VOLUME 22 • NUMBER 1 • 1989

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

///VSP///

UTRECHT, THE NETHERLANDS,
TOKYO, JAPAN

AKADÉMIAI KIADÓ, BUDAPEST

HAEMATOLOGIA

Haematologia is an international journal publishing original papers on all clinical and theoretical aspects of haematology. In addition to regular papers, book reviews and abstracts of selected papers from other periodicals also are a special feature of the journal.

Haematologia, published at quarterly intervals, provides the reader with complex and up-to-date information.

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

Copy Editor:
D. Keszthelyi

Distributor for

Albania, Bulgaria, China, Cuba, Czechoslovakia, German Democratic Republic, Korean People's Republic, Mongolia, Poland, Romania, U.S.S.R., Viet-Nam, and Yugoslavia:

KULTURA
Hungarian Foreign Trading Company
P.O.B. 149, H-1389 Budapest 62, Hungary

For all other countries subscriptions should be sent to

VSP
P.O.B. 346
3700 AH ZEIST, The Netherlands

Subscription price for volume 22:

DM 330,— (including postage)

Haematologia is covered in Current Contents/Clinical Practice, ISI/Biomed and the SCI.

VOLUME 22 · NUMBER 1

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

VSP UTRECHT, THE NETHERLANDS, TOKYO, JAPAN

AKADÉMIAI KIADÓ, BUDAPEST

1989

Printed in Hungary

Akadémiai Kiadó és Nyomda Vállalat, Budapest

Haematologia 22 (1), pp. 3–10 (1989)

Dual Effect of Calcium on Erythrocyte Membrane Contraction

L. MIRČEVOVÁ, L. VIKTORA, H. ŘEHÁČKOVÁ

Institute of Haematology and Blood Transfusion, U nemocnice 1, 128 20 Prague 2, Czechoslovakia

(Received 10 August, 1987; accepted 16 November, 1987)

Effect of different calcium concentrations on erythrocyte ghost shape and size has been studied. Erythrocyte ghosts are smallest at approximately 10 μM of total calcium content and in the absence of EDTA; both decrease and increase of free Ca^{2+} results in expansion of ghosts. Changes in erythrocyte ghost size occur at equal Ca^{2+} concentrations which, as it has been already found, alter ATPase activity of contractile membrane protein. The possibility is discussed that under physiologic conditions contractile membrane protein causes weak membrane contraction which differs from aphysiologic membrane shrinkage brought about particularly by cross-bridges at high Ca^{2+} concentrations.

Keywords: calcium, contractile membrane protein, erythrocyte ghost shape, physiologic membrane contraction

Introduction

Since the 1960-s it has been known that ATP depletion accompanied by Ca^{2+} accumulation is responsible for expressive alterations in erythrocyte shape viz. disc-sphere transformation [1, 2]. Moreover, ATP and Ca^{2+} are known to produce erythrocyte ghost contraction [3–5], and to effect the size of Triton shells [6, 7] and the viscosity of erythrocyte membranes [8, 9].

Our assumption that most of these alterations are caused by conformation and association changes of a contractile membrane protein has been confirmed by the detection of myosin in the erythrocyte membrane [10]. Studying its ATPase activity (previously termed Mg-ATPase or spectrin-dependent ATPase), we observed that it is unusually dependent on Ca^{2+} concentrations. ATPase activity is low in the absence of, or at very low concentrations of Ca^{2+}; with increasing Ca^{2+} concentration it increases towards maximum (apparent dissociation constant K_{Ca} is 0.61 μM). After achieving the maximum, further increase of Ca^{2+} concentration inhibits the ATPase activity – the higher the Ca^{2+} concentration, the stronger the inhibition of the ATPase activity [11].

The aim of our work was to determine whether there is an analogy between the effect of Ca^{2+} on the ATPase activity of contractile protein and its effect on the shape and/or size of erythrocyte ghosts. Our results indicate an analogy – when Ca^{2+} concentration is physiologic, the membrane is slightly contracted.

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Materials and Methods

Preparation of erythrocyte ghosts

Fresh blood from healthy donors was used. Four parts of blood were added to one part of ACD solution (1.4 g sodium citrate, 0.5 g citric acid, 2.5 g glucose, H_2O ad 100 ml). Erythrocytes were isolated by centrifugation and washed with saline. One part of washed erythrocytes was hemolysed with 18 parts of 10 mM Tris-HCl buffer (pH = 7.4) or 10 mM HEPES buffer (pH = 7.3) with 1 mM NaCl and 9 mM KCl and with or without addition of 1 mM ATP, 2 mM $MgCl_2$, 0.1 mM EDTA and $CaCl_2$ at different concentrations. Erythrocytes were hemolysed for 10 minute at 4 °C, then hypotonic hemolysates were adjusted to isotonicity by addition of 2 parts of hypertonic solution and incubated for 30 minutes at 37 °C; the procedure resulted in formation of erythrocyte ghosts with a certain amount of hemoglobin (dark sites on microphotographs). The composition of the final isotonic solutions was 10 mM Tris-HCl buffer (pH = 7.4) or 10 mM HEPES buffer (pH = 7.3), 125 mM NaCl, 25 mM KCl with or without addition of 1 mM ATP, 2 mM $MgCl_2$, 0.1 mM EDTA and $CaCl_2$ at different concentrations. The concentration of calcium given in the "Results" is the calculated concentration of free Ca^{2+} ions (hereafter called Ca^{2+}); the calculation of Ca^{2+} has been described in our previous work [11].

Fixation by glutaraldehyde

Two parts of glutaraldehyde solution (1% glutaraldehyde in 0.135 M of phosphate buffer, pH = 7.2) were added to 1 part of the erythrocyte ghost suspension (see above). After 30-minute standing at room temperature, the ghost suspension was centrifuged and one part of 50% ethylalcohol was added to one part of packed ghost. The obtained suspension was used for the preparation of smears.

Evaluation of preparations

Morphologic evaluation of native preparations was performed using a phase contrast microscope. A light microscope was used for evaluation of stained preparations both fixed and unfixed by glutaraldehyde. Staining was performed according to the Pappenheim technic, using May-Grünwald and Giemsa solutions.

Results

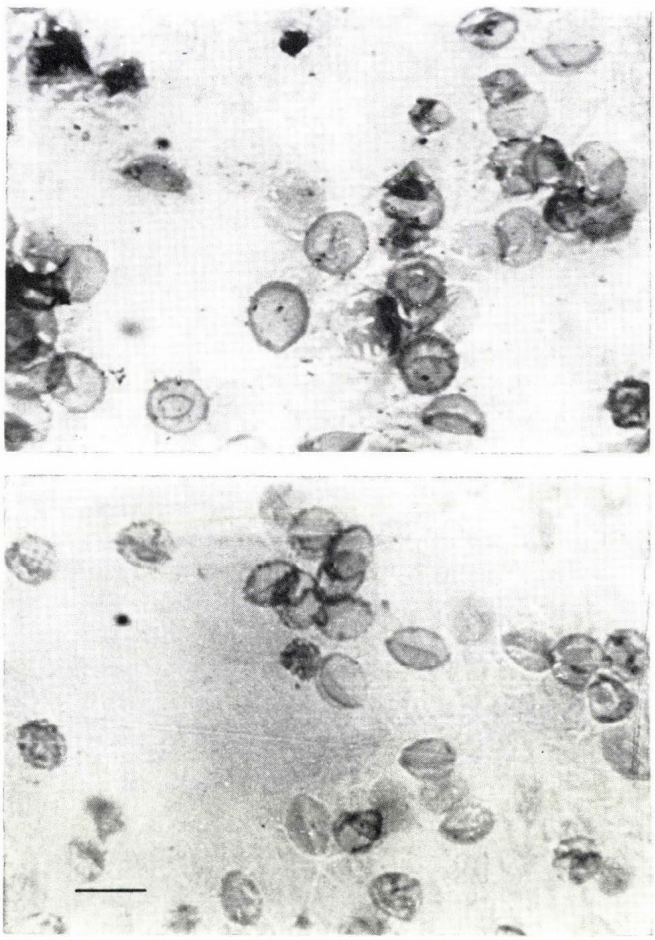
Native preparations of erythrocyte ghosts were studied in a moist chamber and compared with those evaluated after fixation and staining. The latter were more convenient for evaluation, as they were in a static condition and their struc-

ture was more distinct, however, they were less homogeneous than those in the native preparations; the stained preparations without fixation by glutaraldehyde were very similar to those fixed by glutaraldehyde, but less dsitinct.

Further on, erythrocyte ghosts were denominated for simplification as normocytes, microcytes, macrocytes, echinocytes, etc.

In the *presence of ATP and magnesium* changes in Ca^{2+} concentration caused the following changes in the shape of erythrocyte ghosts:

After addition of 0.1 mM EDTA, i.e. at Ca^{2+} concentration lower than



1a

1b

Fig. 1. Effect of 0.1 mM EDTA and 10 mM Ca^{2+} on erythrocyte ghost shape in the presence of 1 mM ATP and 2 mM $MgCl_2$. Given Ca^{2+} concentration is the initial concentration of Ca^{2+} added to erythrocyte ghosts before their resealing and 30-minute incubation (at 37 °C) in Tris buffer (pH = 7.4). Photographs of stained preparations. a) + 0.1 EDTA; b) + 10 mM Ca^{2+}. Bar = 10 μm

10 nM, smooth macrocytes were present in the preparations (Fig. 1a). In some samples, however, the erythrocyte ghosts were rather crenated.

At 0.6 μM and 50 μM Ca^{2+} concentrations, the shape of erythrocyte ghosts was much alike; the smooth erythrocyte ghosts were somewhat larger (Fig. 2b) than the control samples to which no Ca^{2+} and EDTA were added (Fig. 2a). In some preparations echinocytes of the same size as control samples occurred most frequently. At 2 μM and 10 μM Ca^{2+} concentrations, erythrocyte ghosts were slightly crenated normocytes.

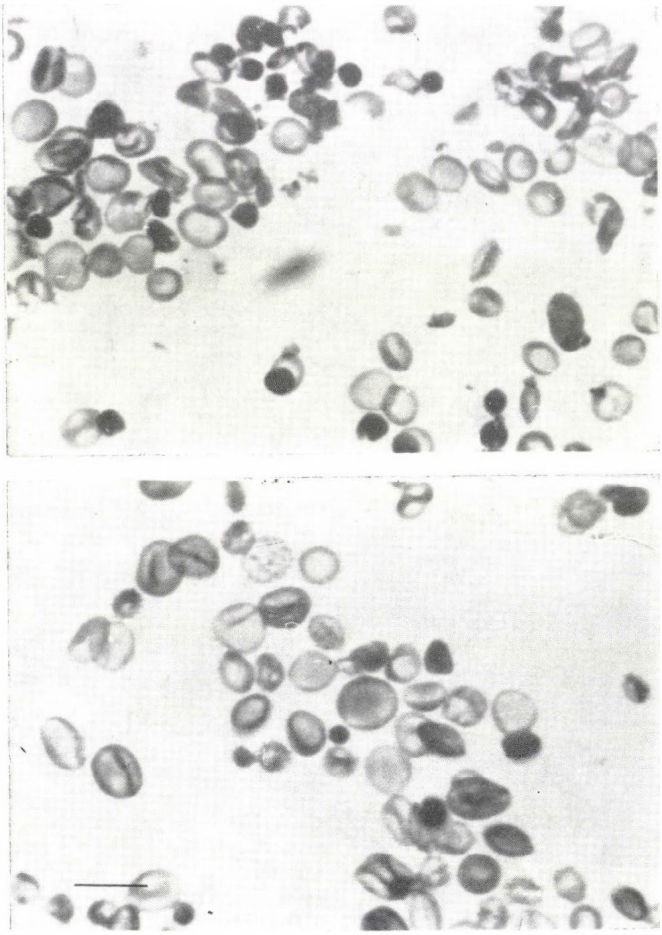


Fig. 2. Effect of 50 μm Ca^{2+} on erythrocyte ghost shape in the presence of 1 mM ATP and 2 mM $MgCl_2$. Given Ca^{2+} concentration is the initial concentration of Ca^{2+} added to erythrocyte ghosts before their resealing and 30-minute incubation (at 37 °C) in HEPES buffer (pH = 7.3). Photographs of stained preparations after fixation by glutaraldehyde. a) control without addition of Ca^{2+}; b) + 50 μM Ca^{2+}. Bar = 10 μm, i.e. the same magnification as in Fig. 1

At 200 μM Ca^{2+} concentration anisocytosis was found. Apart from smooth macrocytes, ghosts of different size and of different degree of crenation occurred. The anisocytosis was even more apparent after addition of 1 mM Ca^{2+}. In the samples macrocytes of different size prevailed; some of them were smooth, others crenated and with some invaginations remindig of erythrocyte ghosts illustrated in Fig. 3a.

At 10 mM Ca^{2+} concentration, smooth macrocytes with atypical cup-like shape were most frequent, some of them were wrinkled (Fig. 1b).

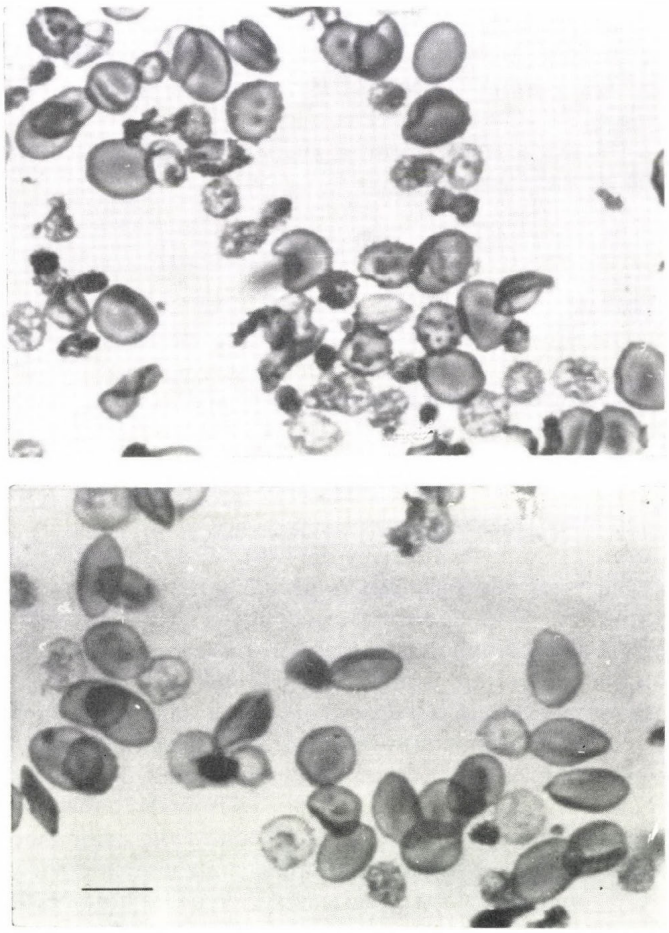


Fig. 3. Effect of 10 μM Ca^{2+} and 100 μM Ca^{2+} on erythrocyte ghost shape; no ATP and $MgCl_2$ added. Given Ca^{2+} concentration is the initial concentration of Ca^{2+} added to erythrocyte ghosts before their resealing and 30-minute incubation (at 37 °C) in HEPES buffer (pH = 7.3). Photographs of stained preparations after fixation by glutaraldehyde. a) + 10 μM Ca^{2+}; b) + 100 μM Ca^{2+}. Bar = 10 μm, i.e. the same magnification as in Fig. 1

In samples with *no ATP and magnesium added*, alterations in the shape of erythrocyte ghosts appeared in response to even smaller changes in Ca^{2+} concentration than is samples with ATP and magnesium.

In the presence of 0.1 mM EDTA, without addition or at a low concentration of Ca^{2+}, erythrocyte ghosts were hardly visible or even desintegrated. Therefore we evaluated only control samples and samples with Ca^{2+}, but without addition of EDTA. Control erythrocyte ghosts incubated without addition of ATP and magnesium were somewhat larger than those incubated in their presence and their diameter was similar to ghosts shown in Fig. 2b.

The addition of 10 μM Ca^{2+} only caused significant changes in the shape and size of erythrocyte ghosts. Macrocytes, either smooth or with some invaginations and protrusions, prevailed in the samples and also atypical echinocytes were present (Fig. 3a). Further increase of Ca^{2+} concentration up to 100 μM Ca^{2+} caused a further enlargement of erythrocyte ghosts (Fig. 3b). Most of the macrocytes had a smooth atypical cup-like shape and were larger than erythrocyte ghosts incubated with 10 μM Ca^{2+}.

Discussion

When calculating the initial concentration of Ca^{2+}, the linkage of Ca^{2+} to different binding sites in the membrane has not been included in the calculation. The membrane has several calcium binding sites; to some of these Ca^{2+} binds already at physiologic concentrations [12–15], and to others only at high aphysiologic concentrations [12, 15].

The actual Ca^{2+} concentrations in erythrocyte ghosts at the time of their morphological evaluation were substantially lower than the initial high Ca^{2+} concentrations stated above since, in the presence of ATP and magnesium, Ca^{2+} is expelled from erythrocyte ghosts by the Ca-pump.

Homogeneity of ghost samples is probably influenced by the difference in age of erythrocytes; older erythrocytes contain a higher amount of calcium in their membrane, and their ghosts are therefore more sensitive to further addition of calcium than the ghosts from younger erythrocytes [16].

Smooth normocytes were prepared in control samples in which total calcium level, measured by an atomic absorption spectrophotometer, was about 10 μM. At this concentration ATPase activity of contractile membrane protein was high [11]. At 0.6 μM Ca^{2+} concentration, which corresponds to the value of apparent dissociation constant for ATPase activity of contractile membrane protein, erythrocyte ghosts were somewhat larger than the control samples incubated without addition of Ca^{2+} and EDTA; in some samples crenated normocytes were found. As we failed to prepare smooth normocytes using Ca-EDTA solutions, we were unable to determine the exact Ca^{2+} concentration needed for their existence.

Our results demonstrate that large, smooth erythrocyte ghosts occur at both low and high Ca^{2+} concentrations when ATPase activity of contractile membrane protein is inhibited [11]. Initial 10 mM Ca^{2+} concentration seems to

be rather high, but due to Ca-pump activity, the actual Ca^{2+} concentration is reduced after 30-minute incubation with ATP and magnesium at 37 °C. At this Ca^{2+} concentration the shape of erythrocyte ghosts is probably not an artefact, since much larger macrocytes can be found in samples incubated at 100 μM Ca^{2+} or even at 10 μM Ca^{2+} concentration without addition of ATP and magnesium.

The formation of macrocytes observed at low concentrations of Ca^{2+} confirms the hypothesis that the membrane, due to Ca^{2+} release, expands [17]. However, the membrane expands also at high Ca^{2+} concentrations. Rand [18] reported that there is a tension in the membrane of normal smooth biconcave erythrocytes. We suggest that this tension is the result of a weak physiologic membrane contraction brought about under physiologic conditions by a contractile membrane protein. Both at low and at high Ca^{2+} concentrations this contraction is weaker or it does not occur at all – and the membrane expands. The physiologic contraction rather differs from aphysiologic membrane shrinkage which at high Ca^{2+} concentrations in erythrocytes is due to the formation of aphysiologic cross-bridges [19] – and probably also to the formation of the bridges among negative charges in the membrane [20].

We suggest that macrocytes and echinocytes do not differ as much as they may seem to at first glance. The membrane contraction is decreased in both macrocytes and echinocytes. This decrease is homogeneous in macrocytes, while in echinocytes protrusions appear on the membrane surface. Of course, there are also other factors responsible for the formation of echinocytes; they may be formed, e.g., by interference with a lipid bilayer [21].

The way in which Ca^{2+} effects the membrane contraction has not been precisely defined yet. In theory, the following events may occur: altered interaction of actin with myosin or with spectrin and band 4.1 [22], the effect of Ca^{2+} on the interaction of myosin with tropomyosin which has been demonstrated in the erythrocyte membrane, too [23]; the effect of Ca^{2+} on actin polymerization [24], which may be of particular importance when actin is present in the membrane in a filamentous form as suggested by Weinstein et al. [25].

The formerly rather static conception of the membrane structure is today gradually replaced by a more dynamic view. We suggest that the shape of a smooth biconcave normocyte depends on dynamic equilibrium among particular membrane components where actomyosin contractile protein plays an important part – and Ca^{2+} is one of the regulators of its activity.

References

1. Nakao, M., Nakao, T., Yamazoe, S., Yoshikawa, H.: Adenosine triphosphate and shape of erythrocytes. *J. Biochem. 49*, 487–492 (1961).
2. Weed, R. I., La Celle, P. L., Merrill, E. W.: Metabolic dependence of red cell deformability. *J. Clin. Invest. 48*, 795–809 (1969).
3. Wins, P., Schoffeniels, E.: ATP + Ca^{++} – linked contraction of red cell ghosts. *Arch. Int. Physiol. Biochim. 74*, 812–820 (1966).

