital cyanosis without obvious cardiac or pulmonary disease was reported by François in 1845 [23]. Familial clusters of idiopathic congenital cyanosis were described by Hitzenberger in 1932 [24]. The classical studies of Gibson in 1948 [3] provided experimental evidence that a NADH-dependent reducing system was deficient in the erythrocytes of patients with idiopathic methemoglobinemia. Scott and his associates [14] identified an enzyme which catalyzed the reduction of methemoglobin with NADH and its markedly decreased activity in the erythrocytes of native Alaskans with methemoglobinemia. Subsequently, the investigations of Hegesh et al. [15], Kaplan et al. [11], Passon and Hultquist [16], Schwartz et al. [2], Sugita et al. [12] and their associates, as well as in our laboratory, have provided considerable insight into the biochemical and genetic alterations that lead to this form of hereditary methemoglobinemia.

Proved or probable cytochrome b_5 reductase deficiency has now been described in more than 250 subjects, and at least another 150 are presumed to have had the same defect because of the apparent recessive mode of inheritance of methemoglobinemia or the prompt disappearance of cyanosis after the administration of methylene blue. The disorder is rare, but distributed world-wide. An unusually high incidence has been reported among Alaskan Eskimos and Indians, Navajo Indians, Puerto Ricans, the natives of the Yakutsk region of Siberia, and individuals of Mediterranean origin.

Enzymopenic hereditary methemoglobinemia may conveniently be divided into two types, even on the basis of the clinical manifestations (Fig. 4). All patients, regardless of type, characteristically have persistent slate-gray cyanosis, often

dating from birth. There is no evidence of cardiac or pulmonary disease. Hardly any adverse effects are discernible when the methemoglobin level is 25 per cent or less. Even with levels up to 40 per cent, the only complaints may be those of easy fatigue and dyspnea after vigorous exercise. There is no hemolysis. In the benign form, Type I, life expectancy is normal and pregnancies are not compro-

ENZYMOPENIC HEREDITARY METHEMOGLOBINEMIA

TYPE I -- BENIGN

Methemoglobinemia well tolerated, readily controlled
Deficiency demonstrable only in erythrocytes
Soluble reductase deficient

TYPE II -- SEVERE, LETHAL

Methemoglobinemia; progressive neurological disorder and mental retardation not affected by therapy
Generalized deficiency demonstrable
Soluble and microsomal reductase deficient

Fig. 4. Principle features of Type I, benign, and Type II, severe, lethal enzymopenic hereditary methemoglobinemia

mised. These patients are really more blue than sick, and treatment is rarely necessary. In both Type I and Type II, control of the methemoglobinemia may be readily achieved by the administration of intravenous or oral methylene blue, oral ascorbic acid, or oral riboflavin.

Infants below the age of three months, and heterozygous carriers of an allelic gene for enzymopenic hereditary methemoglobinemia have intermediate levels of cytochrome b_5 reductase or methemoglobin reductase activity. Their erythrocytes, when challenged with nitrite *in vitro*, demonstrate a slower than normal rate of methemoglobin reduction. In infants, severe gastroenteritis with acidosis, contact with diapers labeled with aniline dyes or washed in disinfectants containing aniline, and ingestion of formulas prepared with nitrate-rich water have been associated with epidemics of methemoglobinemia. The presence in the immature intestine of nitrite-forming organisms, and the immaturity of the erythrocyte enzymatic machinery have been blamed for these cases of toxic methemoglobinemia in which mortality as high as ten percent has been reported. Primaquine, chloroquine, and diaminodiphenylsulfone given as malaria chemoprophylaxis have provoked toxic methemoglobinemia in adults heterozygous for cytochrome b_5 reductase deficiency [25].

As noted, Type I enzymopenic hereditary methemoglobinemia is well tolerated and readily controlled. Cytochrome b_5 reductase deficiency is demonstrable only in the erythrocytes, and only the soluble reductase is decreased in activity. The mutation responsible is presumed to have occurred in that part of the gene regulating only the synthesis or stability of the soluble portion of the enzyme.

Type II enzymopenic hereditary methemoglobinemia, which occurs in 10 to 15 per cent of patients with this aberration, is a much more severe and lethal disorder. In addition to the methemoglobinemia, signs of a progressive neurologic abnormality become apparent at two to three months of age, and death may supervene before the age of one. The fully expressed neurologic syndrome is characterized by severe mental retardation, microcephaly, retarded growth, opisthotonus, attacks of bilateral athetoid movements, strabismus, and generalized hypertonia [5]. Although appropriate therapy can control the methemoglobinemia, treatment has not affected the progressive and generalized disorder. In addition to deficiency of the soluble cytochrome b_5 reductase, nearly total deficiency of microsomal cytochrome b_5 reductase activity has been demonstrated in the leukocytes, muscle, liver, fibroblasts, and brain of such patients [26]. In view of these findings, Kaplan and his associates [11, 22] have postulated that the Type II disorder results from either gene deletion or mutation in paired alleles determining the activity or stability of the entire enzyme polypeptide chain, or the attachment of its hydrophobic segment to the endoplasmic reticulum. Since the microsomal cytochrome b_5/cytochrome b_5 reductase system is known to participate in the desaturation of fatty acids and in some P_{450}-mediated monooxygenase reactions, it has been suggested that the impairment of fatty acid desaturation, especially in the central nervous system, may account for the systemic manifestations.

Definitive diagnosis of enzymopenic hereditary methemoglobinemia requires the demonstration of markedly decreased erythrocyte enzyme activity by direct assay of NADH dehydrogenase I (diaphorase) [14], NADH methemoglobin-ferrocyanide reductase [15], or NADH cytochrome b_5 reductase [16]. Because the Type II form is a generalized disorder, and because deficient cytochrome b_5 reductase activity can be demonstrated in fetal amniotic cells, antenatal diagnosis is now feasible, as shown by Kaplan's group [27].

The Genetics of Enzymopenic Hereditary Methemoglobinemia

The clinical and biochemical data collected over the years are consistent, with very few unexplained exceptions, with the autosomal codominant inheritance of this disorder. Homozygosity is required for full expression of either Type I or Type II, while the heterozygous state is asymptomatic, but may predispose to the development of toxic methemoglobinemia. Cytogenetic analyses and examination of the soluble enzyme obtained from the cytosol of rodent/human fibroblast hybrids have permitted the assignment of the gene for soluble cytochrome b_5 reductase to the human chromosome 22 [28, 29]. The same gene locus is assumed to code for

the full length of the enzyme polypeptide chain, polar (soluble) plus membranous (hydrophobic) segments, originally embedded in the endoplasmic reticulum. Type I is attributed to mutation in the paired alleles which influence the stability or function of the soluble enzyme, or its proteolytic cleavage from the parent molecule in the endoplasmic reticulum. Type II, on the other hand, as noted above, is implied to involve mutation affecting the entire enzyme, or its attachment to the endoplasmic reticulum.

The diaphorase activity of cytochrome b_5 reductase allows its visualization after electrophoresis of hemolysate or tissue extract on starch or polyacrylamide gels [1, 30]. Five electrophoretic variants with normal staining intensity have been reported. The overall incidence of functionally normal variants may be as high as one in one hundred. Similar studies of patients with enzymopenic hereditary methemoglobinemia have revealed at least ten additional phenotypes. The pattern of segregation of the phenotypes within families is consistent with allelism at the enzyme's gene locus.

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Metabolism of the Hexose Monophosphate Shunt in Glucose-6-Phosphate Dehydrogenase Deficiency and Closely Interrelated Reactions*

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The metabolic changes of red blood cells from 25 patients with chronic hemolytic anemia caused by G6PD deficiency were investigated. The average G6PD activity exhibited 5 per cent of the normal control. The glucose oxidation was in most cases reduced even by 50 per cent. Three groups could be distinguished according to their degree of methylene blue stimulation of the oxidative pentose phosphate pathway.

These results are in agreement with changes of the kinetic constants for NADP, NADPH and G6P, respectively. The filtrability of red blood cells decreased in all cases of G6PD deficiency but no correlation was found with the survival time. First results of a preventive medication with D-L-α-tocopherol let assume a reduction of chronic hemolysis.

Key words: G6PD deficiency, hexose monophosphate shunt

The success of a diagnosis of enzymopenic hemolytic anemias depends to a high degree on the reliable and intelligent cooperation of physicians and biochemists.

Enzymopathies of glucose-6-phosphate dehydrogenase and pyruvate kinase are the most common of the enzyme deficiencies leading to hereditary nonspherocytic hemolytic anemia. The clinical symptoms of G6PD deficiency have been described long ago in a poetic manner by the Greek writer and politician Lucian, who recognized the association of the poisonous effect of fava beans and the occurrence of a hemolytic crisis. Many attempts have been made to define the quantitative and qualitative characteristics of G6PD mutants and to investigate the mechanism of hemolysis in the last 25 years [1]. Recently, Persico et al. have succeeded in cloning cDNA sequences of human G6PD [2]. An exact knowledge of the G6PD gene could perhaps help to elucidate in detail the molecular basis of the various forms of G6PD deficiency.

A number of G6PD(-) mutants cause either acute or chronic hemolytic anemia; correspondingly the survival time of red blood cells can be more or less shortened. In this paper I shall outline the attempts to characterize the metabolic changes occurring in the chronic hemolytic anemias and also the treatment of hemolysis.