4. Palek, J., Curby, W. A., Lionetti, F. J.: Effects of calcium and adenosine triphosphate on volume of human red cell ghosts. *Amer. J. Physiol. 220*, 19—26 (1971).
5. Palek, J., Curby, W. A., Lionetti, F. J.: Relation of Ca^{++} — activated ATPase to Ca^{++} — linked shrinkage of human red cell ghosts. *Amer. J. Physiol. 220*, 1028—1032 (1971).
6. Jinbu, Y., Sato, S., Nakao, M., Tsukita, S.: Ca^{2+} — and Mg-ATP-dependent shape change of human erythrocyte ghosts and Triton shells. *Exp. Cell. Res. 151*, 160—170 (1984).
7. Jinbu, Y., Sato, S., Nakao, M.: Reversible shape change of Triton-treated erytrocyte ghosts induced by Ca^{2+} and Mg-ATP. *Nature 307*, 376—378 (1984).
8. Quist, E. E., Roufogalis, B. D.: The relationship between changes in viscosity of human erythrocyte membrane suspensions and (Mg + Ca)-ATPase activity. *Biochem. Biophys. Res. Commun. 72*, 673—679 (1976).
9. Quist, E. E.: Regulation of the shape of unsealed erythrocyte membranes by Mg-ATP and Ca^{2+}. *Arch. Biochem. Biophys. 203*, 123—133 (1980).
10. Fowler, V. M., Davis, J. Q., Bennett, V.: Human erythrocyte myosin: Identification and purification. *J. Cell. Biol. 100*, 47—55 (1985).
11. Mirčevová, L., Kotva, M., Kodíček, M., Řeháčková, H.: Activation of Mg-ATPase (spectrin-dependent ATPase) by Ca^{2+}. *Enzyme 24*, 374—382 (1979).
12. Cohen, C. M., Solomon, A. K.: Ca binding to the human red cell membrane: Characterization of membrane preparations and binding sites. *J. Membrane Biol. 29*, 345—372 (1976).
13. Porzig, H.: Studies on the cation permeability of human red cell ghosts. *J. Membrane Biol. 31*, 317—349 (1977).
14. Porzig, H., Stoffel, D.: Equilibrium binding of calcium to fragmented human red cell membranes and its relation to calcium-mediated effects on cation permeability. *J. Membrane Biol. 40*, 117—142 (1978).
15. Moore, R. B., Manery, J. F., Dryden, E. E.: Calcium-binding groups involved in Ca^{2+} regulation of the structure and function of the erythrocyte membrane. In: *Erythrocyte Membranes: Recent Clinical and Experimental Advances*, vol. 20. W. C. Kruckeberg, J. W. Eaton, G. J. Brewer (eds), Alan R. Liss, Inc., New York 1978, pp. 51—67.
16. Shiga, T., Sekiya, M., Maeda, N., Kon, K., Okazaki, M.: Cell age-dependent changes in deformability and calcium accumulation of human erythrocytes. *Biochim. Biophys. Acta 814*, 289—299 (1985).
17. Seeman, P., Kwant, W. O., Sauks, T., Argent, W.: Membrane expansion of intact erythrocytes by anesthetics. *Biochim. Biophys. Acta 183*, 490—498 (1969).
18. Rand, R. P.: Some biophysical considerations of the red cell membrane. *Fed. Proc. 26*, 1780—1784 (1967).
19. Lorand, L., Siefring, Jr. G. E., Lowe-Krentz, L.: Formation of γ-glutamyl-ε-lysine bridges between membrane proteins by a Ca^{2+}-regulated enzyme in intact erythrocytes. *J. Supramol. Struct. 9*, 427—440 (1978).
20. Burger, S. P., Fujii, T., Hanahan, D. J.: Stability of the bovine erythrocyte membrane. Release of enzymes and lipid components. *Biochemistry 7*, 3682—3699 (1968).
21. Lange, Y., Gough, A., Steck, T. L.: Role of the bilayer in the shape of the isolated erythrocyte membrane. *J. Membrane Biol. 69*, 113—123 (1982).
22. Fowler, V., Taylor, D. L.: Spectrin plus band 4.1 cross-link actin. Regulation by micromolar calcium. *J. Cell. Biol. 85*, 361—376 (1980).
23. Fowler, V. M., Bennett, V.: Erythrocyte membrane tropomyosin. Purification and properties. *J. Biol. Chem. 259*, 5978—5989 (1984).
24. Dancker, P., Löw, I.: Dual effect of Ca^{2+} on ultrasonic ATPase activity and polymerization of muscle actin. *Biochim. Biophys. Acta 484*, 169—176 (1977).
25. Weinstein, R. S., Tazelaar, H. D., Loew, J. M.: Red cell comets: Ultrastructure of axial elongation of the membrane skeleton. *Blood Cells 11*, 343—357 (1986).

Haematologia 22 (1), pp. 11—18 (1989)

Alpha Fetoprotein Inhibits Aggregation of Human Platelets

M. CZOKAŁO[1], M. TOMASIAK[2]

[1]Department of General and Experimental Pathology, and [2]Department of Biochemistry Medical Academy, Mickiewicza 2C, 15—230 Białystok 8, Poland

(Received 25 June, 1987; accepted 15 December, 1987)

The effect of human alpha-fetoprotein (AFP) on platelet aggregation induced by physiological (ADP, PAF, collagen, arachidonic acid) and aphysiological (ionophore A 23 187) activators was investigated

It was found that AFP at the concetrations of 60—750 μg/ml, i.e. far below that observed in human blood, inhibits platelet aggregation induced by physiological activators.

Platelet aggregation induced by arachidonic acid was two-four times more sensitive to inhibition by AFP than that induced by other physiological agonists, whereas aggregation induced by A 23 187 was not affected by AFP even at concentration of 750 μg/ml.

Keywords: human alpha-fetoprotein, human platelets, platelet aggregation

Introduction

Alpha-fetoprotein (AFP) is the major plasma protein of vertebrate embryos including man [1]. Mammalian AFP is a 70.000 dalton glycoprotein mainly synthesized by the fetal liver and yolk sac [1, 2, 3]. AFP present in fetal blood in milligram amounts [2, 4] disappears during the postnatal period, decreasing to nanogram amounts normal in the blood of adults [5].

Structural [6, 7] and immunochemical [8] similarities of AFP to albumin as well as the fact that the concentrations of AFP and albumin in fetal and normal adult blood are in inverse relation [2, 4, 5] suggest that AFP is a fetal analogue of albumin. Like albumin, AFPs from different species, including man, are capable of binding estrogens [9], bilirubin [10], copper [11] and polyunsaturated fatty acids [12, 13, 14].

The biological significance of AFP during embryogenesis has not been completely elucidated. Some authors suggest that it may be related to the fact that this protein shows an extremely high capacity for binding fatty acids, mainly docosahexaenoic (22: 6) and arachidonic (20: 4) acids [15].

Recent observations of Aussel et al. suggest that one of the best ligands binding with AFP is arachidonic acid [16, 17]. Since arachidonic acid is the major substrate in blood platelets for the synthesis of thromboxane A_2 and some prostaglandins, which are crucial for platelet activation, we were interested in whether AFP affects platelet aggregation.

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Materials and Methods

Collagen was the product of Hormonchemia, Munich, FRG. ADP (Na salt), arachidonic acid and crystalline human albumin were from Sigma (St. Louis, MA., USA). Ionophore A 23187 and synthetic PAF (1-0-alkyl-2-acetyl-sn-glyceryl-2-phosphorylcholine) were supplied by Calbiochem (La Jolla, CA., USA).

Alpha-fetoprotein (AFP) was isolated from serum of human cord blood taken during preterm labor, according to the method described previously; purified and tested by polyacrylamide gel electrophoresis, isoelectric focusing and immunoelectrophoresis [18].

Albumin was defatted as described by Chen [19] and dialysed according to Hanson and Ballard [20].

Preparation of platelet rich plasma

Blood from non-pregnant healthy volunteers (25–40 years of age) who had not taken any medication for at least 10 days was collected into 3.8% (w/v) sodium citrate, one volume per nine volumes of whole blood. Platelet rich plasma (PRP) was obtained by centrifugation of the blood in plastic tubes for 20 min at 120 *g* at room temperature. Platelet number in PRP was determined with a phase contrast microscope according to standard laboratory technique and adjusted to 250 000/mm^3 by mixing of PRP with platelet poor plasma.

Assay of aggregation

Platelet aggregation was followed turbidimetrically according to Born [21] by recording the light transmission through a stirred platelet suspension in a plastic cuvette of an aggregometer at 37 °C. Samples of PRP (300 µl) were incubated for 2 min at 37 °C without (control) and with AFP in an aggregometer. Samples were stirred before the inducer was added. For each PRP sample, the threshold aggregating concentrations (TAC), defined as the minimum amount of the stimulus that induced at least 70% increase in light transmission within 3 min was selected. Inducers were used at their threshold aggregating concentrations, or at the concentration which was two times higher than the threshold one. Solutions of arachidonic acid and A 23 187 were prepared as previously described [22, 23]. ADP was dissolved in 0.15 M NaCl and the pH of the solution was adjusted to 7.3 by 0.2 M NaOH. AFP was used as a solution in phosphate buffered saline. No additions exceeded 30 µl. All experiments were performed at least in triplicate, using 7–10 different platelet preparations.

Results

The arachidonic acid-induced aggregation curves are shown in Fig. 1. The aggregation of platelets induced by threshold aggregating concentration of agonist was almost totally inhibited when the concentration of AFP reached 125 μg/ml (Fig. 1A). The aggregation induced by a higher than threshold aggregating concentration of arachidonic acid was inhibited by an approximately two times higher (i.e. 250 μg/ml) concentration of AFP (Fig. 1B).

Figures 2 and 3 show the results of similar experiments with ADP and PAF as agonists. In these cases, a dose-dependent inhibition of platelet aggregation by AFP was observed, and the inhibitory effect was much weaker when the aggregation was induced by higher than threshold aggregating concentrations of ADP (Fig. 2B) and PAF (Fig. 3B). In these cases the total inhibition of platelet aggregation (at threshold aggregating concentrations of ADP and PAF) induced by both agonists was observed at higher concentration of AFP, i.e. about 250 μg/ml (Figs 2A and 3A).

Figure 4 demonstrates similar results, i.e. dose-dependent inhibition of collagen induced platelet aggregation with weaker inhibitory effect at higher than threshold aggregating concentration of agonist. In these case, however, the total

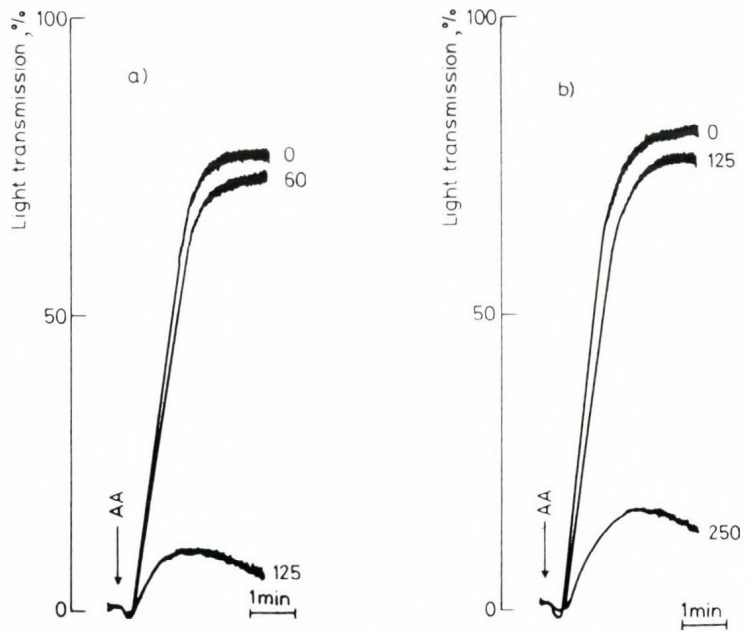


Fig. 1. Arachidonic acid (AA) induced platelet aggregation without (O) and in the presence of AFP (60—250 μg/ml of PRP). Threshold aggregating concentrations (TAC) of arachidonic acid (A) and that double of TAC (B) were used to induce aggregation

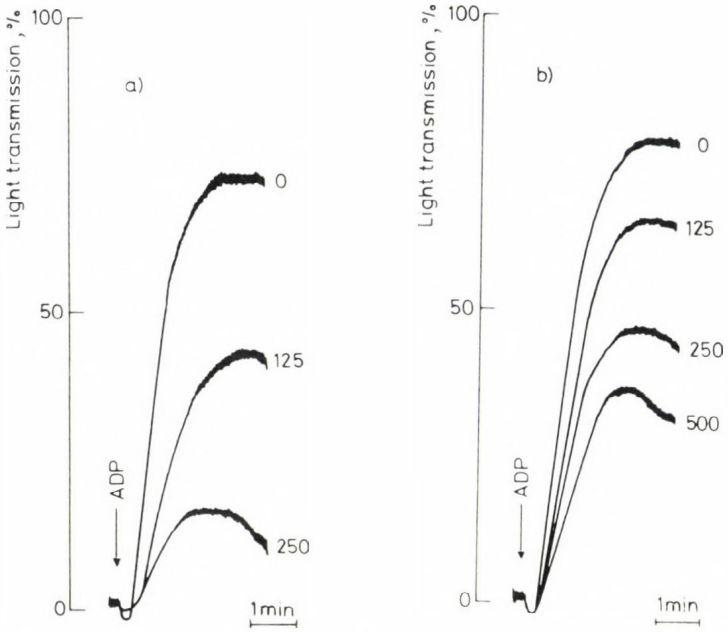


Fig. 2. ADP induced platelet aggregation without (O) and in the presence of AFP (125–500 μg/ml of PRP). Threshold aggregating concentrations (TAC) of ADP (A) and that double of TAC (B) were used to induce aggregation

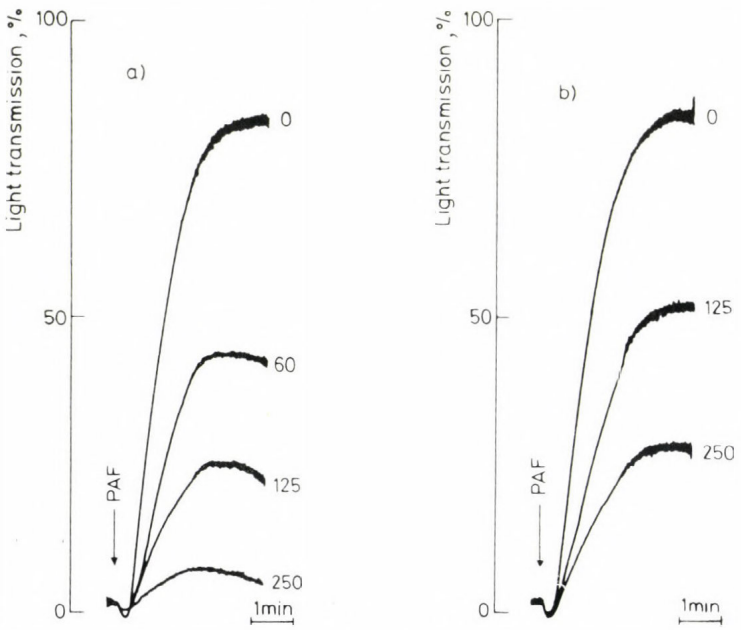


Fig. 3. PAF induced platelet aggregation without (O) and in the presence of AFP (60–250 μg/ml of PRP). Threshold aggregating concentrations (TAC) of PAF (A) and that double of TAC (B) were used to induce aggregation

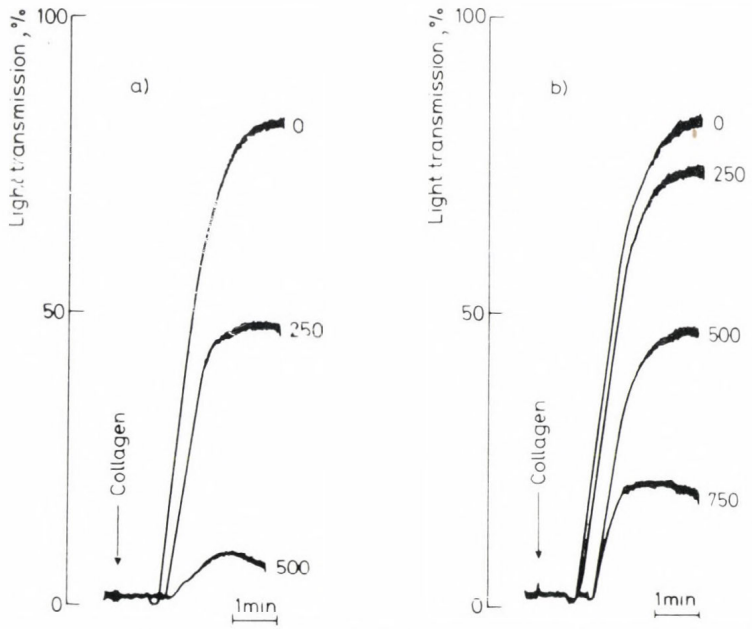


Fig. 4. Collagen induced platelet aggregation without (O) and in the presence of AFP (250—750 μg/ml of PRP). Threshold aggregating concentrations (TAC) of collagen (A) and that double of TAC (B) were used to induce aggregation

inhibition of aggregation (at threshold aggregating concentration of agonists) was observed in the presence of AFP at the concentration of 500 μg/ml.

Table 1 shows that AFP did not affect platelet aggregation induced by ionophore A 23 187 even at very high concentrations. Defatted human albumin at concentrations of up to 1000 μg/ml did not affect platelet aggregation induced by all the tested stimuli.

Discussion

Our results indicate that AFP concentrations much lower than the usual physiological level in fetal blood can inhibit platelet aggregation. The inhibitory effect of AFP on platelet aggregation seems to be specific to this protein since the purified (defatted and dialysed) human albumin did not affect platelet aggregation even at a higher concentration than that of AFP (Table 1). These observations allow us to suggest that the other important physiological function of this protein might be its participation in the regulation of platelet functions. But why does AFP inhibit platelet aggregation induced by physiological agonists? AFP was suggested

Table 1

The effect of alpha-fetoprotein and albumin on platelet aggregation induced by various stimuli

Stimulus	AFP concentration (μg/ml)					Albumin concentration (μg/ml)	
	60	125	250	500	750	750	1000
	Inhibition of aggregation (per cent)						
Arachidonic acid							
0.5 mM*	5	95	100	n.d.	n.d.	0	0
1.0 mM	0	6	90	n.d.	n.d.	0	0
ADP							
4.5 μM*	n.d.	50	82	100	n.d.	0	0
9.0 μM	n.d.	12	41	56	n.d.	0	0
PAF							
0.15 μg/ml*	50	75	95	100	n.d.	0	0
0.3 μg/ml	n.d.	40	70	n.d.	n.d.	0	0
Collagen							
2.0 μg/ml*	n.d.	n.d.	42	95	100	0	0
4.0 μg/ml	n.d.	n.d.	10	45	79	0	0
A 23 187							
15 μM*	0	0	0	0	0	0	0
30 μM	0	0	0	0	0	0	0

n.d. — not determined; * — threshold aggregating concentrations.
The results of one (one out of seven) representative experiment are presented.

to be involved in the regulation of cellular growth [24, 25], lymphocyte [26, 27] and ovarian functions [28]. All the three above mentioned processes are known to be at least partly regulated by prostaglandins. There is also increasing evidence of the transport function of AFP [9–17]. It has been suggested that AFP may transport and deliver polyunsaturated fatty acids to the cell [15].

Taking all this in account and the fact that the aggregation of platelets induced by arachidonic acid was the most sensitive to inhibition by AFP (Fig. 1, Table 1) and the aggregation induced by A 23 187 (which is independent of arachidonate metabolism) was not affected by AFP (Table 1), it is possible that AFP affects platelet aggregation by complex forming with arachidonate or its metabolites. Such a possibility seems to be supported by the fact that the inhibitory effect of AFP on platelet aggregation is at least partially reduced by the increase of the concentration of the platelet activators (Figs 1–4, Table 1).

The interaction of AFP and platelets might be of some importance in post-natal life also. It is known that the synthesis of AFP reappears in adults as a consequence of certain pathological conditions such as hepatocellular carcino-

mas or teratocarcinomas [1, 29]. Although tumor cells have been reported to possess both a platelet-activating material and a procoagulant activity [30, 31], the results presented here suggest that at least some of them that produce AFP might deliver material which is able to inhibit platelet aggregation.

References

1. Ruoslahti, E., Seppälä, M.: Alpha-foetoprotein in cancer and fetal development. *Adv. Cancer Res. 29*, 276 (1979).
2. Gitlin, D.: Normal biology of α-fetoprotein. *Ann. N. Y. Acad. Sci. 259*, 7 (1975).
3. Dziadek, M. A., Andrews, G. H.: Tissue specificity of alpha-fetoprotein messenger RNA expression during mouse embryogenesis. *EMBO J. 2*, 549 (1983).
4. Masseyeff, R., Gilli, J., Krebs, B.-P., Calluaud, A., Bonet, C.: Evolution of α-fetoprotein serum level throughout life in human and rats, and during pregnancy in the rat. *Ann. N. Y. Acad. Sci. 259*, 17 (1975).
5. Ruoslahti, E., Seppälä, M.: Studies on carcino-fetal proteins. III. Development of a radioimmunoassay for α-fetoprotein. Demonstration of α-fetoprotein in serum of healthy human adults. *Int. J. Cancer 8*, 374 (1971).
6. Gorin, M. G., Cooper, D. L., Eiferman, F., van de Rijn, P., Tilghman, S. M.: The evolution of α-fetoprotein and albumin. I. Comparison of the primary amino acid sequences of mammalian α-fetoprotein and albumin. *J. Biol. Chem. 256*, 1954 (1981).
7. Morinaga, T., Sakai, M., Wegmann, T. G., Tamaoki, T.: Primary structure of human alpha-fetoprotein and its mRNA. *Proc. Nat. Acad. Sci. USA 80*, 4604 (1983).
8. Ruoslahti, E., Engvall, E.: Immunological crossreaction between alpha-fetoprotein and albumin. *Proc. Nat. Acad. Sci. USA 73*, 4641 (1976).
9. Uriel, J., Bouillon, D., Aussel, C., Dupiers, M.: Alpha-fetoprotein: The major high-affinity estrogen binder in rat uterine cytosols. *Proc. Nat. Acad. Sci. USA 73*, 1452 (1976).
10. Ruoslahti, E., Estes, T., Seppälä, M.: Binding of bilirubin by bovine and human α-fetoprotein. *Biochim. Biophys. Acta 578*, 511 (1976).
11. Aoyagi, T., Ikenaka, T., Ichida, F.: Copper (II) — binding ability of human α-fetoprotein. *Cancer Res. 38*, 3484 (1978).
12. Parmelle, D. C., Evanson, M. A., Deutsch, H. F.: The presence of fatty acids in human α-fetoprotein. *J. Biol. Chem. 253*, 2114 (1978).
13. Pineiro, A., Olivito, A., Uriel, J.: Fixation d'acides gras polyinsaturés par l'alpha-foetoprotéine et la sérum albumine de rat. Comparison avec l'accumulation de ces acides dans le cerveau au cours du dévelopment post-natal. *C. R. Acad. Sci. Paris 289D*, 1053 (1979).
14. Hsia, J. C., Wong, L. T., Deutsch, H. F.: Determination of the distribution of fatty acids and diethylstilbestrol between serum albumin and α-fetoprotein by concanavalin A affinity chromatography. *Biochim. Biophys. Acta 880*, 117 (1986).
15. Geuskens, M., Naval, J., Uriel, J.: Ultrastructural studies of the intracellular translocation of endocytosed alpha-foetoprotein by cytochemistry and the uptake of ^{3}H-arachidonic acid bound to AFP by autoradiography in rat rhabdomyosarcoma cells. *J. Cell. Physiol. 128*, 389 (1986).
16. Aussel, C., Masseyeff, R.: Human alpha-fetoprotein fatty acid interaction. *Biochem. Biophys. Res. Commun. 115*, 38 (1983).
17. Aussel, C., Massayeff, R.: Comparative binding properties of rat and human alpha-fetoproteins. *Res. Commun. Chem. Pathol. Pharm. 42*, 261 (1983).
18. Czokało, M.: Human alpha-fetoprotein. I. Isolation of homogenous AFP from cord blood serum. *Mol. Cell. Biochem. 25*, 179 (1979).
19. Chen, R. F.: Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem. 242*, 173 (1967).

20. Hanson, R. W., Ballard, F. J.: Citrate, pyruvate and lactate contaminants of commercial serum albumin. *J. Lipid Res. 9*, 667 (1968).
21. Born, G. V. R.: Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature 194*, 927 (1962).
22. Kinlough-Rathbone, R. L., Reimers, H-J., Mustard, J. F., Packham, M. A.: Sodium arachidonate can induce platelet shape change and aggregation which are independent of the release reaction. *Science 192*, 1011 (1976).
23. Kinlough-Rathbone, R. L., Chahil, A., Packham, M. A., Reimers, H-J., Mustard, J. F.: Effect of A 23187 on thrombin-degranulated washed rabbit platelets. *Thromb. Res. 7*, 435 (1975).
24. Schatz, R., Laugier, C., Soto, A. M., Sonnenshein, C.: Alpha-fetoprotein serum levels and the development of estrogen sensitive cell multiplication in the hamster uterus. *Biol. Reprod. 28*, 1148 (1983).
25. Toder, V., Blank, M., Gold-Gefter, L., Nebel, L.: The effect of alpha-fetoprotein on the growth of placental cells in vitro. *Placenta 4*, 79 (1983).
26. Czokało, M., Wisniewski, L.: Culture medium components modify the effects exerted by alpha-fetoprotein (AFP) on lymphocyte response. *Exp. Pathol. 18*, 297 (1980).
27. Gupta, S., Good, R. A.: α-fetoprotein and human lymphocyte subpopulations. *J. Immunol. 118*, 405 (1977).
28. Aussel, C., Laufaurie, M., Masseyeff, R., Stora, C.: In vivo regulation of ovarium activity by alpha-fetoprotein. *Steroids 38*, 195 (1981).
29. Abelev, G. I.: Alpha-foetoprotein in ontogenesis and its association with malignant tumors. *Adv. Cancer Res. 14*, 295 (1971).
30. Donati, M. B., Semeraro, N., Gordon, S. G.: Relationship between procoagulant activity and metastatic capacity of tumor cells. In: *Hemostatic mechanisms and metastasis.* K. V. Honn and B. F. Sloane (eds). Martinus Nijhoff Publishing, Boston 1984, p. 84.
31. Jamieson, G. A., Bastida, E., Ordinas, A. Mechanism of platelet aggregation by human tumor cell lines. In: *Interaction of platelets and tumor cells.* G. A. Jamieson (ed.) Alan R. Liss. Inc., New York 1982, p. 405.

Haematologia 22 (1), pp. 19–24 (1989)

Chromosome Examinations on a Six-hour Culture of Unstimulated Peripheral Blood from a Patient with Childhood Erythroleukaemia

A. SELYPES[1], A. LÁSZLÓ, I. VIRÁG[2]

[1]Department of Hygiene and Epidemiology, University Medical School, H-6720 Szeged, Dóm tér 10, Hungary [2]Department of Pediatrics, University Medical School, Szeged, Hungary

(Received 17 September, 1987; accepted 1 December, 1987)

A six-hour culture of unstimulated peripheral blood from a patient with childhood erythroleukaemia was examined for chromosome karyotype. The characteristic chromosomal abnormalities of erythroleukaemia del (5q) and monosomy 7 were found in this case together with a marker chromosome and other chromosomal abnormalities. The results suggest the usefulness of this simple method in the cytogenetic diagnosis of some types of leukaemias.

Keywords: childhood erythroleukaemia, chromosome examination, short term culture

Introduction

Erythroleukaemia (EL) (FAB type M6) is a relatively rare disease in childhood, occurring in only 0.8–1.0% of childhood leukaemia [1]. EL is a haematopoietic malignancy with an abnormal proliferation of both erythroblasts and myeloblasts. Both the clinical and haematologic pictures suggest that the childhood and adult version of EL represent the same disease [1].

The characteristic chromosomal abnormalities of EL are as follows: −5 or del (5q); −7 or del (7q); −3 and t(3;?); dup (1); +8 [1, 2, 3, 4, 5].

In our study chromosome examinations were made on unstimulated peripheral blood from a nine-year-old boy with EL in the course of chemotherapy. He had an unusually long preleukaemic phase, a rapid progression refractory to the treatment and a very poor prognosis.

Case report

H. P., a nine-year-old boy, was admitted with purpura and extremely low platelet counts in May, 1986. His disease was classified as an amegakaryocytic form of thrombocytopenic purpura, and he was treated with prednisolone and supportive measures. Pancytopenia developed later, with a latent haemolytic process, but no specific cause could be identified on detailed investigation including repeated bone marrow examinations. In December, 1986, progressive anaemia

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

(hematocrit: 26%) and hepatosplenomegaly developed. At this time, the bone marrow contained about 80% erythroid elements (atypical and dyserythropoietic forms in 25%). WBC was 16.0×10^9/l with 5% neutrophils, 7% myelocytes, 3% metamyelocytes, 3% monocytes, 67% lymphocytes, 12% lymphoblasts and 3% erythroblasts; platelet count 25.0×10^9/l. A diagnosis of EL (FAB type M6) was established, in agreement with the cytochemical findings. Results of marker analysis of a bone marrow specimen:

Sm Ig$^+$ lymphocytes:	11%
E-rosettes:	28% (possibly peripheral elements)
VIB-C5$^+$ cells:	0%
VIL-AI$^+$ cells:	0%
VID-I$^+$ cells:	32%
VIP-I$^+$ cells:	27%
VIP-26$^+$ cells:	28%
VIT-6$^+$ cells:	0%
VIT-3b$^+$ cells:	31%
VIM-D5$^+$ cells:	23%
VIM-2$^+$ cells:	26%
VIM-12$^+$ cells:	10%
VIM-13$^+$ cells:	0%
VIE-G4$^+$ cells:	1%
TdT	–
cyt-Ig	–

Immunological analysis and consultations were made at the Hungarian Pediatric Oncology Group, Acute Leukaemia Study Center, Budapest. The investigations and clinical data indicated that this case represents a rare type of acute leukaemia in childhood, with an unusual long preleukaemic phase and a rapid progression refractory to treatment with Alexan, Lanvis and Methipred. Patient died on 3rd February 1987. The post mortem results were fully consistent with the clinical diagnosis.

Cytogenetic study

A six-hour culture of unstimulated peripheral blood cells from the patient was examined for chromosome karyotype using a standard technique [6, 7]. Briefly, 2 ml of peripheral blood was incubated in 10 ml of TC 199 medium at 37 °C for six hours. Vinblastin (0.5 μg/ml medium) was added to block the mitoses one hour before harvest. The fixed cells were transferred to glass slides, treated with trypsin, stained with Giemsa, and examined under a microscope at a magnification of 1600 ×. Karyotypes were analysed according to ISCN [8].

Results

Forty mitoses were investigated under the microscope; 5 were hyperdiploid (containing 47 chromosomes), 4 were hypodiploid (containing 45 chromosomes in 2 cases, and 32 and 39 chromosomes in a single case each), and the remaining 31 were diploid.

Table 1 shows the results of karyotyping. Of the 19 cells examined only 2 karyotypes (11%) were normal; all the others (89%) had numerical or structural

Table 1

Numerical and structural chromosome aberrations

Type of aberration	Number of mitoses
39, X, −7, −8, −10−17, −18−21, −Y, del (3) (p24-pter), del (6) (p11.1-pter)	1
45, X, −18, −Y, +mar, del (6) (p11.1-pter)	1
46, XY	2
46, XY, del (5) (q13−q15)	4
46, XY, −7, +mar, del (5) (q21−q23)	2
46, XY, −7, +mar, del (14) (q21−q24), t (17; ?) (q25; ?)	2
46, XY, −7, +mar, del (5) (q21−q23), inv (14) (q11 q24), t (17; ?) (q25; ?)	1
47, XY, +mar, del (5) (q21−q23)	3
47, XY, +mar, inv (14) (q11 q24), t (17; ?) (q25; ?)	1
47, XY, +mar, del (5) (q21−q23), del (14) (q21−q24), t (17; ?) (q25; ?)	1
48, XY, −7, del(1) (p34-pter), t (17; 21) (q25; q11) +3 mar	1

abnormalities. The most frequent aberrations were as follows: del (5)(q21-q23) in 7 cases (37%); −7 in 7 cases (37%); del (5)(q13-q15) in 4 mitoses (21%); del (14)(q21-q24) in 3 cases (16%); inv (14)(q11q24) in 2 mitoses (9.6%); 17q+ in 5 mitoses (26%), and a marker chromosome was detected in 7 cases (37%).

Discussion

In accordance with earlier data [1−5, 9], we found the characteristic chromosomal abnormalities of EL (del 5q), −7, and abnormalities of chromosome 17 (in the six-hour culture of unstimulated peripheral blood from our patient). At the time of the investigation these dividing leukaemic cells were in the late S or G_2 phases of the cell cycle at the start of culturing. The time of G_2 is four hours in human leukocytes [10], and leukaemic cells have a longer generation time [10, 11] than normal cells. In contrast with our method, the peripheral blood cells were cultured at least for 24 or 48 hours without PHA for conventional

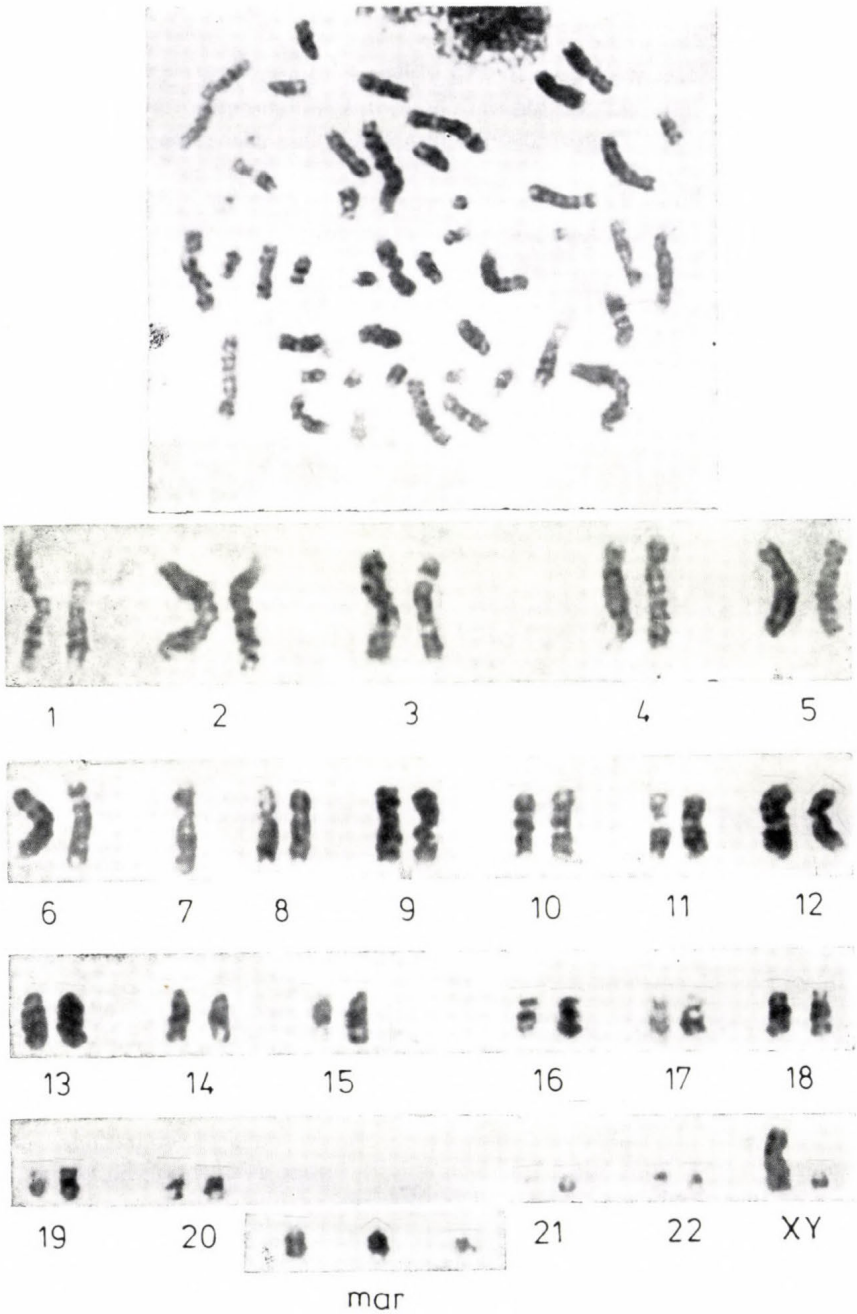


Fig 1. The karyotype shows the aberrations as follows: 48, XY, −7, del(1)(p34-pter), t(17; 21)(q25; q11) + 3 mar

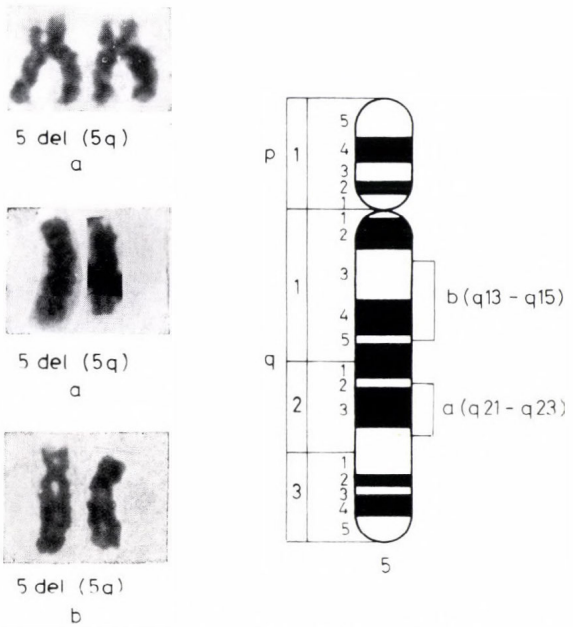


Fig. 2. Aberrations of chromosome 5 in EL: a. del(5)(q21-q23); b. del(5)(q13-q15)

cytogenetic analysis of leukaemic cells from unstimulated peripheral blood of patients with leukaemia [9, 12–14]. Monosomy 5 or del (5q) and monosomy 7 or del (7q) are the most common abnormalities in human preleukaemia [9, 15]. The other changes, such as del (6)(p11.1-pter), del (14)(q21–q24) and inv (14) (q11q24) may be secondary chromosome changes. The same marker chromosome (small acrocentric chromosome) was found in 7 mitoses.

The simple six-hour culture of unstimulated peripheral blood from our patient with EL was suitable for detection of the characteristic chromosomal abnormalities of this disease, which suggests the usefulness of this method in the cytogenetic diagnosis of some types of leukaemias (e.g. in AML and CML, but not in ALL – manuscript is under preparation).

References

1. Freedman, M. H., Chan, H. S. L., Chang, L.: Childhood erythroleukemia. Studies on pathogenesis using colony assays. *Am. J. Ped. Hematol/Oncol. 8*, 2 (1986).
2. Arthur, C. D.: Genetics and cytogenetics of pediatric cancers. *Cancer 58*, 534 (1986).
3. Mitelman, F.: Catalogue of chromosome aberrations in cancer. *Cytogenet. Cell Genet. 36*, 1 (1983).
4. Sandberg, A. A.: The chromosomes in human leukemia. *Semin. Hematol. 23*, 201 (1986).
5. Yunis, J. J.: The choromosomal basis of human neoplasia. *Science 221*, 227 (1983).

6. Autio, K., Schröder, J.: Chromosome break points in clonal and nonclonal chromosome changes in human chronic lymphocytic leukemia. *Hereditas 97*, 221 (1982).
7. Selypes, A., László, A.: A new translocation t(1; 4; 11) in congenital acute nonlymphocytic leukemia (acute myeloblastic leukemia). *Hum. Genet. 76*, 106 (1987).
8. ISCN: An International System for Human Cytogenetic Nomenclature Cytogenet. *Cell Genet. 21*, 313 (1978).
9. Nowell, P. C., Besa, E. C., Stelmach, T., Finan, J. B.: Chromosome studies in preleukemic states. V. Prognostic significance of single versus multiple abnormalities. *Cancer 58*, 2571 (1986).
10. Cronkite, E. P.: Kinetics of leukemic cell proliferation. *Semin. Hematol. 4*, 415 (1967).
11. Killmann, S. A.: Kinetics of leukaemic blast cells in man. *Clin. Hematol. 1*, 95 (1972).
12. Hayata, I., Sakurai, M., Kakati, S., Sandberg, A. A.: Chromosomes and causation of human cancer and leukemia: XVI. Banding studies of chronic myelocytic leukemia, including five unusual Ph^1 translocations. *Cancer 36*, 1177 (1975).
13. Kohno, S.-I., and Sandberg, A. A.: Chromosomes and causation of human cancer and leukemia: XXXIX. Usual and unusual findings in Ph^1 positive CML. *Cancer 46*, 2227 (1980).
14. Levin, M., LeConiat, M., Bernheim, A., Berger, R.: Complex chromosomal abnormalities in acute nonlymphocytic leukemia. Cancer Genet. *Cytogenet. 22*, 113 (1986).
15. Jacobs, A.: Human preleukaemia: Do we have a model? *Br. J. Cancer 55*, 1 (1987).

Haematologia 22 (1), pp. 25–35 (1989)

Human Fetal Liver as a Valuable Source of Haemopoietic Stem Cells for Allogeneic Bone Marrow Transplantation

D. T. PHAN[1], R. MIHALIK[1], M. BENCZUR[1], K. PÁLÓCZI[1], J. GIDÁLI[1], I. FEHÉR[1], J. DÖMÖTÖRI[2], CS. KISS[3], G. GY. PETRÁNYI[1], K. NATONEK[1], S. R. HOLLÁN[1]

[1]National Institute of Haematology and Blood Transfusion, 1502 Budapest, PO Box 44, Hungary; [2]János Hospital, Budapest; [3]Róbert Károly Hospital, Budapest

(Received 28 September, 1987; accepted 15 February, 1988)

The CFU-GM and T cell contents of human fetal livers were studied at various times between 6–14 weeks of gestation. The number of CFU-GM increased parallel to gestational age, especially after week 10. Cells bearing mature T cell markers, however, were found only in one case out of 35 fetal liver samples.

Cryopreservation of fetal liver cells hardly affected the viability and proliferative capacity of CFU-GM in the sample.

According to these findings fetal liver is, at least up to the 14th gestational week, practically free of mature T cells but it does contain a considerable amount of CFU-GM (an accepted indicator of pluripotent stem cell content), consequently fetal liver can be considered as a valuable source of haemopoietic stem cells for allogeneic bone marrow transplantation for children.

Keywords: human fetal liver cells, myeloid progenitors, mature and progenitor T cells, cryopreservation

Introduction

On the basis of animal experiments and clinical experiences, transplantation of fetal liver cells (FLC) seems to offer a promising substitute for MHC-incompatible bone marrow transplantation [1]. In animals, since the first studies of Uphoff 1958, most of the subsequent investigations have shown that FLC induces no or only moderate graft-versus-host disease (GVHD) in strongly histoincompatible donor recipient pairs in mice [2, 3], rabbits [4], dogs [5, 46], horses [6] and monkeys [7].

In man, following the initial trial by Scott et al. 1961, a series of FLC transplantation have been performed in SCID patients with aplastic anaemia [8, 9, 10, 11], severe combined immunodeficiency [12, 13], acute lymphoid and non-lymphoid leukaemia [14, 15], and in individuals suffering from inherited enzym-deficiencies [16]. In general, GVHD in humans occurred rarely, durable engraftment and successful reconstitution of bone marrow functions and cellulae immunity were found in aplastic anaemia patients and SCID, respectively. However, successful FLC transplantations were limited due to insufficient graft size [17].

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Theoretically, as emphasized by Buckley et al. 1976 [18], fetal donors from less than 10 weeks of gestation should be the most suitable sources of FLC transplantation. Heit et al. 1980 [19], on the other hand, proposed fetal liver (FL) as the optimal source for grafting at the gestational age of 18 to 20 weeks since in the number of haemopoietic stem cells obtained from a fetal liver in this period was equivalent to, er even higher than the number of stem cells in a bone marrow graft harvested in the extrauterin life. Most of the clinical reports, however, do not deal with the haemopoietic stem cell content and the relative immune properties of fetal liver grafts. Concerning routine clinical bone marrow transplantation, the correlation between the rate of engraftment and the amount of donor nucleated cells of CFU-GMs infused has been studied. A positive correlation was found with engraftment in some studies [20, 21], but not in others [22, 23]. Most recently, Ma et al. 1987 [24] reported, however, that the correlation between the number of progenitor cells grafted and the haemopoietic recovery was not linear. This implies that there is a threshold for the successful engraftment. To contribute to a better understanding of the optimal conditions for the engraftment of FLC, we studied in the present work the ontogenetic development of T lymphocytes, and the numbers of haemopoietic stem cells (CFU-GM) in livers of 6–14 weeks old human fetuses.

Materials and Methods

Fetal livers: 35 fetal livers were studied. The age of the fetuses ranged between 6 and 14 weeks. The age distribution and the number of livers are listed in Table 1. Fetal livers were removed from the fetuses within 10 min after abortion and placed into cold RPMI-1640 medium supplemented with 10% inactivated FCS, heparin and antibiotics, and stored at 4 °C until cell separation. The livers were washed three times to eliminate blood contamination, then homogenized with gentle aspiration, and the cells were suspended in cold medium. After cell counting the cell suspensions were layered over Ficoll-Hypaque (density 1.077

Table 1

Morphological distribution (%) of liver cells in human fetuses by gestational ages between 6 and 14 weeks

Morphology	Gestational age (weeks)							
	6 (1)*	7 (13)	8 (8)	9 (5)	10 (7)	11 (2)	13 (2)	14 (13)
Blasts	10	11	5	7	8	13	6	6
Lymphoid	2	3	6	5	7	6	7	9
Myeloid	3	5	3	4	4	2	4	5
Erythroid	81	78	83	80	75	78	83	74
Unidentified	4	3	3	4	6	1	0	4

* Number of samples

g/ml) [25], and mononuclear cells were collected and washed two times with cold medium. After these procedures, the ratio of living cells was over 95% as determined by trypan blue dye exclusion.