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The red blood cells of 25 patients were investigated. Most of them exhibited a low G6PD activity attaining about 5 per cent of the normal control. Reticulocytosis varied from 3 to more than 80 per cent. In all cases with low catalytic G6PD activity the CO_2 formation from the oxidative pentose–phosphate pathway was reduced even by 50 per cent. In presence of the electron carrier methylene blue the produced $^{14}CO_2$ from 1-^{14}C-glucose was more than one order of magnitude lower than that of the normal control (Table 1). As it may be seen from the data

Table 1

Activity of the oxidative pentosephosphate shunt

	μmol 1-$^{14}CO_2$ formation/ml cells/h			
	−Mb		+Mb	
	$\bar{x}$	±s	$\bar{x}$	±s
Control n = 5	0.055	0.025	2.310	0.234
G6PD def. n = 13	0.028	0.008	0.106	0.030

Methylene blue: 0.2 mmol l^{-1}
pH 7.35

Table 2

Activity of the pentose–phosphate pathway in comparison with kinetic parameters of G6PD mutants

Proband	1-$^{14}CO_2$ formation		V_{max} G6PD	K_m G6P	K_m NADP	K_i NADPH
	μmol/ml cells/h			μmol/l		
	⊖Mb	⊕Mb				
Control	0.055	2.31	224	50	3.3	21
Ja. K.	0.035	0.265	0–10	—	1.9	5.4
Be. W.	0.035	0.224	0–6	57	—	4.3
Zi. A.	0.031	0.273	0–5	—	—	
Be. M.	0.026	0.169	10–30	52	2	16
We. G.	0.025	0.077	0–5	(71)	(1.4)	(11)
Ko. R.	0.015	0.024	0–5	(96)	(1.4)	(5.8)
Wei. F.	0.026	0.024	0–5	121	—	—
Ma. H.	0.043	0.049	3–10	(86)	(0.4)	(1.1)

Kinetic constants in parentheses resulted from heterozygous women

given in Table 2, 3 groups may be distinguished, one group consisting of red blood cells without any activation of glucose oxidation after the addition of methylene blue and two groups which exhibit some stimulation. Accordingly the properties of the G6PD mutants differ significantly. As might be expected structural changes

caused by point mutations can influence the binding of the ligands. From the point of regulation NADP, NADPH and G6P are likely to be of the greatest importance among the metabolites that may affect the activity of G6PD. On the right side of Table 2 we listed the kinetic constants K_m and K_i respectively for the G6PD mutants with G6P, NADP and NADPH. From these data one may conclude that the interactions of G6PD mutants with NADPH are more often affected than those with NADP and G6P. The NADP/NADPH ratio is very sensitive to oxidizing substances. One might expect that the sum of NADP and NADPH should be constant. Consequently a decrease in the level of NADPH should be accompanied by an increase of the NADP concentration. An increase of the NADP level should change the G6PD from a state of low affinity for NADP to a state of high affinity for this coenzyme since the inhibition of NADPH is competitive with NADP. The resulting activation of glucose oxidation should be reversely proportional to the K_i constant for NADPH. It is more difficult to interpret the group which failed to show a methylene blue stimulation because the kinetic constants of all three substrates seem to be changed. In *in vitro* experiments, Morelli et al. could demonstrate a restoration of glucose oxidation in the presence and in the absence of methylene blue for the genetic defect of the Mediterranean type by the direct entrapment of purified G6PD into the affected red blood cells [3]. In these G6PD deficient cells complete normalization of the activity of the oxidative pentose phosphate pathway was achieved by normalization of the intracellular G6PD activity. They could furthermore detect that the extent of formation of Heinz bodies upon incubation with acetylphenylhydrazine confirmed the full metabolic activity in G6PD-reconstituted Mediterranean erythrocytes compared with the unloaded ones. From these findings one may suppose that the formation of disulfide linked aggregates of polypeptides within the membrane of G6PD deficient red blood cells will also be prevented. To which extent these oxidative damages of the membrane are reversible in cases of chronic hemolytic anemia has not been tested. The precise mechanism leading to hemolysis of G6PD deficient cells has not been fully elucidated. As a rule an intravascular breakdown of G6PD deficient red blood cells occurs after contact with oxidative substances, especially with those which form radicals. The question arises if intravascular hemolysis occurs in G6PD enzymopathies with chronic hemolytic anemia and phagocytosis.

To test the possibility of membrane changes with a reduction of flexibility we have studied the filtrability. The apparatus consisted of four parts (Fig. 1): the filtration tube with the filter (F); the measuring tube (E); the electronic time keeper (G); and an equipment for generating reduced pressure (W). Filtrability was measured at a constant pressure of 10 ml water column. The time which a 2 ml sample needs to pass through the silver filter was recorded by the electronic time keeper. To define a parameter suitable to describe the process of the kinetics of clogging, a mathematical model was derived. It includes 3 premises.

1. There are N_v 'variable' pores to clog and N_c 'constant' pores not being able to clog;

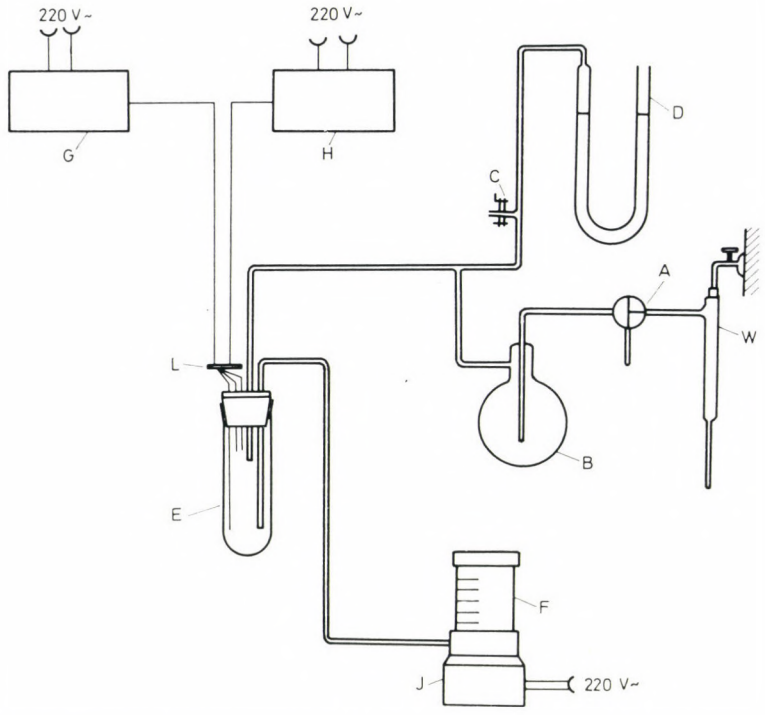

Fig. 1. Apparatus for filtrability studies. E: measuring tube; F: filtration tube with filter; G: electronic time keeper; H: amplifier; J: magnetic stirring; L: electroplating of metals; A, B, C, D, W: pressure regulation

2. the possibility to clog is the same for all 'variable' pores, e.g.

$$\frac{dN_v}{dt} = - k \cdot N_v;$$

3. the rigidity of RBC has no remarkable influence on the fluidity of the suspension in the pores; the flow is only a function of the total number of the free pores

$$v(t) = \frac{d_v}{dt} \Big| t = 0 \cdot \left[Ac \cdot t + \frac{1 - Ac}{k} \cdot (1 - e^{-kt}) \right].$$

The final equation includes 2 parameters, the filtration constant k, and Ac which is the percentage of constant pores. The ability of G6PD deficient blood samples to pass through restricted pores is summarized in Table 3. The filtration constants are increased fivefold on the average in all cases of G6PD deficiency. The decrease of blood filtrability agreed with that observed by Flynn et al. who detected an increase of disulfide concentration in the specific polypeptides of the membrane

Table 3

Filtrability of G6PD deficient red blood cells

	Diluted blood	Washed cell suspension
	← HK 2.0 →	
	k 10^{-3} s^{-1}	
Control	1.96 (0.78–3.21)	1.13 (0.78–1.82)
G6PD def.	9.06 (2.91–16.50)	1.56 (0.83–3.12)

Temperature 20 °C, pH 7.4

and correspondingly a decrease of reactive SH groups in two different cases of G6PD deficiency [4]. Disulfide is known to induce a polymerization of spectrin when approximately 0.3 per cent of the spectrin molecules are bound into a continuous network, when the shear elasticity increases [5].

We could not find a correlation between the increase of the filtration constant for G6PD deficient blood and the severity of the hemolytic anemia as in pyruvate kinase enzymopathies [6]. G6PD deficient red blood cells will probably only be clogged in restricted capillaries if the shear stresses are lowered.

The reduction of filtrability of the G6PD deficient cells nearly ceases after the erythrocytes have been washed 3 times in 0.9 per cent NaCl solution.

The structural matrix of the erythrocyte membrane is provided with a lipid bilayer containing more than one hundred different molecular species of lipids. The phospho- and glycolipids play an important role as a permeability barrier and a variety of proteins is embedded in the bilayer.

Since it is likely that oxidation of SH groups and the aggregation of some of the membrane proteins play an essential role in the mechanism of hemolysis, we

Table 4

Effect of vitamin E on chronic hemolysis in G6PD deficient patients

Proband	Hb g/100 ml	HK %	RBC 10^{12}/l	Retic. ‰	MCV fl	Bilirubin mg/100 ml
Control (n = 6)	13.8	43.5	5.01	9	86.9	<0.9
	13.7	43.7	5.02	26	87.1	<0.9
G6PD deficient	11.8	36.0	3.47	174	99.3	2.47
(n = 15)	12.1	37.7	3.64	62	91.2	1.82

Vitamin E supplementation for 6 weeks

The initial hematologic data are given in the first line, those after 6 weeks of vitamin E supplementation in the second one, respectively

have attempted to prevent it with the antioxidant D-L-α-tocopherol acetate. The dose of vitamin E was 800 IU/day for adult patients. The results observed after 6 weeks of vitamin E supplementation are summarized in Table 4. From the decrease of the reticulocyte count, the level of bilirubin and the mean corpuscular volume of red blood cells one may conclude to a reduction of chronic hemolysis. A small increase in the red blood cell count, the hematocrit value and the hemoglobin level only occurred in cases of mild hemolytic anemia. Catalytic G6PD

Table 5

Influence of vitamin E on phospholipid distribution in red blood cells of patients with G6PD deficiency

	Control	G6PD deficiency	
		before	after 6 weeks of vitamin E supplementation
Total PL (μmol/ml cells)	3.81	2.66	3.48
Sphingomyelin	1.02	0.79	0.93
Phosphatidylcholine	1.17	0.88	1.04
Phosphatidylethanolamine	1.08	0.84	0.97
Phosphatidylserine	0.52	0.11	0.58

PL analysis was carried out by TCC [8]

activity and glucose oxidation were not influenced by vitamin E supplementation. Table 5 shows the distribution of the four major phospholipids in the red cell membrane before and after vitamin E supplementation. The choline containing phospholipids sphingomyelin and phosphatidylcholine are located predominantly in the outer layer, while phosphatidylethanolamine and phosphatidylserine in the inner side of the membrane. The concentration of these phospholipids, particularly that of phosphatidylserine, was low in all cases of G6PD deficiency. The changes in phospholipid distribution could almost be normalized after a 6 week period of vitamin E supplementation. In order to evaluate the results of tocopherol therapy, a period of about 1 year is necessary, as has been done by Corash et al. [7].

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Reactivity of Cold Agglutinins with Subsets of Human Lymphocytes of Various Origins*

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Forty-five cold agglutinins (CA) were tested against various populations of lymphocytes by cytotoxicity and immunofluorescence assays. Marked differences were observed between anti-I and anti-i CA. Thirty-six per cent of anti-I killed preferentially peripheral blood and tonsillary B lymphocytes, whereas only 12 per cent killed preferentially T lymphocytes. Anti-I killed a much higher proportion of B-chronic lymphocytic leukemia cells than peripheral blood lymphocytes, peripheral blood B cells or T-chronic lymphocytic leukemia cells. Forty-three per cent of anti-i killed preferentially peripheral blood T lymphocytes and 54 per cent killed preferentially tonsillary T cells, whereas only 14 per cent killed more peripheral blood B cells and none killed preferentially tonsillary B cells. The kill of thymic lymphocytes and T-chronic lymphocytic leukemia cells by anti-i was very high, whereas the kill of B-chronic lymphocytic leukemia cells was very low. Almost all CA of other than I-i specificities showed preferential kill of peripheral blood B (83 per cent) and tonsillary B (67 per cent) cells. Tonsillary lymphocytes were usually more susceptible to the cytotoxic activity of CA than peripheral blood lymphocytes. Cold agglutinins with κ light chains killed more B cells whereas CA with λ light chains seemed to kill more T cells. Cytotoxicity did not correlate to the utilization of complement. It is suggested that the density and/or the accessibility of membranous antigens may be different on B and T cells, or alternatively that in addition to antigens common to all lymphocytes, anti-I and non-I/i cold agglutinins recognize specific antigenic determinants on B lymphocytes, whereas anti-i cold agglutinins recognize specific antigenic determinants on T lymphocytes.