Indirect immunofluorescence (IF) staining: IF staining was performed by a method described previously [26, 27], with the following reagents: MoAbs specific to CD_2, CD_3, CD_4, CD_8 antigens (Ortho, Raritan, NJ, USA), and rabbit anti-mouse IgG-FITC (DACKO, Denmark). Per cent of positive cells was determined by fluorescence microscopy and flow cytometry-FACS-STAR (Becton Dickinson Mountain View, CA, USA).

E rosette formation: The method was based on that published in the literature [28], using sheep red blood cells (SRBC) treated with AET (2-aminoethylisothiouronium bromide, Calbiochem, USA) [29].

Culture of myeloid progenitor cells: Granulocyte-macrophage progenitors (CFU-GM) were cultured in a semisolid agar medium by the method of Pike and Robinson [30], using human placental conditioned medium (HPCM) as a source of colony-stimulating activity [31]. Colonies grown from FLC were usually larger and denser than those of the adult bone marrow (Fig. 1).

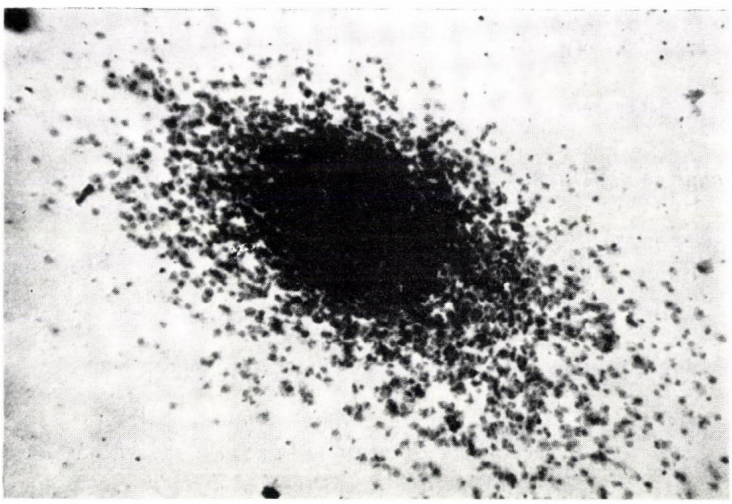


Fig. 1. Granulocyte-macrophage colony obtained from 10-week old fetal liver cells. Magnification × 63

Cryopreservation of FLCs: One ml of FLC suspension (about 5×10^6 to 10^7 viable cells/ml) in RPMI-1640 medium supplemented with 20% FCS and 10% dimethylsulphoxide (DMSO) was put in 2-ml cryotubes (Nunc, Denmark) and frozen in a programmed freezer [32]. The frozen cells were stored in liquid nitrogen for two months.

The protocol of the experiment is shown in Fig. 2.

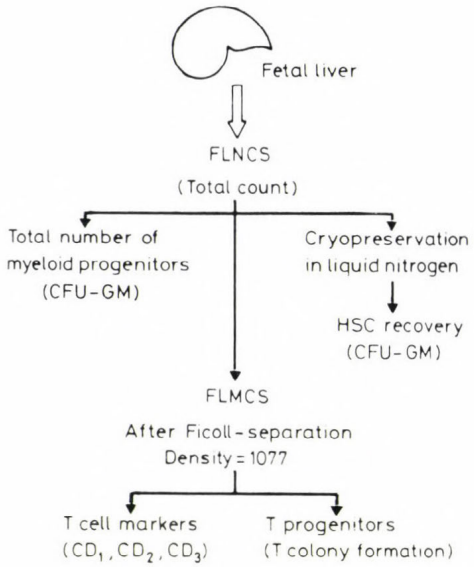


Fig. 2. Scheme of the investigations on human fetal liver cells. FLNCs: fetal liver nucleated cells; FLMCs: fetal liver mononucleated cells

Results

Cellular composition of fetal liver: More than 75% of the FLCs were erythroid and 2–5% were myeloid cells. The number of blast cells decreased parallelly with the gestational age (from 10% to 6%), whereas the ratio of lymphoid cells increased with the maturation of the fetuses (from 2% to 9%) (Table 1).

Table 2

Detection of surface markers of T lymphocytes on human fetal liver cells

Gestational age (week)	n	T lymphocyte markers					
		ER	CD1	CD2	CD3	CD4	CD8
6	1	0	0	0	0	0	0
7	3	0	0	0	0	0	0
8	8	0	0	0	0	0	0
9	5	0	0	0	0	0	0
10	7	0	0	0	0	0	0
11	2	0	0	0	0	0	0
12	4	0	0	0	0	0	0
12	2	0	0	0	0	0	0
14	3	0	0	0	1*	0	0

* 2% of the cells were positive
ER: E rosette

Mature and progenitor T cells: We found mature T cell associated antigen possessing cells in only one liver of a 14 wk old fetus out of the studied 35 fetal livers (Table 2). In this case, 2% of the fetal liver mononuclear cells reacted with anti-CD3 MoAb, whereas in the other cases neither E rosette formation nor positive reaction with anti-T cell MoAbs was found (Table 2). We attempted to establish T cell colonies from the various FLC suspensions, but no T colony forming cells could be detected. Moreover, preincubating fetal liver mononuclear cells with PMA (phorbolmyristateacetate) by the method described by Sazawar et al. [33], neither cells bearing markers of the mature T cells (E rosette, CD_3) nor T-CFCs were found. (Data not shown)

Nucleated cell and granuloid progenitor contents of fetal liver: The number of nucleated cells and GM-CFU were assayed in each FL-s. The amount of nucleated cells increased parallel with the gestational age. The youngest livers (6 weeks old) contained 14.8×10^6 cell which increased to 1.7×10^8 cells per liver at week 14 of gestation (Fig. 3). To study the relationship between the number of plated cells and GM-CFU colonies at the different stage of embryonic development different number of FLC were plated. This experiment was carried out by

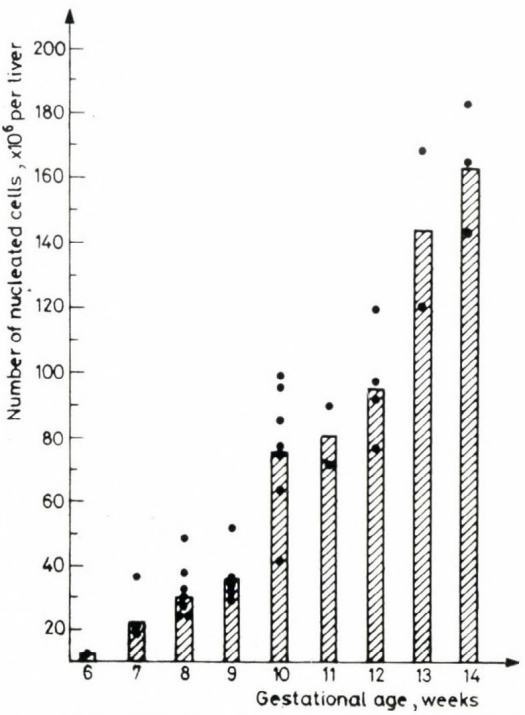


Fig. 3. Nucleated cell content of fetal livers at different ages of gestation. Dots indicate individual livers, the mean for each age-group is presented by a column

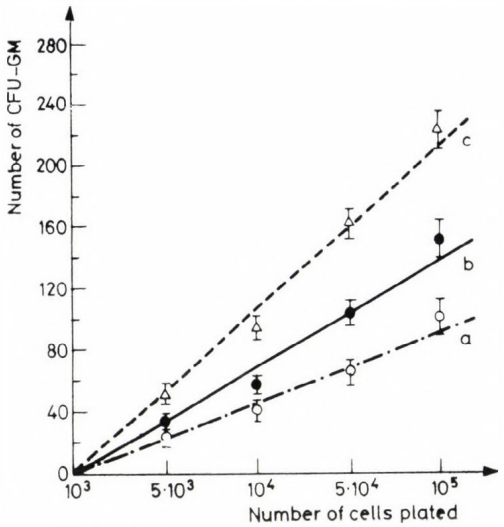


Fig. 4. The relationship between GM-CFU and the number of FL cells plated. Each point represents mean for triplicate culture

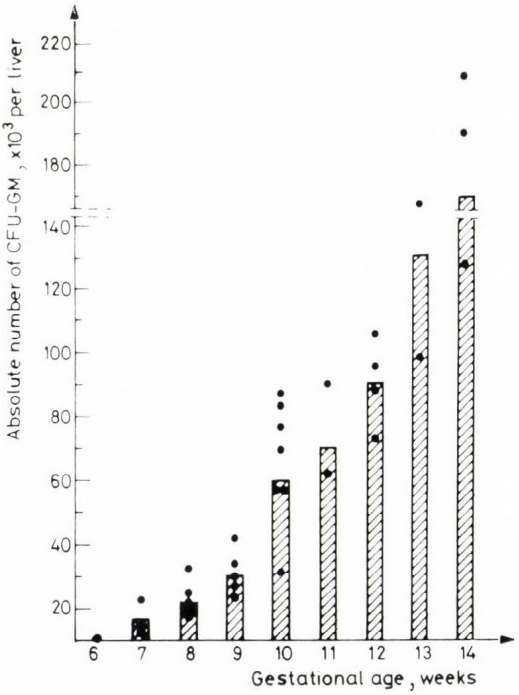


Fig. 5. Total number of GM-CFU obtained from fetal livers at different ages of gestation. Dots indicate individual livers, the mean for each age-group is presented by a column

Table 3

Recovery of cryopreserved fetal liver cells stored in liquid nitrogen for two months

No exp.	Age of fetuses (week)	Number of cells $\times 10^6$ Before/After storage		Recovery (%)	CFU-GM/10^5 plated cells Before/After storage		Recuvery (%)
1	12	5.6	4.8	85	71 ± 2*	65 ± 3*	78
2	8	6.6	6.0	90	66 ± 3	54 ± 6	74
3	12	10.4	8.2	78	97 ± 6	76 ± 5	61
4	8	3.0	2.4	80	62 ± 4	52 ± 2	83
5	8	10.0	9.4	94	89 ± 12	70 ± 5	73
6	10	8.4	8.0	56	92 ± 5	98 ± 3	101
7	14	12.2	6.7	67	125 ± 4	201 ± 20	101
Mean ± SD (%)				78.5 ± 13.2			81.5 ± 14.8

* experiment was performed in triplicate

plating FLC from 10, 11 and 14 week old fetuses. The relationship between plated cell number and colony formation proved to be linear in each case, but the concentration of CFC-GM increased parallel with the age of the FL-s (Fig. 4). The total amount of FL CFU-GM is also increased parallel with the age of the fetuses (Fig. 5).

Recovery of cryopreserved FLCs: In order to obtain data on survival of FLCs after cryopreservation, recovery of CFU-GM was studied. In the average of 7 experiments 78.5% of nucleated cells and 81.5% of CFU-GM could be recovered from cryopreserved FLCs. The results indicate that human FLCs can be stored in liquid nitrogen for long periods without considerable loss of hemopoietic stem cells (Table 3).

Discussion

The aim of this study was to obtain data on the cellular composition of fetal liver as a source for bone marrow grafting. The E rosette formation test, IF-FACS analysis with anti-T specific MoAbs including CD_1, CD_2, CD_3, CD_4, CD_8, and T-colony formation assay were used to detect mature and progenitor T cells and we failed to detect either mature T cells, or T progenitors in the FL of young fetuses. However, it could not be excluded, that our test systems were not sensitive enough to detect premature or T "stem" prethymic cells in the fetal liver. Previous studies, in which T lymphocyte phenotypes were examined, indicate that the earliest fetal thymocytes that show E rosette formation and give proliferative response to PHA and histocompatibility-associated antigens appear about 10 weeks after conception [34], then, from the 15th week on, the thymic cells express antigens

Table 4

Number of granulocytic-monocytic progenitor cells per 10^5 nucleated cells cultured

Adult human bone marrow		Human fetal liver cells		
Gestational age (week)	No of CFU-GM	Donor	Age (yr)	No of CFU-GM
6	66	1	30	43.3 ± 2.0
7	70.0 ± 6.2	2	26	46.9 ± 9.0
8	74.1 ± 11.6	3	10	38.3 ± 7.6
9	83.4 ± 5.5	4	43	48.0 ± 1.5
10	85.7 ± 8.5	5	13	39.3 ± 3.0
11	94.0 ± 9.9			
12	95.7 ± 12.6			
13	90.0 ± 11.3			
14	104.0 ± 18.7			

of mature T cells [35]. In other studies, the fetal liver was found to contain a few (0 – 5%) cells bearing the pan-T antigens during the the most active phase of hemopoiesis in the fetal liver [35, 36]. It has also been reported that fetal liver cells showed a weak response to mitogens or allogeneic cells. This response of FLCs reflects rather a non-specific response of immature hemopoietic cells than a mature T cell function [37].

Morphologically, the lymphocytes in our samples amounted to 2 – 9%, but they proved to be predominantly B-cell and NK-cells [38], corresponding to the fact that experimental and clinical FLC transplants produced GVHD very rarely [2, 8, 9, 10, 15]. These evidences, again, were consistent with the view that fetal liver as well as fetal bone marrow might be human "bursa equivalent" organs [39, 40]. However, it has been reported that the liver of older human and murine fetuses (after 15 weeks or 15 days or more, respectively), contains cells bearing pan-T cell antigens or T progenitor cells [35, 36, 41].

In the present study, we found that the 10 – 14 week old fetal livers contained a considerable amount (about $6-20 \times 10^4$) of CFU-GM (Fig. 5). Based on results reported by Ma et al. 1987 about the requested dose of CFU-GM per kg body weight [24], the number of FL CFU-GM (20×10^4 CFU-GM per liver) detected in our fetuses were sufficient to treat recipients under 20 kg body weight.

We also found morphologically that erythropoiesis prevails in fetal liver. More than 75% of the fetal liver nucleated cells were erythroid cells (Table 1). This is consistent with previous observations [19, 35, 42, 43]. However, 3 – 5% mature myeloid cells (Table 1) and a considerable number of granulocyte-macrophage progenitors (Fig. 5) were also found in our fetuses. Again, this is consistent with the fact that myeloid-associated antigens are early expressed on FLCs, including those of very young (6 wk old) fetuses [44] and that high number of CFU-GM can be obtained from fetuses older than 15 wks [17, 19, 45].

In conclusion, the results of the present studies on FLCs of young fetuses indicate that mature and progenitor T cells were absent, while myeloid progenitors were developed in an early stage of embriogenesis. The FLC of 10–14 wk old fetuses may be considered as a valuable source of haemopoietic stem cells free from mature T lymphocytes for allogeneic BMT, particularly for recipients with low body weight, who have no HLA-sibling donors.

References

1. Prümmer, O., Fliedner, T. M.: The fetal liver as an alternative stem cell source for hemolymphopoietic reconstitution. *Int. J. Cell Cloning 4*, 237 (1986).
2. Uphoff, D. E.: Preclusion of secondary phase of irradiation syndrome by inoculation of fetal hemopoietic tissue following lethal total-body irradiation. *J. Natl. Cancer Inst. 20*, 625 (1958).
3. Bortin, M. M., Saltzstein, F. C.: Graft-versus-host inhibition: fetal liver and thymus cells to minimize secondary disease. *Science 164*, 316 (1969).
4. Porter, K. A.: Use of fetal hemopoietic tissue to prevent late deaths in rabbit radiation chimeras. *Br. J. Exp. Pathol. 40*, 273 (1959).
5. Prümer, O., Werner, C., Raghavachar, A., et al.: Fetal liver transplantation in the dog: II. Repopulation of the granulocyte-macrophage progenitor cell compartment by fetal liver cells from DLA-indentical siblings. *Transplant 40*, 498 (1985).
6. Perryman, L. E., McGuire, T. C., Torbeck, R. L., Magnuson, N. S.: Evaluation of fetal liver cell transplantation for immunoreconstitution of horses with severe combined immuno-deficiency. *Clin. Immunopathol. 23*, 1 (1982).
7. Van-Putten, L. M., Van Bekkum, D. W., DeVries, M. J.: Transplantation of hemopoietic cells in irradiated rhesus monkeys. In: Radiation and the control of the immune response. International Atomic Energy Agency, Vienna 1968, p. 41.
8. Scott, R. B., Matthias, J. Q., Constandoulakis, M., Kay, H. E. M., Lucas, P. F., White-Side, J. D.: Hypoplastic anaemia treated by transplantation of foetal hemopoietic cells. *Brit. J. Med. 2*, 1385 (1961).
9. Kansal, V., Sood, S. K., Batra, A. K., Adhar, G., Malviya, A. K., Kucheria, K., Balakrishnam, K.: Fetal liver transplantation in aplastic anemia. *Acta Haematol. 62*, 128 (1979).
10. O'Reilly, R. J., Pollack, M. B., Kapoor, N., Kirkpatrick, D., Dupont, B.: Fetal liver transplantation in man and animals. In: Recent advances on bone marrow transplantation. Gale R. P. (ed.), Alan R. Ziss Inc., New York 1983, p. 799.
11. Kochupillai, V., Sharma, S., Francis, S., Mehra, N. K., Nanu, A., Kalra, V., Menon, P. S., Bha, F., Gava, M.: Bone marrow reconstitution following human fetal liver infusion (FLI) in aplastic anemia. *Prog. Clin. Biol. Res. 193*, 251 (1985).
12. Githens, J. H., Fulgeniti, V. A., Suvatte, V., Schroter, G., Hathaway, W. E., Pearlman, D. S., Kay, H. E. M., Terasaki, P. I., Hill, G. J., Kempe, C. H. Cox, S. T.: Grafting of fetal thymus and hemopoietic tissue in infants with immune deficiency syndromes. *Transplantation 15*, 427 (1973).
13. Löwenberg, B., Vossen, J. M. J. J., Dooren, L. J.: Transplantation of fetal liver cells in treatment of severe combined immunodeficiency disease. *Blut 34*, 181 (1977).
14. Lucarelli, G., Izzi, T., Porcellini, A., et al.: Fetal liver transplantation in aplastic anemia and acute leukaemia. In: Fetal liver transplantation: current concepts and future directions. Lucarelli, G., Fliedner, T. M., Gale, R. P. (eds), Excerpta Medica, Amsterdam 1980, p. 284.
15. Kochupillai, V., Sharma, S., Francis, S., Mehra, N. K., Nanu, A., Verma, I. C., Takkar, D., Kurna, S., Gokhale, U.: Fetal liver infusion: an adjuvant in the therapy of acute myeloid leukaemia (AML). *Prog. Clin. Biol. Res. 193*, 267 (1985).

16. Touraine, J. L., Malik, M. C., Trager, J., Perrot, H., Maire, I.: Attempt at enzyme replacement by fetal liver transplantation in Fabry's disease. *Lancet 1*, 1094 (1979).
17. Löwenberg, B.: Fetal liver transplantation: alternative. In: *Biology of bone marrow transplantation*. Gale, R. P. and Fox, C. F. (eds), Academic Press, New York 1980, p. 417.
18. Buckley, R. H., Whisnant, J. K. Schiff, R. I., Gilbertson, R. B., Huang, A. T., Platt, M. S.: Correction of severe combined immunodeficiency by fetal liver cells. *New Engl. J. Med. 294*, 1076 (1976).
19. Heit, W., Forzsolt, F., Thiel, E., Kubanek, B.: Fetal liver, a source for hemopoietic reconstitution without GVHD? In: *Biology of bone marrow transplantation*. Gale, R. P. and Fox, C. F., Academic Press, New York 1980, p. 507.
20. Storb, R., Ross., L., Prentice, R. L. and Thomas, E. D.: Marrow transplantation for treatment of aplastic anemia. An analysis of factors associated with graft rejection. *New. Engl. J. Med. 296*, 61 (1977).
21. Niederwieser, D., Gratwohl, A., Oberholzer, M., Osterwalder, B., Nissen, C., and Speck, B.: Bone marrow cell dose and kinetics of recovery following allogeneic marrow transplantation in man. *Blut 47*, 355 (1983).
22. Faille, A., Maranichi, D., Gluckman, E., Deveregie, A., Balitrand, N., Ketels, F., and Dresch, C.: Granulocyte progenitor compartments after allogeneic bone marrow graft. *Scand. J. Haematol. 26*, 202 (1981).
23. Norrie, S., Atkinson, K., Chan, P., and Biggs, J. C.: Reconstitution of hemopoiesis after allogeneic bone marrow transplantation. *Transplant. Proc. 16*, 1021 (1984).
24. Ma, D. D. F., Varga, D. E., Biggs, J. C.: Donor marrow progenitors (CFU-Mix, BFU-E and CFU-GM) and haemopoietic engraftment following HLA matched sibling bone marrow transplantation. *Leukemia Res. 11*, 141 (1987).
25. Boyum, A.: Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest. 21*, (suppl. 97), 77 (1968).
26. Bodger, M. P., Francis, G. F., Delia, D., Granger, S. M., Janossy, G.: A monoclonal antibody specific for immature human hemopoietic cells and T lineage cells. *J. Immunol. 127*, 2269 (1981).
27. Neame, P. B., Soamboonsrup, P., Brownman, G., Barr, R. D., Saeed, N., Cham, B., Pai, M., Berger, A., Wilson, E. C., Walker, I. R., McBridge, J. A.: Simultaneous or sequential expression of lymphoid and myeloid phenotypes in acute leukemia. *Blood 65: 142* (1985).
28. Brown, G., Greaves, M. F.: Cell surface markers for human T and B lymphocytes. *Eur. J. Immunol. 4*, 302 (1974).
29. Madsen, M., Johansen, H. E.: A methodological study of E rosette formation with AET-treated sheep red blood cells. *J. Immunol. Methods 27*, 61 (1979).
30. Pike, B. L., Robinson, W. A.. Human bone marrow colony growth in agar gel. *J. Cell. Physiol. 76*, 77 (1970).
31. Burgess, A. W., Wilson, E. W. A., Metcalf, D.: Stimulation by human placental conditional medium of haemopoietic colony formation by human bone marrow cells. *Blood 49*, 573 (1977a).
32. Douay, L., Gorin, N. C., David, R. et al.: Study of granulocytes-macrophage progenitor CFU-c: preservation after slowfreezing of bone marrow in the gas phase of liquid nitrogen. *Exp. Hematol. 10*, 360 (1982).
33. Sazawar, T., Hyodo, S., Kishi, T., Kashiwa, H., Karawa, T., Kittaka, E., Sakano, T., Usui, T.: Comparison of phorbol myristate acetate and phytohemagglutinin as stimulators of in vitro T lymphocyte colony formation of human peripheral blood lymphocites. *Immunol. 53*, 499 (1984).
34. Hayward, A. R.: Development of lymphocyte responses and interaction in the human fetus and newborn. *Immunol. Rev. 57*, 39 (1981).
35. Rosenthal, P., Rim, T. J., Umiel, T., Griffin, J. D., Osathanondh, R., Schlowwman, S. F.,

Nadler, L. M.: Ontogeny of human hemopoietic cells: analysis utilizing monoclonal antibodies. *J. Immunol. 31*, 232 (1983).
36. Velardi, A., Cooper, M. D.: An immunofluorescence analysis of the ontogeny of myeloid, T and B lineage cells in mouse haemopoietic tissues. *J. Immunol. 133*, 672 (1984).
37. Mukhopahyay, N., Fernbach, D. J., Mumford, D. M., South, M. A.: T and B cells and immune competence in human fetal liver cells. *Clin. Immunol. Immunopathol. 10*, 59 (1978).
38. Phan, D. T., Mihalik, R., Benczúr, M., Dömötöri, J., Kiss, Cs., Petrányi, Gy., Hollán, S. R.: Expression of differentiation- and histocompatibility associated antigens on human fetal liver cells. VIII. European Immunology Meeting, Abstract Book, Zagreb, 1987, p. 9.
39. Owen, J. J. T., Cooper, M. D., Raff, M. C.: In vitro generation of B lymphocytes in mouse fetal liver, a mammalian "bursa equivalent". *Nature 249*, 361 (1974).
40. Gathings, W. E., Kubagawa, H., Cooper, M. D.: A distinctive pattern of B cell immaturity in perinatal humans. *Immunol. Rev. 57*, 107 (1981).
41. Rabinovich, H., Umiel, T. and Globerson, A.: T cell progenitors in the mouse fetal liver. *Transplant 35*, 40 (1983).
42. Thomas, D. B., Yoffey, J. M.: Human fetal hemopoiesis. 1. The cellular composition of fetal blood. *Br. J. Haematol. 8*, 295 (1962).
43. Kelemen, E., Calvo, W., Fliedner, T. M.: *Atlas of human hemopoietic development*. Springer Verlag, Berlin 1979, p. 84.
44. Phan, D. T., Benczur, M., Pálóczi, K., Mihalik, R., Kotlán, B., Gidáli, J., Fehér, I., Dömötöri, J., Kiss, Cs., Petrányi, Gy., Hollán, S. R.: Early expression of HLA-class-I, class-II and myeloid antigens on human fetal liver cells. *Intl. J. Cell Cloning* (Submitted) (1987).
45. Hann, I. M., Bodger, M. P., Hoffbrand, A. V.: Development of pluripotent hemopoietic progenitor cells in the human fetus. *Blood 62 (52)*, 118 (1983).

Haematologia 22 (1), pp. 37—42 (1989)

Glutathione Reductase in the South-Western Province of Saudi Arabia — Genetic Variation vs. Acquired Deficiency

M. A. F. EL-HAZMI[1], A. S. WARSY[2]

[1] Medical Biochemistry Department, College of Medicine & King Khalid Hospital, King Saud University, P. O. Box 2925, Riyad, Saudi Arabia; [2]Centre for Science & Medical Studies for Girls, King Saud University, Riyadh, Saudi Arabia

(Received 22 July, 1987; accepted 8 December, 1987)

The activity of glutathione reductase (GR) is closely associated with the riboflavin level in diet. Dietary deficiency of this water-soluble vitamin causes glutathione reductase deficiency. Furthermore, a variable frequency of GR variants with reduced activity has been reported in several populations. In an attempt to determine GR deficiency due to genetic (GR variant) and acquired causes (riboflavin deficiency), red cell GR activity was estimated in 461 male and female Saudis from the South-Western province of Saudi Arabia. The frequency of genetic GR deficiency (GR variant) was 24.5% in Saudi males and 20.3% in females. The frequency of acquired GR deficiency (riboflavin deficiency) was 17.8% and 22.4%, respectively. Interaction between genetic GR deficiency and other genetic abnormalities, i.e. sickle cell gene and glucose-6-phosphate dehydrogenase deficiency were also estimated. No specific link could be demonstrated.

Keywords: glutathione reductase, riboflavin, Saudi Arabia

Introduction

Glutathione reductase (GR; EC 1.6.4.2) is associated with the hexose monophosphate shunt, and catalyses the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH) using either NADPH or NADH as a proton donor [2]. This reaction is of special significance for the stability and integrity of the red cells, since low GSH levels are shown to be associated with drug induced haemolytic anaemias [3].

Several studies have shown a variable frequency of GR deficiency in different populations. It has been suggested that GR deficiency is either due to the presence of GR variants with slightly reduced or very low activity [14, 21] or to a nutritional deficiency of riboflavin [16, 17]. The close association between GR activity and riboflavin status is due to the fact that flavin adenine dinucleotide (FAD), the co-enzyme form of riboflavin, is required by GR as a co-factor [16]. This latter form of deficiency can be easily managed by the correction of the diet.

We conducted this study on the population of the South-Western province of Saudi Arabia to determine the prevalence of genetic and nutritional GR deficiency and to investigate the frequency of association of GR deficiency with

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

sickle cell and glucose-6-phosphate dehydrogenase deficiency genes which are reported to occur at a high frequency in this population [4, 5].

In this paper our results are presented and discussed in the light of prevailing environmental and genetic factors.

Materials and Methods

A total of 461 male and female Saudis attending outpatient clinics in the Ministry of Health hospitals for minor illnesses were investigated. Two ml of blood was collected by venipuncture in tubes containing acid-citrate dextrose (ACD), and haematological parameters were estimated using Coulter Counter ZF6 with a haemoglobinometer attachment. The plasma was separated from the red cells by centrifugation at 1000 RPM for 5 min and the cells were washed twice with cold physiological saline. Haemolysates were prepared by adding cold distilled water or 0.2% digitonin.

The haemoglobin phenotypes were determined by electrophoresis at alkaline [18] and acid pH [20]. The activity of GR was determined in presence and absence of flavin adenine dinucleotide (FAD) using a spectrophotometric method described by Beutler [1]. The decrease in absorbance was measured for 3 min at 340 nm in a test system at 30 °C containing 2 ml of 0.1 M Tris-HCl buffer (pH 8.0), 0.5 M EDTA, 2 mM NADPH (0.1 ml), 1 mM oxidized glutathione (1mM) and 0.1 ml of the haemolysate.

The activity coefficient (AC) of GR was calculated for each sample as follows:

$$AC = \frac{\text{Decrease in absorbance with added FAD}}{\text{Decrease in absorbance without FAD}}$$

G-6-PD activity was determined in each sample using kits from Boehringer Mannheim GmbH.

Chi square analysis was used to obtain the significance of difference in results between different groups. $P < 0.05$ was considered significant.

Results

The mean and normal range for GR activity, established by using 82 normal individuals, was 3.7 ± 1.3 IU/mg Hb in absence of FAD and 3.9 ± 1.2 IU/gm Hb in the presence of FAD. Samples with low GR activity (less than 2.00 IU/gm Hb) both in the presence and absence of FAD and an AC between $0.9-1.2$ were considered GR deficient. (A change in OD 340 less than 0.01/min was considered partially deficient [9].) Samples with AC equal to or above 1.3 were considered riboflavin deficient [22].

Table 1

Frequency of partial GR deficiency in the Saudi males and females in South-Western province of Saudi Arabia

Sex	No. investigated	Frequency of GR deficiency			
		Due to variant		Due to riboflavin deficiency	
		No.	Frequency	No.	Frequency
Male	269	66	24.5%	48	17.8%
Female	192	39	20.3%	43	22.4%

None of the samples in the male and female populations indicated complete GR deficiency, however, several samples showed a partial GR deficiency. In vitro addition of FAD did not improve the GR activity in these cases. Samples with reduced GR activity due to riboflavin deficiency showed an increase in the GR activity when FAD was added in vitro. The frequency of partial GR deficiency and riboflavin deficiency in the male and female population was calculated and the results are presented in Table 1.

The individuals were classified on the basis of their haemoglobin phenotypes as normal (Hb AA), heterozygotes to sickle haemoglobin (Hb AS) and homozygotes to sickle haemoglobin (Hb SS). The frequency of GR deficiency in the different

Table 2

Frequency of partial GR deficiency in individuals with different haemoglobin genotypes

Hb Genotype	Sex	No. investigated	Frequency of GR deficiency (%)	Frequency of G-6-PD deficiency (%)
AA	M	208	26.4	14.4
	F	155	22.5	7.7
AS	M	41	26.8	14.6
	F	33	12.1	3.0
SS	M	3	—	33.0
	F	4	—	25.0

groups was calculated and presented in Table 2. The frequency of G-6-PD deficiency was also calculated in the groups and the results are presented in Table 2. None of the samples revealed simultaneous G-6-PD and GR deficiency. No GR deficiency was encountered among the sickle cell homozygotes (Hb SS).

Discussion

Riboflavin plays a significant part in the normal functions. Activity of GR and deficiency of riboflavin in diet is a common cause of reduced GR activity [16, 17]. In addition, GR variants with reduced activity have been reported in several populations, these are either partial or complete GR deficiencies [9, 12, 13, 14, 21, 23]. The estimation of GR in presence and absence of FAD and determination of activity coefficient (AC) of GR has been employed successfully for the differentiation of nutritional and genetic GR deficiency [19, 22].

The AC value which represents the degree of saturation of the apoenzyme with riboflavin differentiates between cases of riboflavin deficiency and genetically determined GR deficiency. An AC range of 0.9–1.2 has been considered as the normal range [15], and samples with AC value of 1.3 or more are riboflavin deficient, while the GR deficient samples have low GR activity both in the presence and absence of FAD, and the AC is in the normal range.

The results of this study show that riboflavin deficiency is a common cause of GR deficiency in the population of the South-Western province, which is probably due to their nutritional habits. In addition, gastro-intestinal problems may also influence the absorption of riboflavin.

Furthermore we found a high frequency of partial GR deficiency in this population. This is higher than the frequency of GR deficiency encountered in populations in other regions of Saudi Arabia [9, 11] and confirms our previous suggestion that GR deficiency resulting from partially inactive mutants of GR is probably quite common in different populations. Other populations reported to have partial GR deficiency include South Vietnamese, Iranians, Ethiopians, Afro-Americans, European Americans [13], Sudanese [21] and Spanish [14]. Frequencies ranging between 0.3–22% have been reported. In this respect the population in the South-Western province of Saudi Arabia has the highest frequency of partial GR deficiency compared to all other populations investigated so far.

GR deficiency was also encountered in association with sickle cell gene. However, no specific correlation could be demonstrated between the different red cell genetic abnormalities. In this population the frequency of these abnormal genes is high [4, 5]. As there is a high rate of consanguinity, two or more abnormal genes are often inherited by an individual [6, 7, 8, 9, 10]. This is believed to play a role in the resistance of the carrier of the abnormal genes against the environmental and genetic factors.

The high frequency of partial GR deficiency in the Saudi population, which most likely results from GR variants with reduced activity both in homozygotes and heterozygotes, may, in some unknown way, protect the individual carrier against environmental or genetic factors. Further studies are required to identify the genetic variants of GR and to determine the possible benefit to the carrier of the variants.

Acknowledgement

This study was supported in part by Grant No. AT-04-074 from King Abdul Aziz City for Science and Technology (KACST) and by King Saud University.

References

1. Beutler, E.: Red cell metabolism — manual of biochemical methods. Grune & Stratton, New York 1975, pp. 69—70.
2. Beutler, E., Yeh, M. K. Y.: Erythrocyte glutathione reductase. *Blood 21 (5)*, 573 (1963).
3. Carson, P. E., Brewer, G. J., Ickes, C.: Decreased glutathione reductase with susceptibility to hemolysis (Abstract). *J. Lab. Clin. Med. 58*, 804 (1961).
4. El-Hazmi, M. A. F.: Haemoglobin disorders: a pattern for thalassaemia and haemoglobinopathies in Arabia. *Acta Haemat. 68*, 43 (1982).
5. El-Hazmi, M. A. F.: Abnormal hemoglobins and allied disorders in the Middle East (Saudi Arabia). In: Bowman: *Distribution and evolution of hemoglobin and globin loci*. Elsevier, New York 1983, pp. 239–249.
6. El-Hazmi, M. A. F.: Clinical manifestations and laboratory findings of sickle cell anaemia in association with α-Thalassaemia in Saudi Arabia. *Acta Haemat. 74*, 155 (1985).
7. El-Hazmi, M. A. F.: Studies on sickle cell heterozygotes in Saudi Arabia — Interaction with α-Thalassaemia. *Acta Haemat. 75*, 100 (1986).
8. El-Hazmi, M. A. F., Warsy, A. S.: Aspects of sickle cell gene in Saudi Arabia — Interaction with G-6-PD deficiency. *Hum. Genet. 68*, 320 (1984)
9. El-Hazmi, M. A. F., Warsy, A. S.: Glutathione reductase deficiency in association with sickle cell and thalassaemia genes in Saudi populations. *Hum. Hered. 35*, 326 (1985).
10. El-Hazmi, M. A. F., Warsy, A. S.: On the nature of sickle cell disease in the South-Western province of Saudi Arabia. *Acta Haemat. 76*, 212 (1986).
11. El-Hazmi, M. A. F., Warsy, A. S.: Glutathione reductase deficiency in Saudi Arabia — A study in Riyadh. *Ann. Saudi Med.* (In press).
12. Frischer, H.: Erythrocyte glutathione reductase deficiency in a hospital population in the United States. *Am. J. Hematol. 2*, 327 (1972).
13. Frischer, H., Bowman, J. E., Carson, P. E., Rieckmann, K. H., Willerson, D., Colwell, E. J.: Erythrocyte glutathione reductase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase deficiencies in populations of the United States, South Vietnam, Iran and Ethiopia. *J. Lab. Clin. Med. 81*, 603 (1973).
14. Garcia, S. C., Moragon, A. C., Lopez-Fernandez, M. E.: Frequency of glutathione reductase, pyruvate kinase and glucose-6-phosphate dehydrogenase deficiency in a Spanish population. *Hum. Hered. 29*, 310 (1979).
15. Glatzle, D., Körner, W. F., Christeller, S., Wiss, O.: Method for the detection of a biochemical riboflavin deficiency. *Hum. J. Vitamin, Res. 40*, 166 (1970).
16. Goebel, K. M., Goebel, F. D.: Hemolytic anaemia and pancytopenia in glutathione reductase deficiency: Further experience with riboflavin. *Acta Haemat. 47*, 292 (1972).
17. Lohr, G. W., Blume, K. G., Rindiger, H. W., Arnold, H.: Genetic variability in the enzymatic reduction of oxidized glutathione In: *Glutathione. Proceedings of the 16th Conference of the German Society of Biological Chemistry*. Flohe, L., Benöhr, HCH, Sies, H., Walker, H. D. Wendel, A. (eds) Stuttgart, George Thieme 1974, pp. 165.
18. Marengo-Rowe, A. J.: Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate. *J. Clin. Path. 18*, 790 (1965).
19. Prentice, A. M., Bates, C. J.: A biochemical evaluation of erythrocyte glutathione reduc-

tase test for riboflavin status. Rates and specificity of response in acute deficiency. *Br J. Nutr. 45*, 37 (1981).
20. Robinson, A. R., Robson, M., Harrison, A P., Zuelzer, W. W.: A new technique for differentiation of haemoglobin. *J. Lab. Clin. Med. 50*, 745 (1957).
21. Saha, N.: Erythrocyte glutathione reductase polymorphism in a Sudanese population. *Hum. Hered. 31*, 32 (1981).
22. Tillotson, J A., Baker, E. M.: An enzymatic measurement of the riboflavin status in man. *Am. J. Clin. Nutr. 25*, 425 (1972).
23. Waller, H. D.: Glutathione reductase deficiency. In: Beutler: *Hereditary disorders of erythrocyte metabolism.* Grune & Stratton, New York 1968, p. 185.

Haematologia 22 (1), pp. 43–53 (1989)

A Particular Case of Large Granular Lymphocytes Lymphoma

S. BERCEANU[1], STELA ROMAN[2], ELENA BUTOIANU[1], SORINA LEAHU,[1] ELENA GABRIELESCU[2], A. OLINESCU[3], ELENA MOLDOVEANU[2], I. MORARU[2]

[1]Clinic of Haematology – Fundeni Hospital, 258, Sos. Fundeni, 72437 Bucharest, Romania [2]Department of Immunology, Victor Babeş Institute, 99–101 Spl. Independenţei, Bucharest 35, 76201 Romania [3]Department of Immunology, Cantacuzino Institute, 103 Spl. Independenţei, Bucharest, Romania

(Received 28 April, 1987; accepted 17 December, 1987)

A patient with non-cutaneous lymphocytic lymphoma with leukaemic spread is reported. The large majority of the peripheral blood mononuclear cells formed rosettes with sheep erythrocytes (E), had receptors for the Fc portion of IgG (Fcγ R) and displayed an unusual enzymatic profile. Part of these cells had also labile bound surface membrane IgG. The leukaemic cells were morphologically characterized as large granular lymphocytes (LGL) and consisted of two different types of cells: lymphoid cells and monocytoid cells. The histopathological diagnosis was T-cell lymphoma, pleomorphic type, diffuse. Despite their morphological heterogeneity, all the proliferative cells had the same immunological phenotype, showed normal ADCC activity but no NK activity. After three months, without specific treatment, the surface pattern changed: the cells resembled mature LGL and the NK activity increased. The cytochemical study of the leukaemic cells revealed an enzymatical peculiarity: besides an enzymatic profile characteristic for relatively mature LGL, the cells also displayed peroxidase activity. This unusual aspect – the expression by an individual malignant cell of markers believed to be restricted to a single cellular lineage – might be interpreted as a lineage infidelity or lineage promiscuity. The observation that this lymphoma developed in a patient with a long history of hypogammaglobulinaemia is of particular interest.

Keywords: hypogammaglobulinaemia, LGL lymphoma, lineage infidelity

Introduction

Recent studies on monoclonal antibodies against lymphocyte subpopulations have demonstrated that not all the sheep-rosette forming cells bearing receptors for the Fc portion of IgG (Fcγ R) express the myelomonocytic M_1 antigen and some cells lack both the T-cell and the monocytic determinants [1]. A peculiarity of the Fc receptors is that they bind serum IgG at 4 °C and release it at 37 °C. Due to the presence of these labile IgG determinants, the Fcγ R-positive cells are referred to as "L cells". The mature forms of these cells have a typical morphology characterized by the presence of intracytoplasmic azurophilic granules, and are usually referred to as large granular lymphocytes (LGL). These cells are positive for acide hydrolase and negative for peroxidase. Some of the LGL

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

are unable to form rosettes with sheep erythrocytes, others lack the ability of expressing T-cell antigens. The heterogeneity of their phenotype reflects the existence of a unique cell population in different stages of maturation. The L cells are divided into precursor cells, immature cells and functional competent cells [1]. It has been demonstrated that the natural killer (NK) and antibody dependent cell mediated cytotoxic (ADCC) activities in humans are confined to a LGL enriched fraction. Some authors favour the hypothesis that these cells belong to a separate cellular lineage [1].

In this paper we describe a case of LGL lymphoma presenting unusual laboratory findings.

Materials and Methods

Patient. A 39 year old man was admitted to the hospital in June 1985, with fever and asthenia. A palpable spleen, enlarged liver, and lymph-node involvement were found. The leukocyte count was 11.000/mm^3 with 80% LGL. The patient had a long history (ten years) of bronchopulmonary infections, urinary infections and recurrent arthritis. Determination of serum immunoglobulins showed severe hypogammaglobulinaemia: IgG 353 mg/dl, IgA 107 mg/dl and IgM 12 mg/dl. The patient was investigated both on admission and three months later. The patient received antibiotics and gammaglobulins. No chemotherapy had been administered. After admission the patient's treatment remained the same.

Cell population. The peripheral blood mononuclear cells were obtained from heparinized venous blood, by sodium metrizoate – Ficoll centrifugation [2]. The resulting cell population consisted of over 95% LGL.

Monoclonal antibodies. The murine OKT monoclonal antibodies directed against the T_1, T_3, T_4, T_8, T_{11}, B_1, B_2, B_4, M_1 and HLA-DR lymphocyte membrane antigens were kindly supplied by Dr. Chan-Yi Wang (Memorial Sloan Kettering Cancer Center, New York).

Staphylococcus protein A (SpA) coated erythrocytes (ES reagent). Bovine erythrocytes were coated with SpA as previously described [3].

ES-rosettes assay. Monoclonal antibodies were used in an ES-rosettes assay as already described [4]. Briefly, the suspension of cells in culture medium (2.5×10^5) is incubated for 30 min in ice, with the respective monoclonal antibodies (0.005 ml). After three washings in cold culture medium, the cells were suspended in 0.2 ml culture medium and treated with 0.015 ml ES-reagent. The mixtures were centrifuged at 100 *g* for 10 min and incubated for 2 hours or overnight at 4 °C. The pellett was gently resuspended and treated with 0.030 ml of 0.2% toluidine blue in phosphate buffered saline.

Indirect immunofluorescence assay was performed according to the already described technique [4].

Assay for labile smIgG. Freshly prepared peripheral blood mononuclear cells (10^6/0.5 ml) were mixed with ES reagent and centrifuged at 4 °C for 10 min

at 100 *g*. The samples were stored at 4 °C overnight. The cells preincubated at 37 °C for 2 hours were washed with culture medium and rosetted with ES reagent in the same conditions.

EA_{hu} rosettes assay. Freshly prepared mononuclear cells (10^6/0.5 ml) were mixed with EA_{hu} reagent (human erythrocytes group O, Rh-positive, sensitized with human anti-CD serum 1 : 16.000). After centrifugation at 4 °C for 10 min at 100 *g* the sample was stored for 30 min at room temperature, or overnight at 4 °C.

Cell mediated cytotoxicity assays. Both spontaneous cell mediated cytotoxicity (NK) and antibody-dependent cellular cytotoxicity (ADCC) were carried out at an effector: target cell ratio of 100 : 1 and 10 : 1 as previously described [5]. The results were expressed as specific release of ^{51}Cr. The phytohaemagglutinin (PHA – Difco) was used in a final concentration of 2%. Pokewed mitogen (PWM) was obtained in the Cantacuzino Institute, Bucharest, from Phytolaka Americana, according to the method described by Bodger et al. [6]. This mitogen was used in a concentration of 20 gamma per tube.

Cytochemistry. Smears of leukaemic cells and EA_{hu} rosettes were stained for the following enzymes: acid-naphtol AS-BI phosphatase (ACNP) [7], alpha-naphtol acetate acid esterase (ANAE) [7], naphtol AS-D chloroacetate esterase (NCAE) [8], lysosomal acid glycerophosphatase (AcGP) [9] and endopeptidazic proteolytic enzymes: catepsin B [10], neutral BANA endopeptidase (trypsine-like) [11], alkaline B-PhNA endopeptidase (chimiotrypsine-like) [11] and leucin-aminopeptidase (LANA) [11].