Key words: cold agglutinins, complement, cytotoxicity, lymphocytes, tonsillary cells

Introduction

Cold agglutinins (CA) are cold-reacting antibodies which express cytotoxicity in the presence of complement [1, 2] and coat and interfere with the function of various living cells when no complement is added to the system [3]. CA have been found to interact with membranous antigens on several types of cells including lymphocytes, polymorphonuclears and monocytes, both normal and leukemic [4]. Since CA are immunoglobulins produced by B-lymphocytes and occur mainly in chronic lymphoproliferative disorders and in some infections, it may be impor-

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tant to define specificity of CA against various subsets of lymphocytes. If such specificity was found, it would shed light on the functional heterogeneity of CA, and possibly on the pathogenetic processes of various CA-related conditions. We tested 45 CA against several subsets of lymphocytes, both normal and leukemic, and found differences in the affinity to B- and T-cells related to the I/i specificity and to the light chain type of CA.

Methods

Forty-five sera from patients with a high titre of CA were tested. Twenty-five were anti-I, 14 anti-i and 6 other CA (2 anti-Pr_1, 1 anti-Pr_2, 1 anti-Pr_3, 1 anti-Gd and 1 Sa) [5, 6]. Thirty-seven CA were monoclonal, 27 with κ and 10 with λ light chains. Eight CA were either heterogeneous or the light chain type could not be determined. Total protein, serum electrophoresis, immunoelectrophoresis, immunoquantitation and cryoglobulin precipitation, cold agglutination of red blood cells (RBC) and elution of CA from the appropriate RBC were done, as reported previously [7, 8]. When necessary, the eluates were purified further by column chromatography using Sephadex G-200, DEAE Sephacel and Sephacryl S-300 Superfine (Pharmacia (Canada) Ltd., Dorval, P.Q., Canada). The purity of eluates was tested by Ouchterlony analysis, electrophoresis, immunoelectrophoresis and ultra-low-level immunoquantitation plates obtained from Kallestad, Chaska, Minn., USA.

Peripheral blood lymphocytes (PBL) were isolated by the Hypaque-Ficoll method [9]. Peripheral blood and tonsillary B- and T-cells were separated, as described [10]. The isolated tonsillary cells were diluted to 2×10^7/ml in 20 per cent fetal calf serum (FCS) (Gibco, Grand Island, N.Y.) which was previously absorbed on thrice-washed sheep RBC for 1 h on ice. Mixtures were made of 0.25 ml tonsil cells, 0.25 ml FCS and 0.5 ml 0.2 per cent sheep RBC, previously washed three times. The mixtures were centrifuged at 600 rpm for 5 min and the cells allowed to rosette at room temperature for 1 h. The rosettes were gently resuspended and layered into tubes with 1 ml Hypaque-Ficoll mixture and spun at $400 \times g$ for 40 min at 4 °C. The unrosetted B-lymphocytes were removed from the interface and washed twice in Hank's balanced saline solution (HBSS) (Gibco).

Nylon fibres removed from Leuko-pak leukocyte filters (Fenwall Laboratories, Deerfield, Ill.) were washed in several changes of deionized water. Dried fibres were packed into 10 ml plastic syringe barrels (120 mg/barrel) to form small columns and were rinsed with HBSS. Ten ml of 20 per cent FCS made in HBSS were placed in columns and kept at 37 °C for 30 min. The tonsillary cells were suspended at 5×10^7/ml in 20 per cent FCS. Two ml aliquots of the cell suspension were put into each column and covered with 2 ml of 20 per cent FCS. The suspension was incubated at 37 °C for 30 min. The T-cells were eluted dropwise with HBSS and washed twice in the same buffer.

Peripheral blood B- and T-lymphocytes were purified by the same method as described for tonsillary B- and T-cells. The purity of these and other cells was determined by a combination of Wright, myeloperoxidase [11] and nonspecific esterase stains [12]. Total recovery of PBL was 91 ± 9 per cent, viability was 99 ± 5 per cent and purity was 90 ± 4 per cent. Peripheral B- and T-cells were tested by E, E_{AET} and EAC rosettes, and immunofluorescence for surface immunoglobulins (SIg). Respectively, their recovery rates were 66 ± 33 per cent and 59 ± 23 per cent, viability 90 ± 10 per cent and 97 ± 5 per cent, and purity 77 ± 6 per cent and 99 ± 1 per cent. Viability of tonsillary B- and T-cells was 84 ± 9 per cent and 91 ± 4 per cent respectively and the purity was 87 ± 16 per cent and 99 ± 1 per cent. In preparations of peripheral human and tonsillary B lymphocytes, there was some contamination by monocytes, which were removed by carbonyl iron.

Thymocytes were purified from 7 thymuses obtained fresh from the Department of Surgery of the Hospital for Sick Children, Toronto. The thymocytes were isolated by teasing through copper mesh. The suspension was filtered through a double layer of gauze, red cells were hemolyzed with 0.85 per cent ammonium chloride and the thymocytes were washed twice in HBSS. Their viability varied from 90 to 97 per cent. The E-rosettes of these cells were 80–96 per cent and SIg less than 4 per cent.

Peripheral blood lymphocytes were isolated from 32 patients with B-cell chronic lymphocytic leukemia (CLL), as proven by E, E_{AET} and EAC rosettes and SIg immunofluorescence tests. The yield of B-CLL cells was 83 ± 17 per cent, purity 98 ± 3 per cent and viability 99–100 per cent. Matched control PBL and peripheral B-cells were isolated and tested in parallel to the B-CLL cells. In one case of T-helper-cell CLL, the isolated cells had 97 per cent viability and 99.5 per cent purity.

Monkey lymphocytes were purified by a modified Hypaque-Ficoll method using 10–11 parts of Hypaque 34 per cent and 24 parts of Ficoll 9 per cent (refractive index 1.3595 to 1.3606). The yield of lymphocytes from various monkeys was 68–100 per cent, viability was 94–100 per cent and purity was 85–99 per cent. The control sera, using autologous serum alone, human serum or rabbit complement alone, killed 0–6 per cent of monkey lymphocytes.

Bithermic lymphocytotoxicity assay was used [2]. The lymphocytes were mixed with CA and nontoxic rabbit complement and kept at 4 °C for 60 min, followed by 60 min at 24 °C. CA were used in dilutions 1 : 100 to 1 : 1500. Each serum with CA was tested at several dilutions and the maximum kill, 50 per cent kill and minimum kill were estimated. Each CA was tested against several panels (6 to 20) of peripheral B- and T-lymphocytes, each panel composed of three to four donors. Cytotoxicity assays were performed in duplicate and the controls included lymphocytes with each of the following: CA alone, complement alone, normal human serum and complement, and macroglobulinemic serum without CA activity with complement. The kill of controls was 2.5 per cent ± 0.2 (SEM) (n = 142).

The following criteria were employed to establish whether CA had preferential reactivity with B- or T-cells: At least 20 per cent more of the appropriate cells had to be killed in at least 75 per cent of the panels, and the cytotoxic index (serum dilution killing 50 per cent lymphocytes) had to be higher against appropriate cells.

Direct and indirect immunofluorescence was used, as described [13], with immunofluorescent anti-μ and anti-α antisera (purchased from Meloy Laboratories Inc., Springfield, Va). Utilization of complement was tested by the following technique: Cytotoxic assays were performed using triple volumes of purified CA, rabbit complement and appropriate lymphocytes. After termination of cytotoxic reaction, the mixtures were quickly centrifuged and the supernatant tested for residual complement activity. The reaction mixtures were diluted 1 : 5 to 1 : 20 with veronal buffered saline, μ-0.15, pH 7.4, containing 0.1 per cent gelatin calcium (0.15 mM) and magnesium (0.5 mM) (VBSG). Equal volumes (0.2 ml) of the diluted mixtures and sensitized sheep erythrocytes (10^8 cells/ml) were incubated at 37 °C for 60 min in a constant shaking water bath. The reaction was stopped by addition of 0.6 ml cold VBSG containing 0.01 M EDTA and after centrifugation the degree of hemolysis was determined spectrophotometrically at 412 nm. Sheep erythrocytes (purchased from Woodland Farms, Guelph, Ontario) were sensitized with IgM goat anti-sheep erythrocyte antibody (Gibco). The controls were an admixture of complement with phosphate buffered saline (PBS) and appropriate lymphocytes, lymphocytes alone and complement with PBS. Statistical analysis was performed using Fisher's exact test.

Results

Cytotoxicity of sera with CA against various subpopulations of lymphocytes is summarized in Table 1. In the group of CA which preferentially killed B-cells, the kill of B-cells was on average 108 per cent higher than of T-cells. In the group which killed preferentially T-cells, the kill of T-cells was on average 63 per cent higher than that of B-cells. The corresponding figures for tonsillary B- and T-lymphocytes were 62 per cent and 70 per cent. There was no correlation between the preferential kill of different subpopulations and the titre of CA agglutination of the RBC, thermoamplitude or the concentration of IgM in the serum. Preferential kill was not caused by any cytotoxin unrelated to CA. Neither warm nor cold lymphocytotoxins different from CA were present in the sera after RBC absorption of CA.

The relationship of diagnosis to cytotoxicity was possible to assess in the lymphoproliferative diseases only. Twelve of 27 CA in this group (45 per cent) killed preferentially B-lymphocytes, whereas only 6 (22 per cent) killed more T-lymphocytes ($p < 0.01$). No such difference was found testing tonsillary lymphocytes, 7 (26 per cent) killing more B-cells and 9 (33 per cent) killing more T-cells.

Table 1

Relationship of cytotoxicity to the type of cold agglutinins

Type of lymphocytes	Preferential kill of	Type of cold agglutinins			IgM/κ (27)	IgM/λ (10)
		anti-I (25)	anti-i (14)	others (6)		
Peripheral blood	B	9 (36)+*	2 (14)+	5 (83)	11+ (41)	1 (10)
	T	3 (12)+	6 (43)+	0 (0)	3+ (11)	4 (40)
	Equal kill	13 (52)	6 (43)	1 (17)	13 (48)	5 (50)
Tonsillary	B	9 (36)+	0 (0)+	4 (67)	10+ (37)	2 (20)
	T	3 (12)+	7 (54)+	0 (0)	4+ (15)	4 (40)
	Equal kill	13 (52)	6 (46)	2 (33)	13 (48)	4 (40)

+ Comparison of B vs T, $p < 0.025$
* Number of CA (percentage)

Groups of 5 CA from infectious diseases and 12 CA from miscellaneous conditions were too small to be assessed statistically.