Ultrastructural processing and staining for peroxidase was performed according to the Graham and Karnovski [12] technique.

Results

Clinical evaluation

The clinical state of the patient did not change during the period of observation.

Surface markers

Since the malignant cells expressed Fcγ receptors in a high percent, the possibility of unspecific binding was excluded by using indirect immunofluorescence method in addition to the mAb-ES-rosette assay. The values obtained by both techniques were similar.

Tables 1 and 2 give the results obtained by the investigation of the patient's malignant cells on the first examination and after three months. On admission, the patient's cells presented high levels of E receptors and Fcγ R, smIgG labile bound and were positive simultaneously for T_1, T_3, T_4, T_8, T_{11} and HNK-1 antigens. Immunologic evaluations were done at different bleedings and showed constant results during the first months after admission. Three months later the cellular

Table 1

Reactivity with monoclonal antibodies of the leukaemic cells

Monoclonal antibody	Reported specificity	Patient		Controls*	
		July 1985	November 1985	July	Nov.
OKT1	Pan T	92	42	68.5	70
OKT3	Pan T	94	43	70.2	69.9
OKT4	helper cells	90	25	41.5	42.3
OKT8	cytotoxic/suppressor T cells	98	75	21.9	24.8
OKT11	sheep erythrocytes receptor	96	72	69.8	70
HLA-DR	B-cells, monocytes activated T cells, leukaemic cells	51	10	20.5	24.2
HNK-1	LGL	96	12	16.5	18.2

* Twenty normal donors.

Table 2

Surface markers of the patient's leukaemic cells

Surface markers	Positive cells (%)	
	July 1985	November 1985
SRFC	91.5	67
S. SRFC	24	14
FcγR	72	85
smIgG	14	15
l. IgG	14	14

SRFC = sheep rosette forming cells
S. SRFC = stable (at 45 °C) sheep rosette forming cells
FcγR = cells bearing receptors for the Fc portion of IgG
smIgG = surface membrane IgG
l. IgG = labile bound IgG

phenotype was changed: part of the cells lost the E receptors and T_1, T_3, T_4, T_8, T_{11} and HNK-1 antigens. The malignant cells were negative for monocytic (OKM_1) and B-cell (OKB_1, OKB_2, OKB_4) determinants (data not shown).

Functional activities

Table 3 gives the results of the tests for natural killer and antibody dependent cytotoxicity at different effector: target ratios, in the patient's and in the control's cells. On the first examination, the percentage of target cell lysis was very low, in both the 100 : 1 and 10 : 1 effector : target ratios. On the contrary,

Table 3

NK and ADCC functions of malignant cells

Function	First examination	After three months	Normal*** controls
NK*	100 : 1 = 3.18	100 : 1 = 12.45	40— 50
	10 : 1 = 0	10 : 1 = 0.37	6 — 8
	10 : 1 + PHA = 5.2	10 : 1 + PHA = 37.15	20—100
	10 : 1 + PWM = 17.15	10 : 1 + PWM = 20.24	20—100
ADCC**	100 : 1 = 70.17	100 : 1 = 78.08	50—100
	10 : 1 = 0	10 : 1 = 28.06	10

100 : 1 effector : target ratios
10 : 1

* Percents of specific ^{51}Cr-release from K562 labelled target cells; ** Percents of specific ^{51}Cr-release from RL$^{\circ}$ ♂1 labelled target cells; *** the extreme values of four normal donors tested in concomitance.

the ADCC activity of malignant cells was normal. The patient's LGL showed a weak response to PHA, but a better response to PWM. Three months later, the cellular cytotoxic activity was much improved. At this time the cells were able to kill the targets not only in the 100 : 1, but also in the 10 : 1 ratio. In the presence of PHA, the stimulation index was also improved (from 5.2 to 37.15).

Morphology

The different types of malignant cells were detected in the peripheral blood by light microscopy as well as by transmission electronmicroscopy (TEM):

1) Lymphoid cells with rather abundant cytoplasm. In TEM (Fig. 1b) the plasma membrane showed numerous filiform extensions. The nucleus was irregular, bean-shaped, or indented. The chromatin was concentrated in clusters throughout the nucleoplasm. Occasionally, peroxidase-positive granules were also present.

2) Monocytoid cells (Fig. 1a). The plasma membrane showed numerous fingerlike extensions. The abundant cytoplasm was sometimes vacuolated. The nucleus was irregular, with moderately condensed chromatin. A large number of primary granules were scattered throughout the cytoplasm, most of them peroxidase positive. Both cell types were present at each examination. The proportion of monocytoid cells was 20% and 25%, respectively.

Histopathological evaluation

The normal structure of the examined lymph nodes was almost completely effaced, a diffuse proliferation of malignant cells was observed. The whole lymph node consisted of lympoid tumour cells, reactive cells and numerous blood vessels,

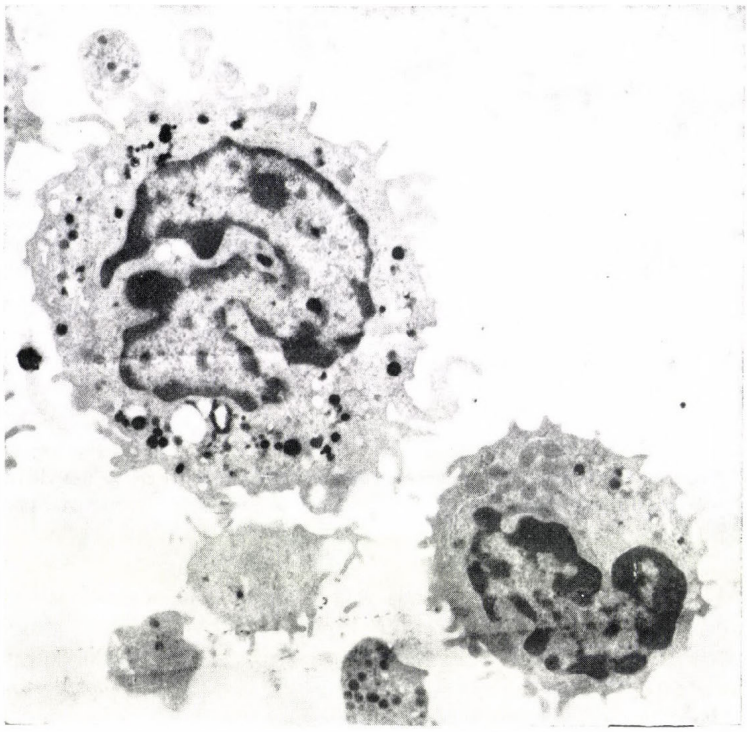


Fig.1. TEM micrograph representing the two different types of peripheral blood leukaemic cells, stained for peroxidase: a) monocytoid cell showing irregular nucleus and a large number of granules, most of them peroxidase positive; b) lymphoid cell, showing a bean shaped nucleus and rather abundant cytoplasm. Note peroxidase positive granules. (original magnification × 4500)

which were lined with thick endotelium. The vessels were full of malignant cells and rare eosinocytes. The tumour cells varied in size and appearance. The smallest cells had dark chromatin and very irregular nuclei. Large cells with irregular nuclei containing 2–3 prominent nucleoli were observed among these cells. The cytoplasm of all tumour cells was abundant and pale. The histopathological diagnosis was T-cell lymphoma, pleomorphic type, diffuse.

Enzymatic equipment

The results obtained in the leukaemic cells and EA_{hu} rosettes are presented in Table 4. Most of the cells showed high positivity for acid phosphatase NCAE and proteolytic enzymes. The enzymatic reactions in the lymphoid cells were granular, especially along the plasma membrane. Only the monocytoid cells displayed alpha-naphthylacetate esterase (ANAE) and acid glycerophosphatase (AcGP) activities, but of very low intensity.

Table 4

Cytochemistry of the leukaemic cells

Enzymes	Positive cells (%)	
	Lymphoid cells	Monocytoid cells
ANAE	2	18
Ac. GP	3	20
Per	20	51
Ac. P.	73	65
NCAE	100	100
PE	100	100

ANAE = alpha naphtyl acetate acid esterase; Ac. GP = lysosomal acid glycerophosphatase; Per = peroxidase; Ac. P = acid phosphatase; NCAE = naphtol AS-B cloroacetate esterase; PE = endopeptidase proteolytic enzymes.

On the basis of the above described enzymatic profile (AcP^+ $NCAE^+$ PE^+ $ANAE\pm$) the malignant cells could have been classified as relatively mature LGL, but part of them also displayed peroxidase activity.

Discussion

A case of non-cutaneous lymphocytic lymphoma with leukaemic spread has been described. On admission, a leukaemic population morphologically characterized as LGL, consisting mainly of two different types of cells was found. The histopathological diagnosis was T-cell lymphoma, pleomorphic type diffuse. The histological preparations also revealed two types of malignant cells. Despite their morphological heterogeneity, all the proliferative cells had the same immunological phenotype. The presence of Fcγ R (among the mononuclear cells only LGL are EACD-positive), of labile $_{sm}$IgG, of HNK-1 antigen and of the T_3, T_8, and T_{11} antigens suggested that the malignant cells belong to the LGL lineage.

The cell surface antigenic pattern showed a particular feature: the coexpression of T_4 antigen in the same percentage as T_1, T_3, T_8 and T_{11} antigens. Three months later the immunologic phenotype had changed: a number of cells had lost E receptors and T_1, T_3, T_4, T_8 and HNK-1 antigens, thus becoming probably a more differentiated leukaemic population. It is known that in the differentiation (maturation) process the normal LGL lose T-cell antigens, and only about 30% of the mature LGL express T-cell markers [1]. Therefore, in our opinion, the patient's leukaemic cells were detected in two discrete stages of maturation. The two morphological types of malignant cells might correspond to the normal counterparts of mature LGL, as described by Semenzatto et al.[15].

The LGL represents a heterogenous cell population, and it is currently not

clear if they derive from T or monocytic lineage, or whether they represent a separate cellular lineage. Therefore, the cells' features are often characterized by ambiguity, as demonstrated by Pandolfi et al. [16] in two cases of LGL lymphoma.

The malignant cells had a normal ADCC activity but a very low NK activity, thus showing a functional discrepancy which has already been reported in malignant cells [17]. At the same time it is still contraversial whether or not in normal subjects NK and ADCC activity is mediated by the same cell with mutiple functions, or is the production of multiple cells with separate specificities. Our data are in agreement with those reported by Abo et al. [16] who sustain that OKT_3^+ HNK-1^+ cells are less effective in NK activity compared to OKT_3^- HNK-1^+. Functional activities of the patient's malignant cells are closely related to those described by Pandolfi et al. [16] in one case of LGL lymphoma with OKT_3^+ OKT_8^+ HNK-1^+ M_1^- cells. The increased NK activity in the presence of PWM might be explained by the fact that T_8-positive cells stimulated with PWM express T_{ac} antigers, thus leading to cell mitosis and therefore to increased functional activity [13]. It seems likely that the increased killing is the result of better binding to the target cells. The preexisting hypogammaglobulinaemia, in our case, might have been enhanced by the suppressor function of the leukaemic cells. Furthermore, the impaired response to PHA might be the effect of an increased "in vivo" suppressor activity. The occurance of the T_4 antigen on the LGL might also have a suppressor function, since it is known that in some circumstances the T_4 antigen may have a suppressor function. Besides, recently, a discrete subpopulation of lymphocytes sharing several phenotypic characteristics with NK cells was identified within the circulating pool of human lymphocytes that bear T_4 marker (22% of the T_4 cells) [14].

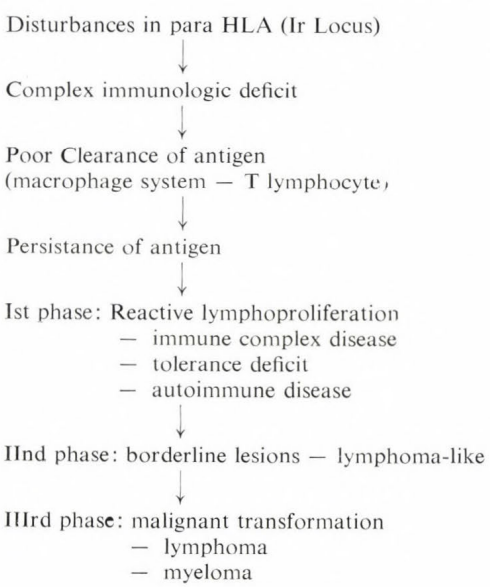
Our cytochemical study revealed some enzymatical peculiarities. Besides the high positivity for AcP, the low levels of ANAE and AcGP are of interest, since these enzymes have been found to be expressed only on mature T-cells and their appearance follows the expression of AcP during cellular maturation [7]. The high naphtol AS-BI acid phosphatase, NCAE and proteolytic enzyme activities are known to be characteristic for mature LGL. According to some authors [15, 16] intense NCAE activity represents a cytochemical marker for the LGL population. The features of these malignant cells resemble those observed by Chan et al. (1984) who identified intense AcP reaction in a LGL subset, characterized by E^+ T_3^+ T_8^+ M_1^- phenotype with marked ADCC activity but no NK activity. The cytochemical phenotype was characterized by ambiguity, since besides the enzymes characteristic for LGL, the malignant cells also displayed peroxidase activity. This peculiarity might be interpreted as a lineage infidelity. An increasing number of authors report cases in which individual leukaemic cells coexpress markers normally believed to be restricted to a single lineage. This has been interpreted by McCulloch et al. as aberrant programming, or lineage infidelity [18]. Graves et al. [19] suggest that lineage infidelity does not reflect genetic misprogramming, but rather, the existence of a transient phase of limited promiscuity of gene expression occuring in normal bipotential or multipotential

progenitors, which may be preserved as a relic in leukaemic cell populations that are in a maturation arrest.

In our case, the immunoenzymatical discrepancy might be the result of the persistence of some features characteristic to a common progenitor for LGL and monocytic lineages.

The observation that this lymphoma appeared in a patient with a long history of hypogammaglobulinaemia is of particular interest. The predisposition for, and the association with malignant lymphoproliferations in immunodeficient subjects is now a well known fact. The first data related to this observation [20, 21, 22, 23, 24, 25] have drawn attention to the fact that hypogammaglobulinaemia creates a predisposition for borderline or lymphomalike lesions (Fig. 2). Dutz et al. [26], Lennert et al. [27], Mathé et al. [28] presented new arguments which confirmed this point of view.

Fig. 2. Mechanisms of malignant transformation in the immune cellular system



The clinical features and course of the disease in our patient was somewhat similar to a non-Hodgkin lymphoma of T_3^+ T_8^+ T_{11}^+ phenotype (Lukes and Meyer classification), with mild, non-progressive evolution, thus showing that the main characteristics of these malignant cells of particular nature, were cytotoxic lymphoid cells, the monocytic features having apparently no influence upon the clinical picture. In agreement with other workers [19], some of our unpublished observations have shown that in acute promyelocytic leukaemia (APL) with malignant cells which displayed peroxidase activity and expressed T-cell markers

(surface antigens), the chemotherapy for APL was inefficient, the prompt recovery of the blood abnormalities being observed only when chemotherapy for acute lymphoblastic leukaemia was started. Therefore in some cases of lineage infidelity the cell surface phenotype seems to be responsible for the evolution of cells and the patients' response to therapy.

Acknowledgements

The authors wish to express their thanks to Dr. V. Ghetie for the helpful advice.

References

1. Horwitz, D. A., Bakke, A.: A Fc receptor bearing third population of human mononuclear cells with cytotoxic and regulatory function. *Immumnol. Today 5*, 148 (1984).
2. Boyum A.: Separtion of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest. 21*,, Suppl. 97 (1968).
3. Ghetie, V., Nilsson, K., Sjöquist, J.: Detection and quantitation of IgG on the surface of human lymphoid cells by rosette formation with protein A coated sheep red blood cells. *Eur. J. Immunol. 4*, 500 (1974).
4. Roman, S., Moraru, I., Ghetie, V.: Detection of human lymphocytes subpopulations by using monoclonal antibodies and protein A coated erythrocytes. *Acta Hematologica (Basel) 73*, 140 (1985).
5. Olinescu, A., Hristescu, S.: The human effector cells of natural cell mediated cytotoxicity and antibody dependent cellular cytotoxicity express a differentiated susceptibility to pronase. *Arch. Roum, Path. Exp. Microbiol. 43*, 447 (1984).
6. Bodger, M. P., McGiven, A. R. Fitzgerald, P. H.: Mitogenic proteins of pokeweed. I. Purification, characterizaton and mitogenic activity of two proteins from pokeweed (Phytolacca Octandra). *Immunology 37*, 785 (1979).
7. Basso, G., Cocito, M. G., Messins, A., Colleselli, C., Zenesco, P.: Study of cytochemical markers Ac. P. and ANAE in childhhod lymphoma and leukemia. *Br. J. Cancer 41*, 835 (1980).
8. Gabe, M.: *Histochemical technique*. Springer Verlag, Berlin 1976.
9. Barka, T., Anderson, P. J.: *Histochemistry*. Harper and Row Publ. Inc., New York 1963.
10. Codoreanu, E., Gabrielescu, E.: Cytochemical investigation of cathepsin B in rheumatoid synovial membrane and fluid. *Rev. Roum. Morphol.* (in press)
11. Pearce, A. G. E.: *Histochemistry, theoretical and applied*. vol. 2. Churchill & Livingston Edinburgh 1972.
12. Graham, R. G., Karnowski, M. C.: The early stages of absorption injected horseradish peroxidase in the proximal tubules of mouse kidney, ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem. 14*, 291 (1966).
13. Ishizaka, A., Sakiyama, J., Takahashi, Y., Matsumoto, S.: The activation of T cells with Staphylococcus aures cowan. *Immunol. Letters 10*, 13 (1985).
14. Velardi, A., Grossi, C. E., Cooper, M. D.: A large subpopulation of lymphocytes with T helper phenotype (LEU-3/T_4^+) exhibits the property of binding to NK cell targets and granular lymphocyte morphology. *J. Immunol. 134 (1)*, 58 (1985).
15. Semenzato, S., Pizzolo, G., Ranucci, A., Agostini, C., Cilosi, M., Quinti, I.: Abnormal expansions of polyclonal large to small size granular lymphocytes. Reactive or neoplastic process? *Blood 63*, 1271 (1984).
16. Pandolfi, F., Pezzutto, A., Rossi, G., Pasqualetti, D., Semenzato, S., Quinti, I., Ranucci,

A., Raimondi, R., Basso, G., Strong, D., Fontana, L., Auete, F.: Characterization of two patients with lymphomas of large granular lymphocytes. *Cancer 53*, 445 (1984).
17. Kadin, M. E., Kamoun, M., Lamberg, J.: Erythrofagocytic T_γ lymphoma. *N. Engl. J. Med. 304*, 648 (1981).
18. McCulloch,: Lineage infidelity in myelogenous leukemias. *Blood 64*, 5 (1984).
19. Greaves, M. F., Chan, L. C., Furley, A. J. W.: Lineage promiscuity in hemopoietic differentiation and leukemia. *Blood 67*, 1 (1986).
20. Berceanu, S., Popescu, I., Gociu, M.: *Volume omagial.* Acad. N. Gh. Lupu. Edit. Acad. R. S. Romania, Bucuresti, 1967.
21. Berceanu, S., Gociu, M., Grozea, P.: *Sistemul reticulo-endotelial.* Edit. Med. Bucuresti (1967).
22. Berceanu, S., Ciocoiu, A., Colita, D.: Meeting of the International Society of Chemo- and Immunotherapy, Vienna. Abstracts, p. 88 (1976).
23. Berceanu, S., Dosius, L., Munteanu, N.: The XVIIth Congress of the International Society of Hematology, Paris. Abstracts p. 694 (1978).
24. Berceanu, S., Paunescu, E.: Biologia şi patologia immunităţii. Edit. Academiei R. S. Romania, Bucuresti (1981).
25. Mesrobeanu, I., Berceanu, S.: *Imunologie si Imunopatologie.* Edit. Medicală Bucureşti 1968, pp. 237, 253.
26. Dutz, W.: *Int. Arh. über Lymphotumoren.* Vienna 1978, p. 17.
27. Lennert, K.: *Malignant lymphomas.* Springer Verlag, Berlin 1978, p. 83.
28. Mathé, G., Belpomme, D., Musset, J. L.: 17th Congress of the International Society of Hematology, Paris. Abstracts II., p. 747 (1978).

Haematologia 22 (1), pp. 55—58 (1989)

Frepuency of Lewis Group Substances in Patients with Cancer of Stomach

E. CSATÓ, E. VASS

Blood Transfusion Centre, "Jósa András" Memorial County Hospital, H-4401 Nyíregyháza, P.O.Box 159, Hungary

(Received 30 June, 1987; accepted 21 April, 1988)

Authors examined the frequency of Lewis group substances in red blood cells (RBC) and saliva of 293 patients with cancer of stomach, and of 760 healthy volunteers. Lewis (a-b-) substances on RBC surfaces were significantly more frequent in patients with cancer of stomach compared to normal controls.

Most patients with cancer of stomach who had Lewis (a-b-) type red blood cells had Lewis -a and Lewis -b substances in their saliva.

The deficiency of Le^a substance and the loss or weakening of Le^b antigen on RBCs may be a prognostic and diagnostic indicator in patients with cancer of stomach.

Keywords: Lewis group substance, cancer of stomach

Introduction

Many reports have been published on the possible relationship between blood groups and diseases. Most of these papers have discussed the relations between the ABO system and certain diseases. Data on the association of other blood systems and diseases are scarce.

Authors studied the possible association between the Lewis blood group substances and the frequency of stomach cancer.

In 1978 E. Csató [1] found a significantly higher ratio of Le (a−b−) type RBCs in patients with stomach cancer. Due to the growing interest in the Lewis system, the authors' newest findings are presented.

The studies of the incidence of Le^a, Le^b antigens in patients with stomach cancer were performed together with those of the ABH antigen frequency and the secretor — nonsecretor character.

Picard et al. [2] and Furukawa et al. [3] examined the incidence of ABH, Le^a and Le^b antigens in extracts of gastric mucosa. As only few samples were examined, no conclusion was drawn regarding the link between Lewis group substances and the cancer of stomach. No report on this subject has been published in the German *Humangenetik* [4].

According to Mourant et al. [5] up to the present no association has been found between the Lewis system and other diseases. This is why no report on this topic was presented in their manual either.

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Mourant discovered the antigens of the Lewis system in 1946 [6]. Lewis group substances may be present on red blood cells, in some tissues and in body fluids.