The relationship of CA specificity to the preferential kill of lymphocytes is shown in Table 1. Anti-I and 'others' showed preference to B-cells, whereas anti-i CA showed preference to T-cells. Anti-I CA usually killed higher percentage of tonsillary than PBL cells. Anti-i and 'other' CA killed more PBL B-cells than tonsillary B-cells. The kill of tonsillary T-cells was usually higher than that of PBL-T cells.

Comparison of 27 IgM/κ CA with 10 IgM/λ CA (Table 1) showed that in the former group more CA were killing preferentially B-cells than those killing T-cells ($p < 0.01$). In the latter group, there was preferential kill of T-cells. This group was too small to be assessed statistically.

Comparison of cytotoxicity to immunofluorescence (IF) was done using chromatographically pure CA. Five anti-I and five other CA (one each, i, Pr_1, Pr_2, Pr_3, Gd) were tested. In 9 of 10 instances the number of IF positive PBL greatly exceeded the comparable number of killed cells. Whereas the average number of killed cells was 27 per cent (range 1–97 per cent), the number of fluorescent positive cells was 60 per cent (range 29–100 per cent). Control cells treated with pure IgM and IgA without CA activity showed fluorescence in 1–2 per cent.

Comparing cytotoxicity and immunofluorescence of B- and T-lymphocytes it was found that preferential coating corresponded to the preferential kill.

Thymocytes were killed more efficiently by anti-i CA. All anti-i, but only 60 per cent of anti-I CA tested killed more than 50 per cent of the cells. Nine of 15 anti-I, ten out of 10 anti-i and one out of 4 other CA tested against T-CLL cells killed more than 50 per cent of the cells. This contrasted greatly with B-CLL cells which were killed much more efficiently by anti-I and much less efficiently by anti-i CA, as compared to the PBL or the peripheral B-cell (Table 2). Lympho-

cytes from two kinds of monkey (squirrel monkey and marmoset) behaved similarly to B-CLL cells, whereas in four others (stump-tail monkey, *Macaca fascicularis*, *Macaca rhesus mulatta* and *Cercopithecus aethiopis*) the cells were more susceptible to anti-i CA.

Table 2

Cytotoxicity of cold agglutinins against B chronic lymphocytic leukemia lymphocytes*

Cold aggl.+	CLL	PBL§	P
anti-i (Den)	45±33	66±16	<0.025
anti-i (Nic)	25±15	60±17	<0.001
nati-I (Ver)	81±28	53±22	<0.005
anti-I (Step)	82±20	46±15	<0.001
anti-I (Bul)	95± 5	54±12	<0.001

* thirty-two cases of B-cell CLL
+ dilution 1 : 600
§ PBL — peripheral blood lymphocytes

To assess whether cytotoxicity is related to the utilization of complement, 11 pure IgM CA (9 anti-I and 2 anti-i) were tested for cytotoxic activity against peripheral and tonsillary B- and T-cells and the residual activity of complement was assayed in the supernatants. Residual complement activity expressed as a percentage of the initial total complement activity varied from 5 per cent to 28 per cent; the means being for PBL-B 16 per cent, PBL-T 19 per cent, tonsillary-B 11 per cent, and tonsillary-T 12 per cent. In some cases the utilization of complement was lower, i.e., residual complement activity was higher in the assays with T-lymphocytes. Therefore, 4 pure CA which killed predominantly B-cells were tested against B- and T-lymphocytes, increasing the amount of complement threefold in the assays with the T-cells. Such increase did not change the preferential kill of B-cells.

Discussion

Cytotoxicity of CA against PBL has been well documented [1, 2]. Sera and purified CA exhibit cytotoxicity to PBL-B and PBL-T, tonsillary lymphocytes (TL), thymocytes (Th) and B-chronic lymphocytic leukemic (B-CLL) cells [2, 14]. It was noted that anti-i CA are more toxic than anti-I to PBL, TL and Th, and less toxic to B-CLL cells. Thomas suggested that sera with CA, especially those with anti-i, have in addition to antibodies against I/i antigens another cytotoxin directed uniquely against T-lymphocytes [15]. Others were unable to find such separate anti-T specificity [2, 14, 16], some differences between individual CA in

their reactivity against B- and T-cells have, however, been observed. Shumak et al. using radiolabelled CA found differences in the attachment to T- and B-cells. Anti-i (Hog) bound equally to B- and T-cells, whereas the binding of anti-I (Step) to B-cells was greater than to T-cells [14]. Biberfeld et al. tested anti-I CA from patients with *Mycoplasma pneumoniae* infection (MPI) using immunofluorescent assays, and found no selective reactivity against B- or T-PBL. MPI sera did not, however, react with Molt-4 (T-cell leukemia lymphoid line) but did react with lymphoblastoid B-cells and one Burkitt line [16]. In another study employing two purified, radiolabelled CA, one anti-I and one anti-i, B-cells bound more anti-I and anti-i than did T-cells [17].

The differences in the results of the above studies could be explained by incidental selection since only a few CA and a small number of panels of lymphocytes were used. It was obvious, however, that some CA react preferentially with PBL-B and others with PBL-T. Since CA interact with a group of glycoproteins and glycolipids responsible for antigenic specificity [5, 18, 19], it seems possible that these antigens are different on B- and T-cells. Indeed, the basic glycoprotein pattern on T-PBL was found to be quite different from that on B-PBL [20, 21]. Therefore, it was of interest to test a large number of CA of various specificities against different subsets of lymphocytes. If found, preferential activity of CA would shed light on heterogeneity of the antibody and/or on the differences in quality and/or quantity of antigenic determinants on various cells.

Our results showed that anti-i often kill preferentially PBL-T and tonsillary T-cells, thymocytes and T-CLL cells. Thus, anti-i CA seem to recognize some antigenic determinants on these cells which are either not present or have lesser density on B-cells. Another possibility, that some antigenic determinants which react with anti-i are more exposed on T- or more hidden (cryptic) on B-cells, cannot be ruled out. Unfortunately, treatment of B- and T-cells with ficin and papain resulted in a substantial death of the cells, and thus was not suitable for the analysis of preferential cytotoxicity (W. Pruzanski, unpublished). The importance of the cellular membrane in interaction with CA was evident when a large group of B-CLL was tested. In keeping with our observation that anti-i are preferentially directed against T-cells, these CA killed very few B-CLL cells. On the other hand, the kill of B-CLL cells by anti-I was much higher than that of normal PBL or of PBL-B cells. This observation confirms our own preliminary results and those of others [21, 22]. CLL cells have less i antigen [14, 22], different surface glycoproteins as compared to normal PBL-B and PBL-T cells [23] and low density or qualitative differences in the surface receptors to complement [24]. In similar to B-CLL, preference in kill by anti-I CA was noted in 2 of 6 kinds of monkey lymphocytes. The significance of this observation cannot be assessed without further functional characterization of these cells.

Tonsillary lymphocytes were usually more susceptible than PBL to CA. This observation adds to the list of differences between these two types of cell. Different proportions of T- and B-cells and variable responses to antigenic stimulation were observed when tonsillary lymphocytes and PBL were compared [25].

The fact that CA with κ light chains had preferential reactivity against B-cells, whereas CA with λ light chains reacted more with T-cells, is of considerable interest. CA with λ chains have more often been found in malignant lymphomatous diseases and are usually directed against i antigen [8]. Thus, biologically they are different from the more common variety of CA with κ chains.

Since one of the explanations of the differences in interaction of CA and lymphocytes could be the different utilization of complement, we estimated residual activity of complement in the supernatants after cytotoxicity assays. Indeed, some variations in complement utilization were observed, but a threefold increase in complement added to the cells which were less efficiently killed by CA did not change the preferential kill.

The fact that anti-i behave differently from anti-I CA may have a biological significance. It may mean that in addition to antigens common to all lymphocytes and recognized by all CA, anti-I and unrelated to I/i CA recognize certain specific antigenic determinants on B-lymphocytes, whereas anti-i CA recognize different antigenic determinants on T-cells. Alternatively, the density and/or accessibility of various antigenic structures in the membrane may be different in B- and T-lymphocytes. On the other hand, quite a marked heterogeneity was evident and may be interpreted as an expression of individual specificities of CA. Heterogeneity and differences in expression of I and i antigens on the RBC have been well recognized [5, 18, 19]. The requirement for multivalent attachment may also play a role in the reactivity of CA with various cells [26]. Structural requirements for anti-I and anti-i CA to recognize various domains within the membranous glycolipids or glycoproteins may vary [27–29]. Some receptors may be hidden deep in the cellular membrane [26, 30] and their nature has to be investigated to explain the differences in reactivity of CA with various cells.

*

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The Prevention of Rhesus Haemolytic Disease: the Present Position*

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It is about 12 years since anti-D was introduced and the four principal speakers are going to outline how they see the successes and the failures of the prophylaxis. In my ten-minute introductory talk I am going to deal with Rhesus deaths in England and Wales, concentrating mainly on the 35 neonatal deaths and 55 stillbirths (these were the final figures) which occurred in 1979 [4]. Then the four principal speakers will tell of their experiences outlined in their abstracts. To some extent we shall be telling the same story, with the theme of 'levelling out' being common to us all, but there will be different views about ante-natal anti-D, plasmapheresis and oral antigen [2]. After these four papers there will be an additional one by Professor Jörg Schneider, concerned with a special problem, namely immunization after abortion brought about by prostaglandin therapy.

First, the 1979 deaths. Figure 1 shows the neonatal death rate from Rhesus disease going back to 1951, and since 1977 the figures have been tending to level out. In that year there were 40 liveborn deaths and 70 stillbirths, in 1978 the figures were 26 and 67 and in 1979 35 and 55.

Why should be this so? The answers are not difficult and are detailed in Table 1, which gives the circumstances in which the mothers of the 35 neonatal deaths became immunized. It is interesting that category 1 has changed very little over the last four years, though it is bound to do so in the long run. Category 2 could be reduced by ante-natal anti-D but the numbers are small, and our criteria for being immunized during pregnancy are very strict. Category 3 is unlikely to be altered and 4 may remain much the same, but category 5 could certainly be improved.

The 55 stillbirths in 1979 have a similar type of breakdown.

What has improved in England and Wales is the death certification. There are far fewer inaccurate ones in 1979 than there were in the previous two years and we think this is due to our earlier papers [1, 3].

My personal view about ante-natal anti-D is that it should be restricted to certain classes and that it is much more important to improve category 5. Plasmapheresis I am not happy about – there has been no proper control series – and as

* Introductory lecture presented at the International Congress ISH–ISBT, Budapest, August, 1982.

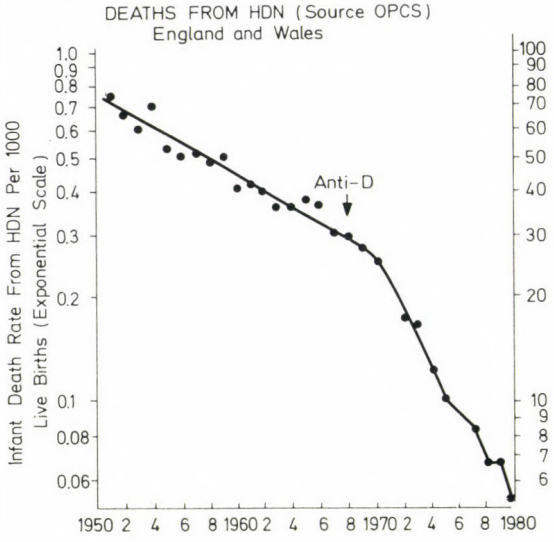

Fig. 1. Accelerated decline in deaths from Rhesus haemolytic disease since the introduction of prophylactic anti-D

Table 1

Neonatal Rhesus deaths, England and Wales, 1979

Category	Circumstances in which mothers became immunized	No. of patients
1	Before anti-D introduced	17
2	Immunized *during* 1st pregnancy	4
3	Immunized by non-D antigens, e.g. $\bar{c}$ or E or both	3
4	Failures of anti-D	2
5	No record of anti-D being given	9
	Total	35

regards oral antigen in my view the basic work has not yet been undertaken. I expect these points will come up among the other speakers and if not they can be dealt with in the discussions following the main papers.

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Prevention of Rhesus Haemolytic Disease in Finland*

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I was asked to present a summary of the present position of the prevention of Rhesus haemolytic disease in Finland. The data are based on a continuing follow-up of all Rh-negative women receiving anti-D immunoglobulin and a registration of all pregnant Rh-negative women, including those who already had an Rh-positive pregnancy before anti-D immunoglobulin had become available.

Anti-D immunoglobulin has been used in Finland since 1969 and we have indeed seen a striking decline in the number of immunized women although it has remained almost static for the last two years (Fig. 1). The reasons for this failure to reduce the incidence of Rhesus haemolytic disease further have already been discussed here today. They are failure to administer anti-D immunoglobulin; failure of protection, and ante-partum immunization.

All immunized women identified during the programme are analyzed to ascertain the circumstances in which they had become immunized. I will start with the reasons for immunization which are not associated with failure of protection.

This category consists of women who had already had a sensitizing Rh-positive pregnancy before anti-D was available. Their number has fallen dramatically. During the first 10 years of the programme their number was 556 and during the last three years only 15 (Table 1).

Immunization due to failure to administer anti-D immunoglobulin when indicated is not a major problem, but it is a continuing one (Table 2). Although the

Table 1

Number of Rh-negative women sensitized before anti-D was available

Origin of sensitization	No. of immunized women	
	1969–78	1979–81
Rh-positive pregnancy before anti-D available	556	15

* Lecture held at the International Congress ISH–ISBT, Budapest, August, 1982.

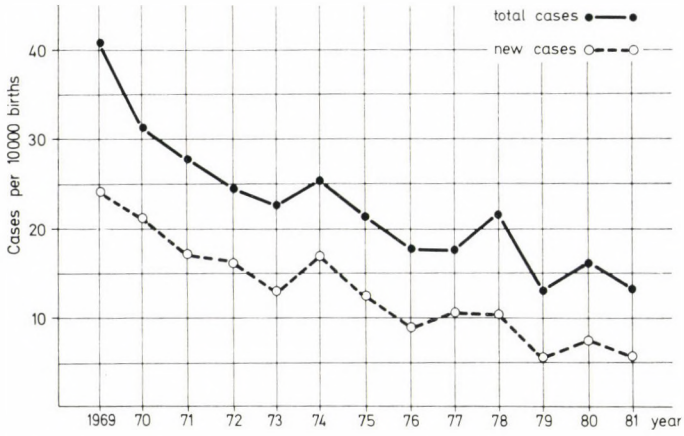

Fig. 1. Frequency of Rh immunized women giving birth in Finland 1969–1981

Table 2

Non-administration of anti-D immunoglobulin, 1969–81

No. of women not given anti-D when indicated	No. of women immunized
259	86

total number of women immunized due to oversight is fairly small, it is not negligible in association with deaths from Rhesus haemolytic disease in subsequent pregnancies, which will be discussed later.

Immunization by blood transfusion is no longer a topical problem, but during the first 10 years of the programme 27 women were still found to be immunized due to mismatching and in some few cases it was the cause of stillbirth.

Immunization during a first pregnancy, which by definition cannot be prevented by post-partum prophylaxis, is the most important cause of immunization. Since the introduction of Rh prophylaxis in 1969 we have encountered 160 primigravidae with antibodies in their sera during a first pregnancy, which gives an immunization rate of 0.52 per cent (Table 3).

Table 3

Rh immunization during the first pregnancy, 1969–81

No. of primigravidae	Immunized No.	Immunized %
30 915	160	0.52

I will now proceed to the failure of protection. Post-delivery failures occur, but their incidence is very low – less than 0.1 per cent. Failure of protection detected in a subsequent pregnancy in a woman who had received anti-D immunoglobulin after an Rh-positive pregnancy is of more importance (Table 4). In 11 cases Rh antibodies were detected before the 28th week of gestation and eight infants required exchange transfusions. Fifty-eight women developed antibodies late in pregnancy and 23 infants required treatment. Rh antibody was first detected at delivery in the last 48 women and only one infant in this group was exchange transfused. The incidence of antibody development and even the time of the initial appearance of antibodies during a subsequent Rh-positive pregnancy is very similar to that appearing in women during their first pregnancy. It is evident that in both these groups immunization occurred during that pregnancy and therefore represents a primary immunization and not a booster effect.

In Table 5 are summarized all the new cases of immunization during the programme according to the original causes of immunization. The first two cate-

Table 4

Results of the test for Rh antibodies during subsequent pregnancies, 1969–81

No. of women given anti-D more than once	Immunized No.	Immunized, %
17 005	117	0.69

gories are of no importance today, but the other four represent realities which we must accept in anti-D prophylaxis after delivery. During the prophylaxis programme, the incidence of Rhesus haemolytic disease in Finland has declined from 3.3 to 0.9 per 1000 live births and the mortality rate has fallen from 0.26 to 0.02. Only one infant was lost last year and in this case the mother had been immunized before 1969.

An analysis of all stillbirths and neonatal deaths due to Rhesus haemolytic disease between 1969 and 1981 showed that most of the women with perinatal deaths were immunized before 1969. Eighty-two perinatal deaths belong to this category (Table 6). In six cases the underlying cause was a failure to administer anti-D when indicated. Three stillbirths were a consequence of blood transfusion in earlier years. The origin of the remaining eight neonatal deaths was ante-partum immunization. There was no mortality due to failure of protection with anti-D immunoglobulin.

Unfortunately, post-partum administration of anti-D immunoglobulin has no effect on sensitized primigravidae, neither has it any effect in modifying the severity of Rhesus haemolytic disease in subsequent Rh-positive pregnancies.

The perinatal mortality rate secondary to Rhesus haemolytic disease is low in Finland – much lower than in many other countries with post-partum prophy-

Table 5

Analysis of all identified new cases of immunization, 1969–81

Origin of immunization	Immunized women, No.
Rh-positive pregnancy before anti-D available	571
Mismatched blood transfusion	27
Non-administration of anti-D immunoglobulin	86
Ante-partum immunization	160
Post-delivery failure	55
Failure in subsequent pregnancies	117
Total new cases	1016

Table 6

Perinatal deaths due to Rhesus haemolytic disease, 1969–81

Origin of immunization	No. of stillbirths and neonatal deaths	
	1969–78	1979–81
Antibodies detected before anti-D available	78	4
Non-administration of anti-D when indicated	5	1
Mismatched blood transfusion	2	1
Ante-partum immunization	7	1
Total No. of Rhesus deaths	92	7

laxis. Whatever the reason might be, the low birth rate, small families, a liberal abortion law and last but not least a good educational standard, the good results today do not justify the initiation of an antenatal anti-D programme.

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Nonenzymatic Glycosylation of Normal and Abnormal Haemoglobin Alpha Chains*

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The total amount of haemoglobin-bound glucose is determined by the chemical method for quantification of glycosylated haemoglobin, used in the control of diabetic patients. About 40 per cent of the measured amount of glucose is bound by the alpha chains of haemoglobin. The effect of substitution of alpha chains on the glycosylation of the abnormal and normal chains was investigated in a J Buda and G Pest heterozygote. Intact red blood cells were incubated in the presence of labelled glucose of 0.20 MBq/μmol specific activity and 35 mM concentration, and nonenzymatic glucose incorporation into normal and abnormal alpha chains was followed. By the help of fingerprinting and autoradiography of separated chains the distribution of the incorporated radioactivity was investigated. Based on the results we may conclude that (i) 65 per cent of incorporated glucose is bound to five peptides; (ii) the abnormal chain does not affect the glycosylation of the normal alpha chain; (iii) the substitution of a potent glucose binding site (α 61) does not affect the glycosylation of the other residues in the abnormal chain; (iv) the presence of the abnormal chain does not interfere with the diagnostic use of the chemical method for quantification of glycosylated haemoglobin.

Key words: abnormal haemoglobin α-chain, diabetes, nonenzymatic glycosylation

Introduction

The human haemoglobin α-chain is the product of four genes [1]. The sequence studies of the two abnormal and the normal α-chains of a double heterozygote for two distinct α-chain abnormalities (Hb J Buda: α 61 Lys–Asn, Hb G Pest: α 74 Asp–Asn) have shown that apart of these two point mutations the products of the two α-chain loci have a completely identical primary structure [2, 3]. No heterogeneity has been detected either in the structure of mRNA or in that of the coding DNA of the human haemoglobin α-chain [4, 5].

Our previous results have revealed a slight difference in immunochemical behaviour of the normal haemoglobin α-chain of the double heterozygote (A^x) as compared with the α-chain of normal donors. Quantitative complement fixation assays have shown a difference between the two chains at the limit of significance [6].

* Lecture held at the International Congress ISH–ISBT, Budapest, August, 1982.

Since this result cannot be explained by structural differences we have surmised a difference in the postsynthetic glycosylation of the chains, which may alter the immunological activity of proteins of the same primary structure. The glycosylation can mask antigenic sites or can form new ones through the keto-group of the sugar, as it was shown with Hb A_{1c} [7].