According to our present knowledge, antigens of the Lewis system consist of water soluble mucoids. Japanese authors [7] state that Lewis substances may be produced in the gastric mucosa.

In the last decade the importance of the Lewis system has been recognized in connection with organ transplantation [8]. The Lewis antigens are considered to be additive transplantation antigens. In cases of kidney transplantation it has been proved that when the recipients were Le (a − b −) and their donors were Le antigen positive the tolerance to the transplanted organ was significantly lower.

Sneath and Sneath [9] stated that Lewis antigens present in plasma are passively attached to red blood cell surfaces. This is attributed to an enzymatic function. Results of recent studies indicate that the presence of Lewis substances can be determined from samples of saliva.

In 1948 Grubb [10] observed a correlation between the Lewis system and the secretion of ABH blood group substances in body fluids.

Subjects of Le (a + b −) type secrete Le^a but not ABH substances into the body fluids.

Subjects of Le (a − b +) type secrete ABH, Le^a and Le^b substances in to their body fluids.

The majority of Lewis (a − b −) type subjects secrete ABH and Le^{bH}, while Le^a substance is not detectable in their body fluids.

The Le (a − b −) type is the only group where the occurance of the Le^a substance is exceptionally low.

Ceppelini [11] reported two cases in which Le^a substance was detected in the saliva of a Le (a − b −) subject. In 1957 Iseki, Masaki and Shibasaki [12] discovered Le^o antigen in Le (a − b −) subjects.

According to Gunson and Latham [13] Le^a substance exceptionally detected in the saliva of Le (a − b −) persons is identical to Le^c antigen.

In 1970 Potapov [14] discovered Le^d antigen on the surface of RBC of nonsecretor Le (a − b −) subjects. Thus, the RBCs of Le (a − b −) nonsecretors are Le (a − b − c − d +).

According to Race and Sanger [15] the so-called European average of the frequency of the best known antigens Le^a and Le^b were as follows:

Le (a + b −) = 22%; Le (a − b +) = 72%; Le (a − b −) = 6%.

Materials and Methods

Between 1966 and 1984 the blood and saliva samples of 293 patients with cancer of stomach were studied. Lewis group substances were determined in saliva and blood samples, ABH antigens were detected in the saliva of the patients. 206

males and 87 females were included in the study. Samples were obtained from patients treated in the 1st and 2nd Department of Surgery of the County Hospital Nyíregyháza, 2nd Department of Medicine of the the Medical School at Debrecen, and the County Oncological Care Centre at Nyíregyháza, Hungary.

The diagnosis was established by endoscopical and histological examinations. Our studies were completed by similar ones performed on 760 heatlhy controls (480 males and 380 females), donors at the County Blood Transfusion Centre in Nyíregyháza.

Lewis antigens were determined with antisera supplied by Biotest Serum Institute GmbH, Frankfurt am Main (FRG), Ortho Diagnostics Inc. (USA) and Cilag AG, Schaffhausen (Switzerland).

ABH antigens were detected by serotypes provided by Human Co., Budapest (Hungary).

Assays on saliva and blood samples were performed within three days after sampling.

Lewis group substances and ABH antigens in saliva were detected by the agglutination inhibition test.

Le^{a+} and Le^{b+} test cells served as controls.

Results

Table 1 demonstrates the incidence of Le (a−b−) types in patients with stomach cancer and in healthy donors. The data show that the frequency of Le (a−b−) was 6.3% in healthy controls and 16% in patients. Le^a substance was

Table 1

The frequency of the Lewis group in healthy persons and in patients with gastric cancer

Red blood cell	Le^{a+b-}	Le^{a-b+}	Le^{a-b-}	
PATIENTS n = 293	19 6.4%	227 77.4%	47 16%	
			SALIVA Le^{a+}	SALIVA Le^{a-}
			26 55.3%	21 44.7%
HEALTHY n = 760	150 19.7%	562 73.9%	48 6.3%	
			SALIVA Le^{a+}	SALIVA Le^{a-}
			2 4.3%	46 95.7%

x^2 probe = 46.769

detected in 2 cases (4.3%) in healthy Le (a−b−) controls, and in 26 cases (55.3%) among patients. This was interesting because Le^a substance occurs rather rarely in the saliva of Le (a−b−) subjects.

To our best knowledge no report was published on a comparison of the incidence of Le^a substance in the saliva of patients with stomach cancer and in healthy controls.

Discussion

Judd et al. [16] assume that malignant tumours secrete steroidhormone-active substances which diminish the expression of Le^b genes on RBCs. Two such cases have been reported. The tumour of the oesophagus has also been listed in this group.

A similar mechanism is assumed to explain the inagglutinability of Lewis antigens on the RBC surfaces of patients with gastric cancer, altough to our best knowledge the malignant tumours of the stomach do not produce steroid-active substances.

In our opinion the examination of Lewis antigens may be diagnostic and prognostic indicators in cases of stomach cancer.

H. Koprowski et al. [17] assumed that the incidence of Le (a−b−) is higher in patients with gastric cancer.

In 1986 we found that the incidence of Le^{a+} in patients with gastric cancer was 8.8% lower, in nonsecretor type than in healthy controls whose mean value was 22% [18].

The absence of Le^a on RBCs, the diminished expression of Le^b gene on RBC appears to be much more frequent in patients with stomach cancer. This fact supports the probability of an association between the Lewis system and gastric cancer.

Further investigations are neede to solve this problem.

References

1. Csató, E.: A Lewis csoportanyagok vizsgálata gyomorrákban. *Transfusio 11*, 52 (1978) (in Hung.)
2. Picard, J., Edward, D. W., Feizi, T.: Changes in the expression of the blood group A, B, H, Le^a and Le^b antigens and the blood group precursor associated I (MA) antigen in glycoprotein — rich extracts of gastric carcinomas. *J. Clin. Lab. Immunol. 1*, 119 (1978).
3. Furukawa, H., Takizawa, H., Iseki, S.: Immunochemical studies on human cancer. I. Cancer-associated antigen reacting with anti — stomach cancer immune serum. *Proc. Japan Acad. 53*, 1616 (1977).
4. Helmbold, W., Schwarzfischer, F., Vogel, F.; *Humangenetik* .Georg Thieme Verlag, Stuttgart 1972.
5. Mourant, A. E., Kopeć, A. C., Domaniewska−Sobczak, K.: *Blood Groups and Diseases*. Oxford University Press, London 1978, p. 5.

6. Mourant, A. E.: A new human blood group antigen of frequent occurence. *Nature 237*, 158 (1946).
7. Takizawa, H., Iseki, S.: ABH and Lewis blood group activities in glycolipid and glycoprotein fractions from human erythrocytes gastric mucosa and saliva. *Proc. Japan Acad. 53*, 163 (1977).
8. Oriol, R., Carlton, J., Yvarti, J. I.: The Lewis system, new histocompatibility antigens in renal transplantation. *Lancet i*, 574 (1978).
9. Sneath, J. S., Sneath, P. H. A.: Transformation of the Lewis groups' human red cells. *Nature 172*, 176 (1965).
10. Grubb, E.: Correlation between Lewis blood group and secretor character in man. *Nature 1933*, 162 (1948).
11. Ceppelini, R., Dunn, L. C. and Inella Filomena.: Immunogenetica II. Annalisi genetica formale de carrateri Lewis conparticolare riguardo alla natura epistatica della specifita serologica Le^b. *Fol. Hered. Path. 8*, 261 (1959).
12. Iseki, S., Masaki, S., and Shibasaki, K.: Studies on Lewis blood group system. Le^c blood group factor. *Proc. Imp. Acad. Japan 33*, 492 (1957).
13. Gunson, H. H., Latham, Valerie: An agglutinin in human serum reacting with cells from Le (a-b-) non secretor individuals. *Vox Sang. 22*, 344 (1972).
14. Potapov, M. I.: Detection of the antigen of the Lewis system characteristic of the erythrocytes of the secretory group Le (a-b-). *Probl. Haemathol. (Moscow) 11*, 45 (1970).
15. Race, R. R., Sanger, R.: *Blood Groups in Man*, Sixth Edition. Blackwell Scientific Publications, Oxford 323 (1975).
16. Judd, W. J., Steiner, F. A., Friedman, B. A., et al.: Anti-Le^a as an autoantibody in the serum of a Le (a−b+) individual. *Transfusion Aug.* 436 (1978).
17. Koprowski, H., Blaszczyk, M., Steplewski, Z.: Lewis blood-type may affect the incidence of gastrointestinal cancer. *Lancet i 1332* (1982).
18. Csató E., Vass, J.: Frequency of non-secretor types among stomach cancer patients. *Haematologia 19*, 147 (1986).

Abstracts

Thrombin-induced membrane depolarization of plateles and its inhibition by cetiedil. A. Yamaguchi, H. Azuma, S. Sekizaki, H. Suzuki, K. Tanoue, and H. Yamazaki (Department of Cardiovascular Research, The Tokyo Metropolitan Institute of Medical Science, Bunkyo-Ku, Tokyo 113, Japan). *J. Biochem. 103*, 787 (1988).

When 50 μM cetiedil alone was added to a platelet suspension, increase in Na^+ content, decrease in K^+ content, and depolarization of platelet membrane were observed without change in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) or in the morphology of platelets. The cetiedil-induced depolarization was attenuated by the reduction of extracellular sodium concentration, while sodium transport inhibitors such as procaine and tetrodotoxin failed to modify the depolarization. On the other hand, thrombin caused such changes in platelets as increases in Na^+ content, ^{22}Na space and $[Ca^{2+}]_i$, decrease on K^+ content, and membrane depolarization. All these changes caused by thrombin were inhibited by cetiedil. It is suggested that cetiedil brought the increased ion transport and subsequent partial depolarization, which might lead to modification of the reaction of platelet membrane induced by thrombin. *G. Gárdos*

Ca^{2+} mobilization can occur independent of acceleration of Na^+/H^+ exchange in thrombin-stimulated human platelets. G. B. Zavoico and E. J. Cragoe (Hematology Division, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts). *J. Biol. Chem. 263*, 9635 (1988).

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and pH (pH_i) were measured simultaneously by dual wavelength excitation in thrombin-stimulated human platelets double-labeled with the fluorescent probes fura-2 and 2', 7'-bis (carboxyethyl)-5, 6-carboxyfluorescein to determine the relationship between changes in $[Ca^{2+}]_i$ and pH_i, respectively. At 37 °C, thrombin (0.5 or 0.1 units/ml) increased $[Ca^{2+}]_i$ with no detectable lag period to maximum levels within 13 s followed by a slow return to resting levels. There was a transient decrease in pH_i within 9 s that was immediately followed by an alkalinization response, attributable to activation of Na^+/H^+ exchange, that raised pH_i above resting levels within 22 s. At 10—15 °C, thrombin-induced changes in $[Ca^{2+}]_i$ and pH_i were delayed and therefore better resolved, although no differences in the magnitude of changes in $[Ca^{2+}]_i$ and pH_i were observed. However, the increase in $[Ca^{2+}]_i$ had peaked or was declining before the alkalinization response was detected, suggesting that Ca^{2+} mobilization occurs before activation of Na^+/H^+ exchange. In platelets preincubated with 5-(N-ethyl-N-isopropyl)amiloride or gel-filtered in Na^+-free buffer (Na^+ replaced with N-methyl-D-glutamine) to inhibit Na^+/H^+ exchange, thrombin stimulation caused a rapid, sustained decrease in pH_i. Under these conditions there was complete inhibition of the alkalinization response, whereas Ca^{2+} mobilization was only partially inhibited. Nigericin (a K^+/H^+ ionophore) caused a rapid acidification of more than 0.3 pH unit that was sustained in the presence of 5-(N-ethyl-N-isopropyl)amiloride. Subsequent stimulation with thrombin resulted in slight inhibition of Ca^{2+} mobilization. These data show that, in human platelets stimulated with high or low concentrations of thrombin, Ca^{2+} mobilization can occur without a functional Na^+/H^+ exchanger and in an acidified cytoplasm. Therefore, Ca^{2+} mobilization does not require activation of Na^+/H^+ exchange or preliminary cytoplasmic alkalinization. *G. Gárdos*

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Immunocytochemical localization of the Ca^{2+}-ATPase polypeptide in human platelets. G. H. Herbener and W. L. Dean (Departments of Anatomical Sciences and Neurobiology and Biochemistry, University of Louisville School of Medicine, Louisville, KY 40292). *Biochem. Biophys. Res. Comm. 153*, 848 (1988).

Specific polyclonal antibodies raised against purified human platelet Ca^{2+}-ATPase were used with protein A-gold immunocytochemistry to localize this protein in human platelets. Immunolabeling specifically detected Ca^{2+}-ATPase over the surface connected membrane system (SCS) in sections of paraformaldehyde-fixed, Lowicryl-embedded platelets. The maximum density of label, determined by quantitative morphometric techniques, was observed over electron-dense regions within the SCS which may represent specialized structures for uptake and release of Ca^{2+}. Less intense immunolabeling was observed over cytosol and may represent localization over the dense tubular system (DTS) which was not readily visualized under the processing procedures employed.

G. Gárdos

Effect of PCR 4099 on ADP-induced calcium movements and phosphatidic acid production in rat platelets. R. Feliste, M. F. Simon, H. Chap, L. Douste-Blazy, G. Defreyn and J. P. Maffrand (Inserm Unité 101, Biochimie des Lipides, Hopital Purpan, Toulouse, Toulouse, France). *Biochem. Pharmacol. 37*, 2559 (1988).

Antiplatelet activity of PCR 4099, an analogue of ticlopidine, resides in its specific effect against exogenous as well as released ADP. This study investigated in rat platelets the effects of the drug on ADP-induced shape change, elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and hydrolysis of inositol phospholipids, monitored as $[^{32}P]$ phosphatidic acid formation. Shape change and influx of Ca^{2+} ions across the plasma membrane were not modified after PCR 4099 administration using aspirin-treated platelets. On the other hand, phosphatidic acid formation and calcium mobilization from internal stores were strongly inhibited. These results suggest that PCR 4099 leaves intact the machinery involved in ADP-induced platelet shape change and influx of calcium ions, but inhibits an early step in the ADP--response coupling leading to inositol phospholipid hydrolysis and aggregation.

G. Gárdos

Fura-2 used as a probe to show elevated intracellular free calcium in platelets of Dahl-sensitive rats fed a high salt diet. S. Vasdev, P. Thompson, C. Triggle, P. Fernandez, P. Bolli and V. S. Ananthanarayanan (Disciplines of Medicine, Basic Sciences and Biochemistry, Memorial University, St. John's, Newfoundland, Canada). *Biochem. Biophys. Res. Comm. 154*, 380 (1988).

Elevated intracellular free calcium concentration $[Ca^{2+}]_i$ in vascular smooth muscle cells has been implicated in the pathophysiology of hypertension. Platelet $[Ca^{2+}]_i$ was measured using the fluorescent indicator, Fura-2, in Dahl sensitive (DS) and resistant (DR) rats given high (8% NaCl) and low (0.4% NaCl) salt diets, as well as in the spontaneously hypertensive (SHR) and Wistar--Kyoto (WKY) rats. The aim of this study was to show whether $[Ca^{2+}]_i$ is elevated in salt induced hypertension. Platelet $[Ca^{2+}]_i$ and systolic blood pressure (SBP) were higher ($p < 0.001$) in DS rats given a high than low salt diets. In contrast, no changes in platelet $[Ca^{2+}]_i$ and SBP were observed in DR rats. In SHR, platelet $[Ca^{2+}]_i$ and SBP were higher ($p < 0.001$) than in the WKY rats. Platelet $[Ca^{2+}]_i$ correlated with SBP in all groups of rats ($r = 0.929$; $p < 0.001$, $n = 38$). The parallel increase in SBP and $[Ca^{2+}]_i$ in the DS high salt rats and the SHR suggests that an increased $[Ca^{2+}]_i$ is involved in the pathophysiology of hypertension in the two models which differ with respect to the pathogenesis of their hypertension. This increase in $[Ca^{2+}]_i$ therefore seems to reflect an abnormality on $[Ca^{2+}]_i$ handling in hypertension regardless of its cause.

G. Gárdos

The effect of ATP, intracellular calcium and the anion exchange inhibitor DIDS on conductive anion fluxes across the human red cell membrane. P. Bennekou and P. Stampe

(August Krogh Institute, Zoophysiological Laboratory B, University of Copenhagen, Copenhagen, Denmark). *Biochim. Biophys. Acta 942*, 179 (1988).

The influence of ATP depletion, the intracellular ionized Ca-concentration, anion substitution and DIDS on the conductive anion fluxes across the human red cell membrane has been examined. Under physiological or near physiological conditions it is not possible to observe conductive anion fluxes across the erythrocyte membrane in that anions totally dominate the membrane conductance. Consequently anions are at electrochemical equilibrium and the netflux is zero. However, conductive anion fluxes can be induced by raising the potassium conductance, either by addition of valinomycin, or by triggering the native calcium activated potassium channel by addition of the Ca^{2+} ionophore A23187 to cells suspended in a calcium containing medium. The interpretation of data from experiments with valinomycin induced netfluxes has normally been done according to a constant field model, and the results have consequently been given as permeabilities. Since it has been demonstrated recently, that these cation pathways do not conform to a constant field scheme, it has been chosen, instead of permeabilities, to calculate the ion conductances from net efflux data, using an independent estimate of the membrane potential. The main result reported, is that only component is found for the conductive anion fluxes in the presence of DIDS using the latter theoretical framework, whereas a sizeable DIDS-insensitive component is found when the constant field analysis is used. Furthermore it is found that ATP and intracellular calcium do not influence the anion conductances.

Ilma Szász

Red blood cell membrane microviscosity correlates with posttransfusion survival. L. R. McLean,. C. Grote, E. B. Silberstein, and M. McGill (Merrell Dow Research Institute, and Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA). *Biochem. Biophys. Res. Comm. 154*, 387 (1988).

The cholesterol/phospholipid and sphingomyelin/phospholipid ratios of red blood cells stored for 42 days were unchanged after storage. However, the total phospholipid concentration in the red cells decreased suggesting a loss of red cell membrane during storage. The 24 h survival of the stored red cells was assessed by the ^{51}Cr technique in homologous donors. A decrease in fluorescence polarization of diphenylhexatriene incorporated into the membrane was observed following storage which correlated with survival. Thus, molecular defects in the lipid bilayer are associated with long-term storage of red blood cells.

Ilma Szász

Sites of p-chloromercuribenzene sulfonate inhibition of red cell urea and water transport. D. M. Ojcius and A. K. Solomon (Biophysical Laboratory, Department of Physiology and Biophysics, Harvard Medical School, Boston, MA). *Biochim. Biophys. Acta 942*, 73 (1988).

The mercurial sulfhydryl reagent, p-chloromercuribenzene sulfonate (pCMBS), inhibits water and urea fluxes across the human red blood cell membrane. The kinetics and affinities for pCMBS binding to separate water transport and urea transport inhibition sites were previously determined by Toon and Solomon (1986) in red cells that had been treated with N-ethylmaleimide (NEM) to block five of the six sulfhydryls on the red cell anion exchange protein, band 3. It has been used autoradiographs of gels from NEM -treated cells, labeled with ^{203}Hg-pCMBS, to localize these water and urea transport inhibition binding sites separately and find that both are on band 3. Each site is saturable and the time course of each uptake can be fitted to the equation for a bimolecular association (with negligible dissociation) with time constants in agreement with those of Toon and Solomon. Determination of the binding stoichiometry shows one urea inhibition site and three water inhibition sites for every four band 3 molecules. These results inidicate that band 3 plays a role in both urea and water transport and suggest that the functional unit may be a tetramer.

Ilma Szász

Platelet Survival Studies

The International Committee for Standardization in Haematology has revised its earlier recommendations for Platelet Survival Studies. Radioactive indium (^{111} In) has advantages over ^{51}Cr as a platelet label because of its short half life, higher photon yield and greater affinity for platelets. Standardized methods are described for labelling an autologous platelet sample with indium oxine or tropolone and for analysing the data by an iterative "multiple hit" method.

This document has been published in Journal of Nuclear Medicine, *29*, 4: 564–566 (April 1988).

Reprints are available from ICSH Secretariat, c/o R. L. Verwilghen, University Hospital, Herestraat 49, B-3000 Leuven, Belgium.

A computer program for estimating the mean platelet survival is also available on request.

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 brackets. 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.

Authors are entitled to 50 reprints free of charge.

HAEMATOLOGIA
VOLUME 22 · NUMBER 1

Contents

Mirčevová, L., Viktora, L., Řehačková, H.: Dual effect of calcium on erythrocyte membrane 3

Czokalo, M., Tomasiak, M.: Alpha-fetoprotein inhibits aggregation of human platelets 11

Selypes, A., László, A., Virág, I.: Chromosome examinations on a six-hour culture of peripheral blood from a patient with childhood leukaemia 19

Phan, D. T., Mihalik, R., Benczur, M., Pálóczi, K., Gidáli, J., Fehér, I., Dömötöri, J., Kiss, Cs., Petrányi, G. Gy., Natonek, K., Hollán, S. R.: Human fetal liver as a valuable source of haemopoietic stem cells for allogeneic bone marrow transplantation 25

El-Hazmi, M. A. F., Warsy, A. S.: Glutathione reductase in the south-western province of Saudi-Arabia — Genetic variation vs. acquired deficiency 37

Berceanu, S., Roman, S., Butoianu, E., Leahu, S., Gabrielescu, E., Olinescu, A., Moldoveanu, E., Moraru, I.: A particular case of large granular lymphocytes lymphoma 43

Csató, E., Vass, E.: Frequency of Lewis-group substances in patients with cancer of stomach 55

Abstracts 61

Index: 26.861

VOLUME 22 • NUMBER 2 • 1989

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

VSP

UTRECHT, THE NETHERLANDS,
TOKYO, JAPAN

AKADÉMIAI KIADÓ, BUDAPEST

HAEMATOLOGIA

Haematologia is an international journal publishing original papers on all clinical and theoretical aspects of haematology. In addition to regular papers, book reviews and abstracts of selected papers from other periodicals also are a special feature of the journal.

Haematologia, published at quarterly intervals, provides the reader with complex and up-to-date information.

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

Copy Editor:
D. Keszthelyi

Distributor for

Albania, Bulgaria, China, Cuba, Czechoslovakia, German Democratic Republic, Korean People's Republic, Mongolia, Poland, Romania, U.S.S.R., Viet-Nam, and Yugoslavia:

K U L T U R A
Hungarian Foreign Trading Company
P.O.B. 149, H-1389 Budapest 62, Hungary

For all other countries subscriptions should be sent to

VSP
P.O.B. 346
3700 AH ZEIST, The Netherlands

Subscription price for volume 22:

DM 330,— (including postage)

Haematologia is covered in Current Contents/Clinical Practice, ISI/Biomed and the SCI.