To test our hypothesis, we have followed the incorporation of radioactivity from D-(^{14}C)-glucose into the α-chains in intact red blood cells of the double heterozygote for α Buda and α Pest, and of a normal control donor.

Materials and Methods

Blood samples of the double heterozygote patient and that of the normal control donor were incubated in sterile standard ACD anticoagulant solution at 37 °C in the presence of 35 mM uniformly labelled D-(^{14}C)-glucose of 20 MBq/μmol specific activity.

D-(^{14}C)-glucose was the product of IIPPRI (Prague).

After one week of incubation the cells were haemolyzed, the haemoglobin was converted to globin and the chains were separated. The haemolysates were prepared according to Drabkin [8], followed by the reduction of glucose onto the haemoglobin with $NaBH_4$ as suggested by Bunn et al. [9]. Haemoglobin was converted to globin by hydrochloric acid–acetone precipitation and the chains were separated on carboxymethyl cellulose (Whatman CM 52) in the presence of 8 M urea, by the method of Clegg et al. [10].

Fingerprints were made after tryptic digestion on cellulose thin layer (Merck 5716), by using pyridine–acetic acid buffer pH 6.4 for electrophoresis, and pyridine–butanol–acetic acid–water (40 : 60 : 12 : 48) for chromatography. About 1 mg of hydrolysate was loaded onto the cellulose thin layer. The fingerprints were developed by ninhydrin staining.

Autoradiographs were prepared by direct exposure on X-film (Agfa) for three weeks. Radioactive spots were scraped off from the plates, and the peptides were eluted with diluted HCl. Specific radioactivity (cpm/E_{220}) was determined after filtration through a 3 μm Millipore filter; radioactivity measurement was performed as described by McKenzie and Gholson [11].

Results and Discussion

During the one week incubation 0.020 mol glucose/monomer and 0.014 mol glucose/monomer were bound by the normal α-chain of the double heterozygote and by the control one respectively, as it was calculated from the radioactivity attached to the separated chains.

To find out if there was any difference in the distribution of the bound glucose on the different α-chains, we have compared the fingerprints and their autoradiographs made from the tryptic digest of the chains.

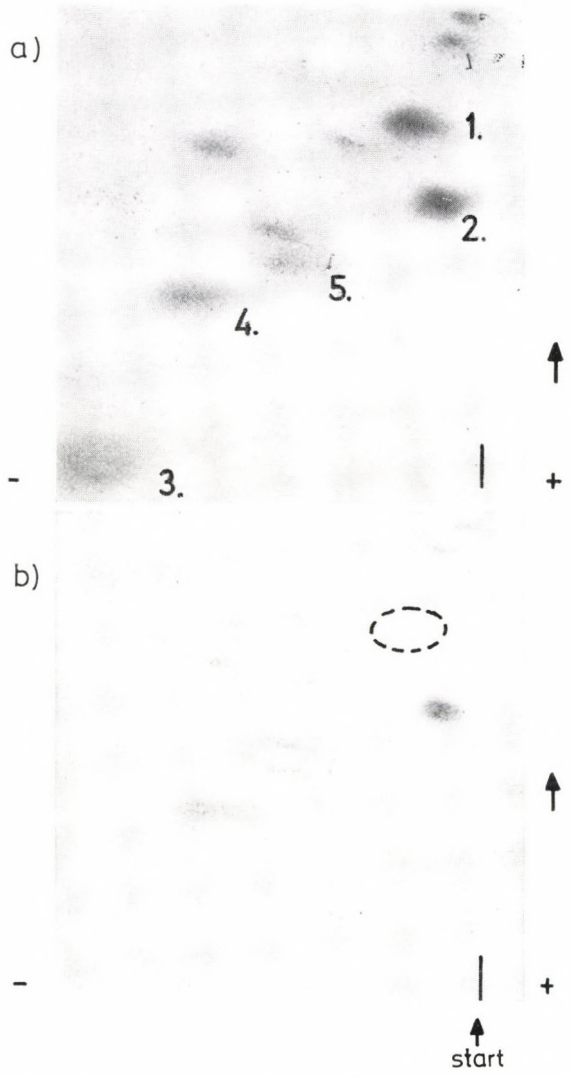

Fig. 1. *a.* Autoradiograph of the fingerprint of human haemoglobin α-chain incubated with D-(^{14}C)-glucose in intact red cells. *b.* Autoradiograph of the fingerprint of αJ Buda chain. Incubation, and preparation of the fingerprints and autoradiographs are detailed in the text. The number of spots corresponds to the peptides listed in Table 1

In order to prevent the loss of weakly labelled peptides a three-week exposure time was used to prepare the autoradiographs. Figure 1*a* shows a representative autographic pattern. The α-chain of the normal control, Hb A^x, and of Hb G Pest gave a similar picture. The autoradiograph of α J Buda in which the strongest

glucose acceptor residue (Lys 61) is substituted by Asn, differed from those of the other chains. As a consequence of the substitution no radioactivity could be detected in the Tp VIII–IX peptide of the α J Buda chain (Fig. 1*b*, dotted circle).

In contrast to Bunn et al. [9] we have found numerous radioactive peptides. The difference must have resulted from the long exposure time of the autoradiographs or from the different incubation of haemoglobin with glucose.

The various metabolites of glycolysis display different affinities toward the amino groups of haemoglobin [9, 12–14]. In a living system, i.e. where the glycolytic enzymes are active, the proteins incubated with labelled D-glucose react with the mixture of radioactive metabolites. The distribution of incorporated radio-

Table 1
Specific radioactivity of tryptic peptides of haemoglobin α-chains incubated with D-(^{14}C)-glucose in intact red cells

	α^N		α^{A^X}		αG Pest		αJ Buda	
	a	b	a	b	a	b	a	b
1. Tp VIII–IX	704	1.00	1006	1.00	931	1.00	—	—
2. Tp III–IV	386	0.55	564	0.56	516	0.55	496	(0.55)
3. Tp VII–VIII	65	0.09	94	0.09	83	0.09	—	—
4. Tp II–III	325	0.46	442	0.44	372	0.40	376	(0.42)
5. Tp I–II	140	0.20	202	0.20	180	0.19	176	(0.20)
total chain	2456	—	3587	—	3206	—	2127	—

α^N = α-chain of normal control; α^{A^X} = normal α-chain of double heterozygote
a = specific activity: (cpm: E_{220}) × number of peptide bonds
b = specific activity in the unit of the Tp VIII–IX peptide

activity will depend on the different affinities and concentrations of these metabolites. This might explain the difference between the data in the literature and our own findings.

To compare quantitatively the glycosylation of chains, the specific radioactivity of the labelled peptides was determined (Table 1). Of the bound radioactivity 65 per cent was in the five strongly labelled peptides. The ratio of the specific activities of these peptides was the same in the α-chains. It follows that the slight quantitative difference in glucose binding of the A^x α-chain and of the normal control chain (α^N) was not combined with a change of the distribution of glucose on these chains.

The slight quantitative difference in glycosylation of the two chains may have been caused by small differences in the concentration of the metabolites as of 2,3-DPG being present in the blood, as it was shown by Haney and Bunn [12]. Such a concentration difference may be the result of the individual glucose metabolism in the normal population, too.

As glycosylation may affect the immunological properties of haemoglobin it has been concluded that the small immunochemical difference found earlier [6]

between the normal α-chain of the double heterozygote for α J Buda and α G Pest, and the α-chain of a normal control originates most probably from the slight quantitative difference in glycosylation of these chains, resulting in an alteration in the number of the same antigen.

Fitzgerald and Cauchi [15] have found that the substitution of Hb S (β 6 Glu–Val) does not alter the glycosylation of the N terminal valine of the β_S chain. No data have so far been available concerning the effect of other substitutions. Our studies have shown a lack of glycosylation as a result of the mutation at the strongest glucose receptor site of the α-chain (61 Lys–Asn), therefore the total amount of the glucose bound by the J Buda α-chain is reduced. This change in the glycosylation of Hb J Buda did not change the distribution of glucose on other residues of the same chain or the reaction of the two other α-chains (α G Pest, α A^x) being present in the blood of the double heterozygote (Table 1). From the same data we may conclude that substitution of the α G Pest chain had no effect on the glycosylation.

Taking into account that abnormal α-chains are present generally in 25 per cent, and that the α-chains bind about the 40 per cent of the haemoglobin-bound glucose, the data of Table 1 allow to calculate, that the presence of an abnormal α-chain cannot reduce the total amount of bound glucose by more than 3 or 4 per cent.

All this may have some importance in the diagnostics of diabetes. The presence of an abnormal haemoglobin can drastically change the result of the chromatographic method for fast-moving haemoglobins. As regards the colorimetric method, determining the total bound glucose the change in the amount of glucose caused by the presence of the abnormal α-chain is comparable with the error of the method. That is why in the case of haemoglobinopathies we recommend to use the colorimetric method for the determination of glycosylated haemoglobin for the diagnosis or control of diabetes.

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Acknowledgement to Reviewers

The Editors of *Haematologia* acknowledge the valuable help of the guest referees who have reviewed the papers submitted for publication in *Haematologia* during 1982.

Joint Meeting of
The 19th Congress of the
International Society of Haematology

and

The 17th Congress of the
International Society of Blood Transfusion

Budapest, August 1–7, 1982

How (not) to Organize a Haematology Congress?

We are not quite sure after closing the Budapest Congress of ISH–ISBT, 1982, what the best way to organize such a huge international meeting is, but we can certainly provide you with some information about the basic pitfalls of this work. Here are some tips that future organizers may find helpful:

1. Never hold a Congress in a large round-shaped building. If this is unavoidable, however, do not forget to install numerous 'lost and found' offices to take care of the participants. Proper medical staff is also needed to treat claustrophoby, nausea and vertigo of desperate scientists who spend their time rushing round and round trying to find their way.

Isn't the Sporthall an ideal place for a congress?!

Drawings by I. Szórády; Photos B. Sarkadi

2. Never ever plan more than 125 symposia, 20 plenary lectures, 400 poster presentations and 25 discussions to start simultaneously and on the same location. If this still happens, make sure to organize a lovely, sunny weather to keep congress participants away from the programmes.

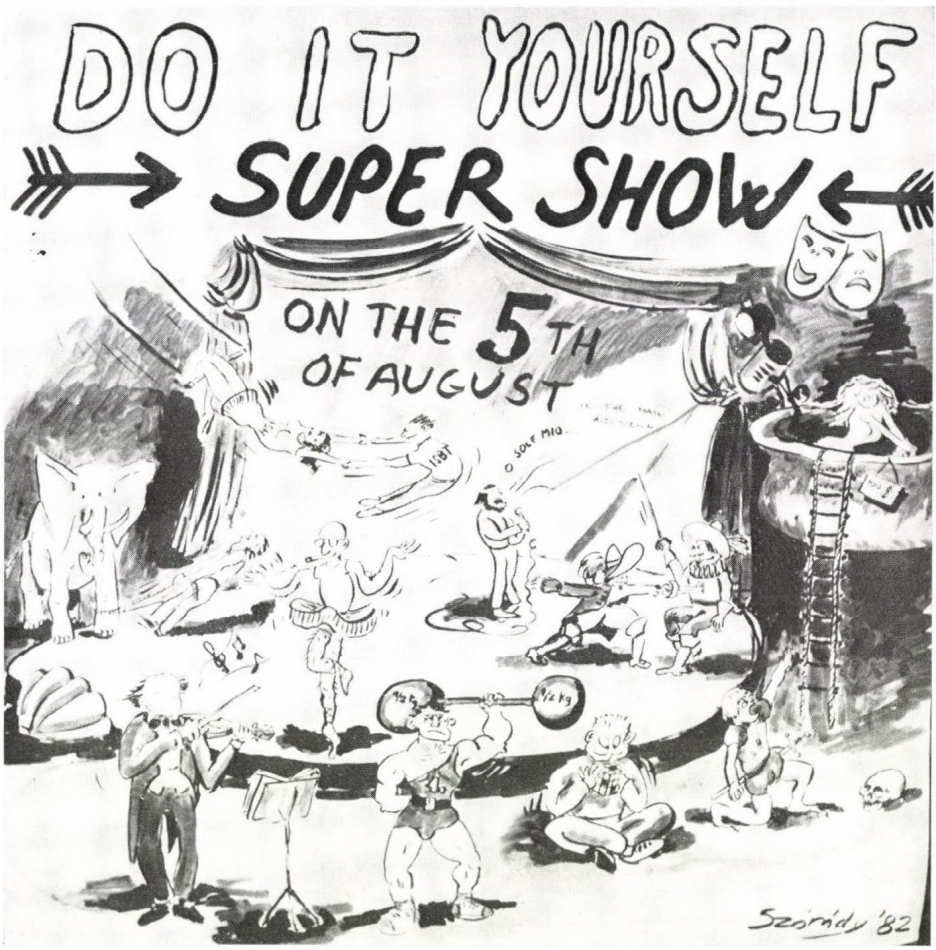
3. Never put down into the Programme booklet the exact starting time and location for any of the scientific or social programmes, as the chance is very small that they will start at the given time and location. Business meetings of various working parties and scientific councils should not be planned in advance as a lunch-meeting often turns into a dinner-conference.

4. If you organize an 'Ask the expert' party, do not provide large quantities of cheese and red wine for the participants as both experts and non-experts will

"Ask the experts" while participating at the wine and cheese party

begin to converse more and more casually, but less and less about science, as the level of alcohol in their blood increases. Rather, use such an occasion to make up a 'Do it yourself' show, as good red wine brings hidden talents into motion and creates a fantastic atmosphere for joy and laughter.

These few suggestions may help the organizers of future congresses in their work, but we still believe that in August we had a useful and memorable congress in Budapest. In the present volume of *Haematologia* you find the scientific programme of the Congress, some photographs taken at the various congress-events, the reproduction of caricatures that attempted to inform the audience, and the texts of a song and a poem written and presented beautifully in the 'Do it yourself' show



by Professor Humphrey Kay. (The song was accompanied on the piano by Professor George Klein).

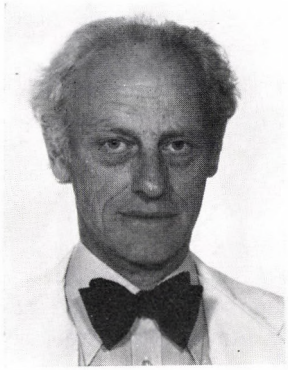
We hope that our readers will enjoy the short remembrance to the Budapest Congress and fruitfully use the results presented in the articles in their future work in laboratories and hospitals.

They really went for blood ... The soccer game between Haematologists and Transfusiologists as our cartoonist saw it

Stem–Cells

by

HUMPHREY E.M. KAY



Dr. H. E. M. Kay

Two stem-cells, call them Abraham and Moses,
In their first-class compartment, filled with roses,
Feeding on royal jelly one supposes.
Brought by their stromal resident supporters,
View with disdain the overcrowded quarters
Where dwell their teeming progeny of daughters
And ask the question, fateful for each stem,
'When I should next divide' says A to M,
'Will both of me become like two of them?'
'These things' said Moses after a solemn pause
'Must be determined by almighty cause
and not by chance. We must define our laws;
They should be codified and set in stone
As you, O Abraham, I'm sure will own,
Being the scion and founder of our clone'.
Abraham nodded, winked to himself and said
'It may be so' then, seeing his daughters sped
From out their home and many others dead,
Began to feed, and Moses too, until replete
Roundly distended, mitotic and discrete,
Each for a moment left his stromal seat
And swiftly consummating that mitotic burst,
Knowing that two of them must be dispersed,
Two Abrahams were fractionally first
And pushed both Moses through the compartment door,
Saying as they did so 'What a tedious bore!
Why should they think we need to have a law?'

A Song for Haematologists

(To the tune of the Hippopotamus song by Flanders and Swann)

HUMPHREY E. M. KAY

An old haematologist was standing one day
In an old haematology lab.
He thought what a lot I've missed of the life that is gay
Through pursuite that are dreary and drab.
I've sat counting cells by the billion or more
Till the corpuscles swim through my gaze,
And yet though my story be tedious and gory
I'll sing to the end of my days:

Blood, blood, glorious blood,
Thicker than water and nicer than mud,
It's sweeter, it's neater, more pure and completer,
We'll live by the litre on glorious blood.

The old haematologist whose story we've told
Looked back to the days of his youth
To his work of a quality quite uncontrolled
But when no one would question his truth,
When clotting was simple, and blood groups were few,
Only God knew what lymphocytes did,
You could say 'all that's new is in Dacie and Lewis'
And buy one for only a quid.

Blood, blood, glorious blood,
Thicker than water and nicer than mud,
It's sweeter, it's neater, more pure and completer,
We'll live by the litre on glorious blood.

The old haematologist looked through the notes
Of the case for the next CPC,
There was no cause of death though the studies they'd done
Were as thorough as thorough could be,
Every test had been carefully repeated and checked
In their efforts to keep him alive,
Each sample spectacular, more drastic than Dracula,
The total blood volume times five.

Blood, blood, glorious blood,
Thicker than water and nicer than mud,
It's sweeter, it's neater, more pure and completer,
We'll live by the litre on glorious blood.

So remember, whoever you happen to be,
The moral that comes from all that:
Though your blood may be blue as the sunniest sea
Or as red as a cardinal's hat,
If it's warm and it's wet and it's still gurgling round,
It's far better than any champagne
So never mind whether a dearth or a plethora
Let's all sing together again:

Blood, blood, glorious blood,
Thicker than water and nicer than mud,
It's sweeter, it's neater, more pure and completer,
We'll live by the litre on glorious blood.

British Society of Haematology, Oxford, March 1982.

Dr. Kay as choir leader

The opening ceremony

The poster sessions

Pictures from the exhibition

The 'wine and cheese' party

Dr. T. M. Dexter and Dr. E. C. Gordon-Smith, the most outstanding haematosingers

The Haematologists are coming ... But where's B.P.L. ("Paddy") Moore looking!? Incidentally, the final score: Haematologists 9, Transfusiologists 3

A Short Review of the Programme of the International Congress ISH–ISBT, Budapest 1982

Plenary Lectures

Frei, III. E. (USA): The present state of the art in the treatment of acute leukemia

Freireich, E. J. (USA): Factors predicting for response and survival in adult acute leukemia

Galton, D. A. G., Goldman, J. M., Catovsky, D. (UK): Treatment of chronic granulocytic leukaemia

Koscielak, J. (Poland): Modern concepts of structure and function of blood cell membrane glycosphingolipids

Goldstein, J. (USA): Enzymatic conversion of red cell ABO groups for transfusion

Bergelson, L. D., Dyatlovitskaya, E. V. (USSR): Shedding of glycolipids from leukemic lymphocytes and other tumor cells

Curling, J. M. (Sweden): Current practice and future possibilities in plasma protein fractionation

Brummelhuis, H. G. J. (The Netherlands): Present state and future of the preparation of plasma components

Weatherall, D. J. (UK): Molecular basis of the heterogeneity of haemoglobin disorders. **Stratton Award Lecture**

Bernard, A., Boumsell, L. (France): An overview of the actually definable human leukocyte differentiation antigens: a prelude to the first international workshop on human leukocyte differentiation antigens (Report)

Rapoport, S. M. (GDR): Regulation of red cell metabolism

Finch, C. A., Huebers, H. A. (USA): Iron deficiency and iron overload

Engelfriet, C. P., Beckers, Th. A. P., van 't Veer, M. B., Ouvehand, W. H., Kr. von dem Borne, A. E. G. (The Netherlands): Recent advances in the pathophysiology and diagnostics of immune haemolytic anaemias

Anstee, D. J. (UK): Monoclonal antibodies – tools for the characterization of blood group antigens

Koskimies, S. (Finland): Specific antibody production by EBV-transformed human lymphocytes

Waldenström, J. (Sweden): Monoclonal immunoglobulins and solid tumors

Friedman, R. M. (USA): The structure, functions and use of interferons

Nevanlinna, H. R. (Finland): The production of leukocyte interferons. **Jean Juillard Award Lecture**

Thomas, E. D. (USA): Bone marrow transplantation. **Stratton Award Lecture**
van Rood, J. J. (The Netherlands): Blood transfusion and transplantation
Vyas, G. N. (USA): Molecular immunobiology of the hepatitis B group of viruses
Barker, L. F. (USA): Status and future prospects for hepatitis control
Ratnoff, O. D., Gordon, E. M., Douglas, J. G. (USA): Some recent advances in surface-mediated reactions
Előd i, Susan (Hungary): Surface oriented enzyme reactions in blood coagulation
Lorand, L. (USA): Physiological controls in the clotting of fibrinogen. Relevant molecular diseases
Borsos, T., Circolo, A. (USA): Effect of cell surface hapten density and immunoglobulin class on complement lysis and agglutination of cells
Dierich, M. P. (FRG): Physiological and pathological effects of activated complements
Orkin, S. H., Kazazian, H. H. Jr., Goff, S. C., Antonarakis, S., Sextion, J., Boehm, C., Michelson, A., Felber, B., Hamer, D. (USA): The molecular genetics of human globin genes and thalassaemias
Kan, Y. W. (USA): Gene transfer
Luzzatto, L., Pereico, G. M., Toniolo, D., D'Urso, M., Nobile, C., Martini, G. (UK): Molecular cloning of sequences of human glucose-6-phosphate dehydrogenase cDNA
Beutler, E. (USA): Advances in enzyme replacement therapy
Mitchison, N. A. (UK): Membrane receptors and cell cooperation
Klein, G. (Sweden): Tumour antigens and neoplastic transformation
Schlossman, S. F. (USA): Human lymphocyte differentiation: characterization by cell surface markers
Sachs, L. (Israel): Self renewal and normal differentiation of leukemic stem cells
Neuwirt, J. (Czechoslovakia): Stem cells in circulation
Chertkov, J. L., Gurevitch, O. A. (USSR): Hemopoietic microenvironment and hemopoietic stroma precursors
Fliedner, T. M. (FRG): Use of blood derived stem cells for transplantation
Kelemen, E. (Hungary): Antenatal haemopoiesis in man: its tale for a haematologist
Huestis, D. W. (USA): Current status of granulocyte (PMN) transfusion
Gregoriadis, G. (UK): Liposomes in haematology: tools for research and clinical application
Masouredis, S. P. (USA): Some mechanisms involved in immunomediated hemagglutination
Levy, M. (Israel): The international study of agranulocytosis and aplastic anaemia (Report)
Baumelou, E., Najean, Y. (France): Retrospective analysis of etiology in aplastic anaemia from a study of 498 cases (Report)

Symposia

Advancement in diagnostics and treatment of polycythemia
Chairman: Erslev, A. J. (USA); Co-chairman: Gráf, F. (Hungary)

Cold agglutinins, antigens and antibodies
Chairman: Pruzanski, W. (Canada); Co-chairman: Hughes-Jones, N. C. (UK)

Strategic planning for blood service management
Chairman: Moore, B. P. L. (Canada); Co-chairman: Brodheim, E. (USA)

Rh haemolytic diseases
Chairman: Clarke, C. A. (UK); Co-chairmen: Mollison, P. (UK), Bowman, J. M. (Canada)

Platelet heterogeneity
Chairman: Penington, D. G. (Australia); Co-chairman: Cserháti, I. (Hungary)

Geographical haematology
Chairman: Bernard, J. (France); Co-chairmen: Ruffie, J. (France), Tokarev, Yu. N. (USSR)

Hereditary disorders of erythrocytes associated with enzyme deficiencies
Chairman: Jaffé, E. R. (USA); Co-chairman: Jacobasch, G. (GDR)

Biochemistry, physiology and genetics of factor VIII
Chairman: Bloom, A. L. (UK); Co-chairman: Soulier, J. P. (France)

Biological action and clinical significance of antithrombin III
Chairman: Abildgaard, U. (Norway); Co-chairman: Sas, G. (Hungary)

Plasma cell neoplasms
Chairman: Bergsagel, D. E. (Canada); Waldenström, J. G. (Sweden)

Selection of patients for early transplantation in acute leukaemia
Chairman: Kay, H. E. M. (UK); Co-chairman: Feinstein, F. E. (USSR)

Pharmacologic modulation of erythropoiesis
Chairman: Fisher, J. W. (USA); Co-chairman: Fehér, I. (Hungary)

Bone marrow biopsy
Chairman: Rozman, C. (Spain); Co-chairman: Frisch, Bertha (Israel)

Identification of blood group and HLA antigens from blood spots and tissues
Chairman: Dodd, Barbara (UK); Co-chairman: André, A. (Belgium)

Anemia of the astronautes
Chairman: Crosby, W. (USA); Co-chairman: Hollán, Susan R. (Hungary)

Blood bank component production: Blood bag systems
Chairman: Cash, J. D. (UK); Co-chairman: Harsányi, Veronika (Hungary)

Liability and reality in haemotherapy
Chairman: André, A. (Belgium); Co-chairman: David-West, A. (Nigeria)

Chemistry and structure of red cell surface antigens
Chairman: Salmon, Ch. (France); Co-chairman: Lisowska, E. (Poland)

Intravenous immunoglobulin treatment and prophylaxis
Chairman: Hässig, A. (Switzerland); Co-chairman: Abe, T. (Japan)

Platelet activation reactions
Chairman: Caen, J. P. (France); Co-chairman: Muszbek, L. (Hungary)

Characteristics and indications of factor VIII concentrates
Chairman: Soulier, J. P. (France); Co-chairman: Mannucci, P. (Italy)

Design of haemophilia care programs with limited resources
Chairman: Barker, L. F. (USA); Co-chairman: Britten, A. F. H. (USA)

Present and future research challenges in hemophilia treatment
Chairman: Aledort, L. M. (USA); Co-chairman: Hilgartner, Margaret W. (USA)

Plasma fibronectin: biological and clinical significance
Chairman: Lundsgaard-Hansen, P. (Switzerland); Co-chairman: Eriksen, H. O. (Denmark)

Progress in standardization in haematology
Chairmen: Lewis, S. M. (UK), Beutler, E. (USA); Co-chairman: Hollán, Susan R. (Hungary)

Screening methods for markers of hepatitis viruses
Chairman: Seidl, S. (FRG); Co-chairmen: Prince, A. (USA), Freisleben, E. (Denmark)

Cellular proliferation characteristics and prognosis in acute leukemia
Chairman: Mauer, A. M. (USA); Co-chairman: Stryckmans, P. (Belgium)

Activation of coagulation factors on the surface of platelets
Chairman: Walsh, P. N. (USA); Co-chairman: Előd, Susan (Hungary)

Early erythroid differentiation in human leukemias
Chairman: Andersson, L. C. (Finland); Co-chairman: Karhi, K. (Finland)

Environmental modification of hemoglobin structure and function
Chairman: Bunn, H. F. (USA); Co-chairman: Winterhalter, K. H. (Switzerland)

Advance and retreat in Hodgkin's disease
Chairman: Eckhardt, S. (Hungary); Co-chairman: Stobbe, H. (GDR)

Genetics of thalassaemia
Chairman: Luzzatto, L. (UK); Co-chairman: Fessas, Ph. (Greece)

Recent advances in paternity testing
Chairman: Mayr, W. R. (Austria); Co-chairman: van Loghem, Erna (The Netherlands)

Transferrin
Chairman: Aisen, P. (USA); Co-chairman: Egyed, A. (Hungary)

Iron overload
Chairmen: Hershko, C. (Israel), Wickramasinghe, S. N. (UK); Moderator: Modell, B. (UK)

Roundtable on red blood cell deformability
Chairman; Ross, D. W. (USA); Co-chairman: Szelényi, Judith (Hungary)

T cell growth factors and long-term cultures of T cell
Chairman: Bonnard, G. D. (USA); Co-chairman: Petrányi, G. Gy. (Hungary)

New antisickling agents
Chairman: Labie, Dominique (France); Co-chairman: Nagel, R. L. (USA)

Critical evaluation of erythropoietin assays
Chairman: van Assendelft, O. W. (USA); Co-chairman: Anger, G. (GDR)

Blood group antigens and antibodies in malignant diseases of haemopoietic or lymphoreticular tissues
Chairman: Bird, G. W. G. (UK); Co-chairman: Watkins, W. M. (UK)

Recent advances in diagnostics of non-Hodgkin's lymphomas
Chairman: Rák, K. (Hungary); Co-chairman: Boiron, M. (France)

Enzymology of blood coagulation
Chairman: Hemker, H. C. (The Netherlands); Co-chairman: Váradi, Katalin (Hungary)

Poster Sessions

1269 posters dealing with the actual topics were presented

Summing-up Lectures

Dexter, T. M. (UK): Stem cells
Herberman, R. B. (USA): Leukemia antigens and immunity in man
Kan, Y. W. (USA): Abnormal haemoglobins
Mann, K. G. (USA): Hemostasis
Sloviter, H. A. (USA): Artificial blood
Högman, C. F. (Sweden): New trends in blood component therapy
Meryman, H. T. (USA): New trends in cryopreservation
Steinbuch, M. (France): New plasma fractionation products

INDEX TO VOLUME 15

CONTENTS

Author Index

Subject Index

INSTRUCTIONS TO CONTRIBUTORS

HAEMATOLOGIA is designed for the publication of original papers, preliminary reports, and reviews which contribute to the advancement in all fields related to haematology and blood transfusion. Manuscripts should be submitted in correct English and are accepted on the condition that they have not been published or accepted for publication elsewhere. Case reports, technical notes, and Letters to the Editor are also welcomed and will be published if appropriate.

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24
H-1113 Budapest, Hungary

Three copies of the manuscript should be submitted. They should be typed double-spaced on one side of a good quality paper with proper margins. The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which the work has been carried out; (4) name and full postal address of the author to whom communications regarding the manuscript should be directed; (5) a short title not to exceed 40 characters (including space) to be used as a running head. The second page should contain an **abstract** of 50—100 words, summarizing the reasons for the study, the methods used, the results, and the major conclusions. This page should also contain 4—8 **keywords** placed in alphabetical order. Original papers should not exceed 15 printed pages including tables, figures, and references. Case reports should not be more than four, technical notes and Letters to the Editor not more than two printed pages in length. In the manuscripts the approximate location of tables and figures should be indicated in the margin. The manuscript of original papers should be divided into summary, introduction, materials and methods, results, discussion, acknowledgements and references. Review articles should also be appropriately divided. SI units should be used in the manuscript, except that, for the time being, litre (l) may be used as a unit of volume.

References: Only papers closely related to the author's work should be referred to. In the text citations should include the name of the authors and/or the reference number in parentheses. A list of references, numbered according to the order of their occurrence in the text, should be compiled at the end of the paper. It should be typewritten, double-spaced, on a separate sheet.

References to periodicals should be typed double-spaced and mention: (1) name(s) and initials of all the authors; (2) title of the paper; (3) international abbreviation of the periodical; (4) volume; (5) first page number; (6) year of publication in parentheses. Thus: 4. Liebermann, J. E., Gordon-Smith, E. C.: Red cell pyrimidine 5'-nucleotidase and glutathione in myeloproliferative and lymphoproliferative disorders. *Brit. J. Haemat. 44*, 425 (1980).

References to books should include: (1) author(s)' name(s); (2) title; (3) editor(s); (4) publisher; (5) place and year of publication. Thus: 5. Guyton A. C., Taylor, A. E., Granger, H. J.: *Circulatory Physiology. II. Dynamics and Control of Body Fluids.* Saunders, Philadelphia 1975. Or: 6. Rappaport, J. M.: Granulocyte transfusion. In: *Haematology of Infancy and Childhood.* D. G. Nathan, F. A. Oski (eds). Saunders, Philadelphia 1974, p. 875.

Illustrations must be cited and numbered in the order they appear in the text. All line drawings should be submitted as clear, glossy, black and white originals or good quality photographs. The author's name and the title of the paper together with the serial number of the figure should be written on the back of each print. Legends for illustrations should be typewritten, double-spaced, on a separate sheet, and included at the end of the manuscript. Tables, each bearing a title, should be self-explanatory and numbered consecutively.

Authors will receive one set of proofs which must be corrected and returned to the Editor-in-Chief *within three days of receipt*. Major alterations of the text cannot be accepted.

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Contents

Index: 26861