VOLUME 22 · NUMBER 2

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

VSP UTRECHT, THE NETHERLANDS, TOKYO, JAPAN

AKADÉMIAI KIADÓ, BUDAPEST

1989

Haematologia 22 (2), pp. 65–68 (1989)

The Effect of Alkaline Phosphatase on Platelet Aggregation

A. B. WEITBERG

Division of Haematology/Oncology, Roger Williams Cancer Center, 825 Chalkstone Avenue, Providence, Rhode Island 02908, USA, and the Department of Medicine, Brown University, Providence, Rhode Island

(Received 14 July, 1987; accepted 21 December, 1987)

Elevated concentrations of alkaline phosphatase occur in a variety of diseases. The effect of alkaline phophatase on platelet aggregation was tested. Adenosine diphosphate-induced aggregation was inhibited at the highest concentrations of alkaline phosphatase employed. Similar results were observed when epinephrine, collagen and arachidonic acid were used as aggregating agents. A dose-dependent inhibition of ristocetin-induced aggregation by alkaline phosphatase was observed. These results were confirmed using plasma from patients with cholestatic disease and elevated concentrations of alkaline phosphatase similar to those used in the in vitro studies. These data indicate that patients with significantly elevated levels of alkaline phosphatase may be predisposed to a thrombocytopathy.

Keywords: adenosine diphosphate, alkaline phosphatase, platelet aggregation, ristocetin, thrombocytopathy

Introduction

Serum contains alkaline phosphatase, an enzyme which hydrolyzes synthetic phosphate esters at pH 9. This enzyme is produced by many tissues (especially liver, bone, intestine and placenta) and is excreted in bile. Increased alkaline phosphatase occurs in patients with a variety of diseases, including hepatitis, cirrhosis, biliary obstruction, Paget's disease, bone metastases and metastatic liver disease [1].

The physiologic agent responsible for platelet aggregation is adenosine diphosphate (ADP), which is released from platelets during exposure to collagen or ADP [2]. Other effectors of platelet aggregation include epinephrine, ristocetin, thrombin and products of arachidonic acid metabolism [3].

Deaggregation occurs when local ADP is degraded by plasma as well as platelet ATPase and ADPase [4]. Because alkaline phosphatase is also capable of hydrolyzing ADP, platelet aggregation may be affected in patients with elevated concentrations of this enzyme.

Materials and Methods

Platelet-rich plasma was prepared from venous blood, anticoagulated with 3.8 per cent (wt/vol) trisodium citrate, and centrifuged at 200*g* for seven minutes.

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

The platelet count was then adjusted to 300×10^9 per liter, by dilution with autologous platelet-poor plasma. In some experiments, plasma from patients with extrahepatic cholestasis was used. Several plasma samples from different patients were used for this purpose. Platelet-rich plasma of a healthy donor was mixed with the patient's plasma sample to achieve the platelet count as noted above. Platelet aggregation was recorded in a Payton dual-channel aggregometer. The aggregating agents used were adenosine diphosphate, epinephrine, collagen, ristocetin and sodium arachidonate (Sigma Chemical Co., St. Louis, MO) alone or in the presence of increasing concentrations of alkaline phosphatase (Type I, 1 – 3 units/mg solid, Sigma Chemical Co.). Alkaline phosphatase was measured on an American Monitor KDA using a modification of the method of Bowers and McComb.

Student's *t* distribution was used for all statistical analyses.

Results

As shown in Table 1, ADP-induced aggregation was reduced significantly in the presence of the highest concentrations of alkaline phosphatase tested. Similar results were observed with epinephrine, collagen and arachidonic acid. In the presence of alkaline phosphatase, a dose-dependent inhibition of ristocetin-induced aggregation was observed. When aggregation was not inhibited, alkaline phosphatase had no effect on the rate of aggregation.

Table 1

The effect of alkaline phosphatase on platelet aggregation as measured by the maximal change in optical density (%) ~

Alk. Phos. (IU/L)	ADP1*	ADP2*	Epi*	Coll*	AA*	Risto*
None	86%	88%	90%	90%	88%	95%
63	86%	88%	90%	89%	85%	94%
126	85%	87%	89%	88%	80%	36%**
315	82%	87%	89%	88%	55%**	32%**
630	54%**	64%**	86%	86%	50%**	22%~~
1260	50%**	58%**	36%**	41%**	46%**	12%~~

Abbreviations: Alk. Phos.-Alkaline Phosphatase, ADP-Adenosine diphosphate, Epi-Epinephrine, Coll-Collagen, AA-Arachidonic Acid, Risto-Ristocetin.

~ Pooled data from 3 separate experiments

* ADP1: 10^{-6}M, ADP2: 2×10^{-6}M, Epi: 2×10^{-6}M, Coll: 10^{-5}M, AA: 5×10^{-5}M, Risto: 2×10^{-5}M

~~ $p < .0005$ vs. control (none)

** $p < .001$ vs. control (none)

Similar results are shown in Table 2 when patients' plasma containing elevated concentrations of alkaline phosphatase was added to normal platelets and aggregation measured.

Table 2

The effect of alkaline phosphatase on platelet aggregation using patients' plasma as measured by the maximal change in optical density (%) ~

Alk. Phos. (IU/L)	ADP1*	ADP2*	Epi*	Coll*	AA*	Risto*
None	85%	87%	90%	90%	87%	94%
62	86%	88%	91%	90%	86%	95%
138	86%	87%	88%	89%	81%	35%**
324	81%	87%	88%	88%	54%**	31%**
628	53%**	62%**	86%	87%	50%**	20%~~
1280	49%**	57%**	35%**	40%**	45%**	10%~~

Abbreviations: Alk. Phos.-Alkaline Phosphatase, ADP-Adenosine diphosphate, Epi-Epinephrine, Coll-Collagen, AA-Arachidonic Acid, Risto-Ristocetin.

~ Pooled data from 3 separate experiments

* ADP1: 10^{-6}M, ADP2: 2×10^{-6}M, Epi: 2×10^{-6}M, Coll: 10^{-5}M, AA: 5×10^{-5}M, Risto: 2×10^{-5}M

~~ p, .0005 vs. control (none)

** $p<.001$ vs. control (none)

Discussion

This study demonstrates that elevated concentrations of alkaline phosphatase, similar to those observed in vivo, inhibit platelet aggregation in vitro. The fact that high concentrations of the enzyme were necessary to abrogate the effect of ADP may be related to the fact that small concentrations of ADP are capable of inducing platelet aggregation. Concentrations of alkaline phosphatase <630 IU/L may not hydrolyze enough ADP to inhibit aggregation.

The results with epinephrine and collagen are consistent with the observation that they induce platelet aggregation by stimulating ADP release. The effect of alkaline phosphatase on arachidonate- and ristocetin-induced aggregation cannot be explained by the enzymatic degradation of ADP. These substances cause platelet aggregation by mechanism usually not involving ADP. However, it is known that alkaline phosphatase decreases the negative platelet surface charge which may be involved in aggregation using these agents [6].

These data suggest that in disease states characterized by elevated levels of alkaline phosphatase, platelet function may be impaired. Using plasma from pa-

tients with elevated levels of alkaline phosphatase, platelet aggregation was inhibited in a manner similar to the results observed in vitro. Patients with significantly elevated concentrations of alkaline phosphatase, therefore, may be at increased risk for bleeding.

Acknowledgement

This work was supported by the Rhode Island Foundation.

References

1. Isselbacher, K. J., LaMont, J. T.: Diagnostic procedures in liver disease. In: *Principles of Internal Medicine*. G. W. Thorn, R. D. Adams, E. Braunwald, K. J. Isselbacher, R. G. Petersdorf (eds), McGraw-Hill, Inc., New York 1977, Vol. 2, p. 1580.
2. Hovig, T.: Release of platelet aggregating substance (ADP) from rabbit blood platelets induced by saline extraction of tendons. *Thromb. Diath. Haemorrh.* 6, 86 (1963).
3. Karpatkin, S.: Metabolism of platelets. In: *Hematology. W. J. Williams, E. Beutler, A. J. Erslev, R. W. Rundles (eds)*, McGraw-Hill, Inc., New York 1977, p. 1190.
4. Spaet, T. H., Lejnieks, I.: Studies on the mechanism whereby platelets are clumped by adenosine diphosphate. *Thromb. Diath. Haemorrh. 15*, 36 (1966).
5. Gaarder, A., Jonsen, J., Laland, S., Hellem, A., Owren, P. A.: Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature 192*, 531 (1961).
6. Mehrishi, J. N.: Phosphate groups (receptors ?) on the surfaces of human blood platelets. *Nature 226*, 452 (1970).

Haematologia 22 (2), pp. 69–78 (1989)

Microspectroscopy of Red Blood Cells

M. BRUNORI[1], M. COLETTA, [1]A. BELLELLI[1],. V. EVANGELISTA[2], P. A. BENEDETTI[2], M. BRUMEN[3]

[1]Dipartimento di Scienze Biochimiche e Centro di Biologia Molecolare del C. N. R., Università "La Sapienza", Piazza Aldo Moro 5, 00185 Roma, Italy, [2]Istituto di Biofisica del C. N. R., Pisa, Italy, [3]Institute of Biophysics, Faculty of Medicine and Josef Stefan Institute, Ljubljana, Yugoslavia

(Arrived 19 June, 1987; accepted 28 December, 1987)

Spectroscopic techniques have been widely employed to analyze properties of macromolecules and dynamics of intracellular events on bulk preparations of cells. The development of computer controlled microspectrophotometers has made possible the study of the same events in single cells, often providing significant and unexpected results.

This paper briefly reviews experimental works carried out in our laboratories on single red blood cells. Microspectrophotometric techniques were applied which make use of the fact that ligand binding to intracellular haemoglobin is associated with optical changes.

Information on the relative abundance of different haemoglobin components inside single erythrocytes of trout blood was obtained from spectra of air equilibrated samples, taking advantage of the extreme pH sensitivity of one of the four haemoglobin components.

The kinetics of oxygen and carbon monoxide binding to haemoglobin has been followed and demonstrated to correspond to a zero order process, with a rate much slower thant that characteristic for haemoglobin in solution. These results demonstrate that the process is diffusion limited; computer simulations suggest that ligand uptake is limited by the time required for the diffusion from the extracellular space of enough ligand molecules for total saturation of intraerythrocytic haemoglobin.

Finally, oxygen dissociation curves in single red blood cells can be obtained by means of particular flow cell, with promising results for the study of physiological and pathological processes (namely red cell sickling in drepanocytosis).

Keywords: single cell microspectroscopy, erythrocyte, ligand diffusion

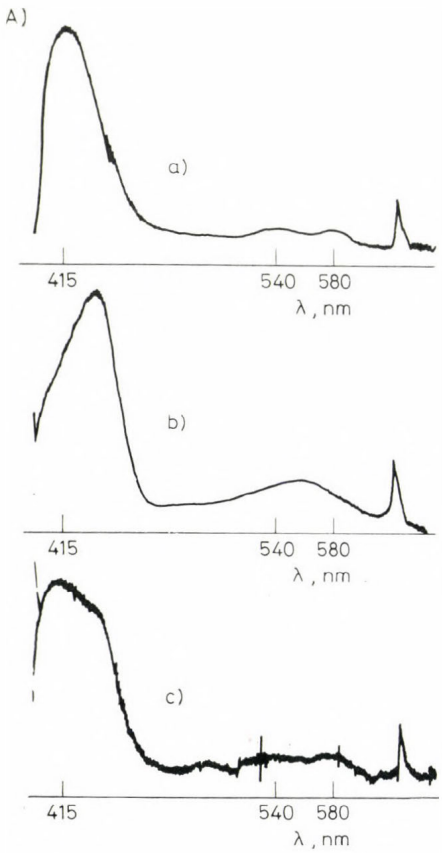
Introduction

In the last decade, quantitative investigations of biochemical processes have been progressively extended from the study of the isolated micro and macromolecular components to the study of interactions of more integrated systems, such as the whole cell. Often the classical approach of cell disruption is required to follow the biochemical event under examination. Optical microscopy is generally used only to acquire morphological information on fixed specimens with limited applicability to the in vivo situation.

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

In this review we present some results obtained by a combination of high resolution optical microscopy with quantitative spectrophotometry to follow time-dependent intracellular phenomena in intact cells under physiological conditions (see also Hofrichter, 1979, Hofrichter and Eaton, 1976, and Coletta et al., 1982). This approach, as applied to single red blood cells, makes use of a computer controlled scanning microspectrophotometer (Benedetti et al., 1976) coupled with a photochemical device to induce gas exchange and thus to follow reactions in single erythrocytes starting from the photolabile complex of haemoglobin (Hb) with carbon monoxide (CO) (Antonini et al., 1978, Antonini et al., 1982). The studies described in this paper deal with two principal lines of research which were undertaken in our laboratories during the last decade and are still in progress, namely:

1) spectrophotometric and functional investigations in single red blood cells to determine the distribution of haemoglobin components among individual erythrocytes whenever the haemolysate is known to contain several molecular species;



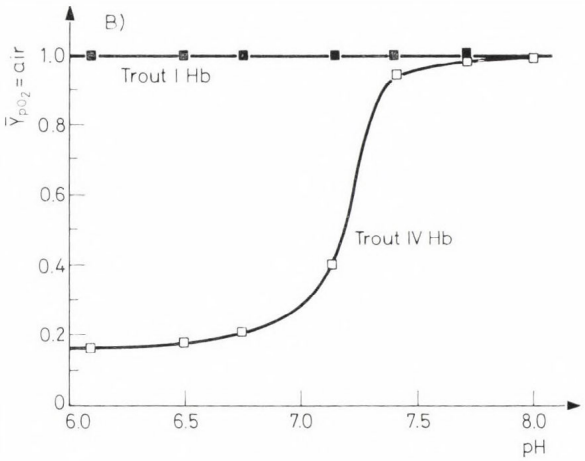


Fig. 1. Panel A: Absorption spectra of single red blood cells from trout (Salmo irideus) blood. Conditions: A: air equilibrated isotonic phosphate buffer pH 7.6. B: isotonic phosphate buffer pH 7.6 plus sodium dithionite (no oxygen present). C: air equilibrated isotonic phosphate buffer pH 6.
Panel B: Oxygen saturation in air of the two main hemoglobin components from trout blood, i.e. Hb I (pH insensitive) and Hb IV (displaying the Root effect)

2) kinetic events involved in gas dissociation and uptake by haemoglobin in single erythrocytes, and the effect of these processes on varying cellular morphology and intracellular conditions. Such measurements have been carried out in erythrocytes of species other than human, such as *Camelus dromedarius*, the trout *Salmo irideus* and the clam *Scapharca inaequivalvis*.

The present results suggest future developments of dynamic microspectroscopy for the study of normal and abnormal processes in red blood cells.

Methods

Measurements in single erythrocytes have been undertaken with a computer-controlled microscpectrophotometer equipped with a fast moving condenser similar to the prototype described elsewhere (Benedetti et al., 1976). The diameter of the monochromatic analysis beam from a Xenon arc lamp XBO75W2 (Osram) can be changed, with a diaphragm, to $1-2\ \mu m^2$. Furthermore, during wavelength scanning, the moving condenser under the control of a PDP 11/23 Digital computer allows the analysis beam to be shifted into two positions (i.e. outside and inside the single cell). Light transmittance in the two different locations can be compared, thus allowing a measure of optical density. With this method, absorption spectra of haemoglobin in single red blood cell can be collected over a wavelength region ranging from 360 to 760 nm (see Fig. 1).

The photolysis source necessary to carry out the kinetic experiments is a mercury arc lamp HBO 100W (Osram), rendered monochromatic by a proper interference filter (Balzers, λ = 546 nm). Steady state photodissociation can be achieved by opening a shutter (rise time = 1 ms).

The optical and mechanical setup is connected "on-line" to the computer for real time control, data aquisition and processing.

Applications

A) Intracellular distribution in various species

The collection of absorption spectra in the single red blood cell can be very important in detecting inhomogeneous distribution of components in heterozygotes, or, in species containing different types of haemoglobins, in the haemolysate. If the various components display different spectral features, such as met Hb Fe (III) and oxy Hb Fe (II), the most straightforward way of quantitating such a heterogenous distribution is by optical absorption of several individual erythrocytes over the whole spectral range. However, in most cases, the intrinsic spectroscopic features of the haemoglobin components are similar, and therefore the solution to the problem must reside in determining different functional properties of the various haemoglobin components.

A very interesting example of the latter approach is represented by the blood from the trout *Salmo irideus*, which contains four types of haemoglobin; three of these, namely component I and II on the one hand (30%) and component IV on the other (65%) represents 95% of the proteins in the haemolysate. Exploiting the different functional properties of these two components, the problem of determining their distribution in individual erythrocytes has been solved. In fact, trout Hb I and II are characterized by a pH-independence of oxygen affinity, whereas in the case of trout Hb IV a decrease in pH decreases oxygen affinity to such an extent that at pH 7.0 the protein is incompletely saturated with the gas even at atmospheric pressure (Brunori, 1975). Therefore, comparison of the optical absorption spectrum of oxygenated red blood cells from trout between pH 8.0 and pH 6.0 makes possible the estimation of the fraction of deoxygenated sites within any particular cell (Fig. 1). If the expected oxygen saturation of the various haemoglobin components in the solution is independently known, the percentage of each component in the single erythrocytes can be calculated. The conclusion of this analysis is that (1) the percentage of each component is similar in every cell examined; (2) each type of haemoglobin is present in every red blood cell in a proportion similar to that of the haemolysate. Such a conclusion is relevant to the physiological role played by these functionally different haemoglobins in the oxygen transport process, as discussed by Brunori et al. (1974).

As a recent and more sophisticated development of this approach we have set up a method to determine oxygen equilibrium binding curves by collecting spectra in single red blood cells under controlled gas pressure, employing a flow

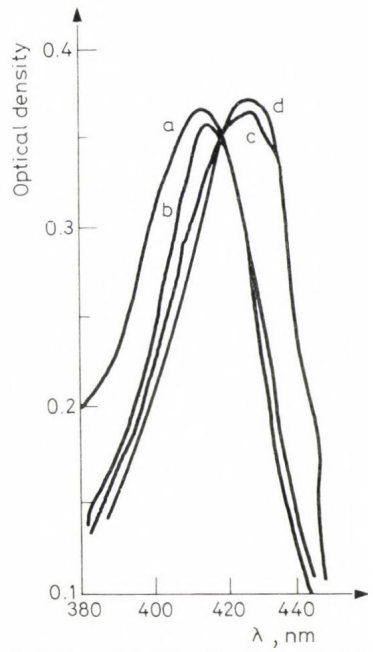


Fig. 2. Absorption spectra of a human red cell equilibrated at different oxygen partial pressures: A: 152 mm Hg; B: 10 mmHg; C: 7 mmHg; D: 2.8 mmHg. Other conditions: isotonic phosphate buffer pH 7.4, t = 27 C

cell to change conditions at will. An example of spectra collected at various oxygen concentrations is given in Fig. 2. This method, which is being developed in our laboratories, will prove to be very useful in analyzing the functional properties of red blood cells at various stages of maturation and for testing the effect of drugs on the oxygen carrying properties in living cells.

B) Intracellular uptake and release of gaseous ligands

The study of intracellular ligand binding kinetics in single erythrocytes makes use of the photosensitivity of the complex between haemoglobin (Hb) and carbon monoxide (CO), according to the following equations:

$$\text{Hb CO} \xrightarrow{+\text{hv (light)}} \text{Hb} + \text{CO} \quad (1)$$
$$\text{Hb} + \text{CO} \xrightarrow{\text{(dark)}} \text{HbCO} \quad (2)$$

Scheme I

When a bright light is switched "on", starting from a system completely saturated with CO, deoxygenated haemoglobin is rapidly produced (reaction 1); this process, driven by light, achieves completion because of the outward diffusion of CO due to the concentration gradient between the inside ([Hb] = [CO] = 20 mM) and

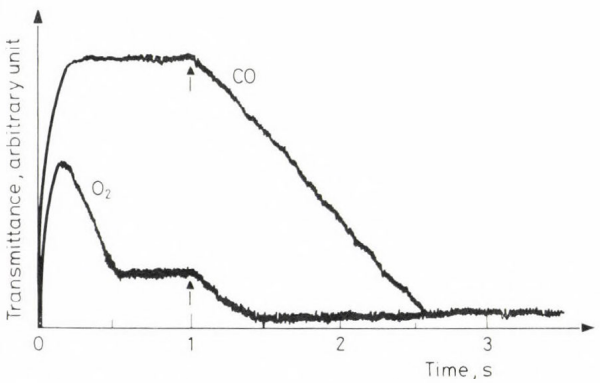


Fig. 3. Time course of transmittance changes at 434 nm following irradiation (546 nm) of CO equilibrated human red blood cells. The top trace is obtained in the absence of oxygen, the bottom one in the presence of 0.08 mM oxygen; CO was present in both experiments at a concentration of 0.1 mM. Upward deflection (at the left) indicates accumulation of deoxy Hb, which in the presence of oxygen (bottom trace) disappears to yield oxyhemoglobin (see scheme II); the photodissociation beam is kept "on" from time zero to 1 sec, and then switched off (arrow). Conditions: isotonic phosphate buffer pH 7.4, T = 27 C

the outside ([Hb] = 0; [CO] = 1 mM) of the RBCell. When the photodissociating light is turned "off", rebinding occurs (reaction 2) (see Fig. 3), at a rate which is dependent on the concentration of free CO in solution.

Similarly, kinetics of oxygen binding to intraerythrocytic haemoglobin can be followed taking advantage of the very low photosensitivity of the HbO_2 complex and of the higher affinity of haemoglobin for CO (Antonini and Brunori, 1971). Thus, under appropriate CO/O_2 concentration ratios, the intracellular haemoglobin is fully saturated with CO in the dark, while irradiation with a stationary light brings about the following reaction (see also Fig. 3):

$$\underset{(1)}{\text{Hb CO} + \text{O}_2} \overset{+h\nu}{\rightleftarrows} \underset{(2)}{\text{Hb} + \text{CO} + \text{O}_2} \overset{+h\nu}{\rightleftarrows} \underset{(3)}{\text{HbO}_2 + \text{CO}} \quad \text{(Scheme II)}$$

Thus, under these conditions deoxy Hb is transiently accumulated in the cell (state 2), and formation of the oxygenated complex (HbO_2) under stationary light can be followed (state 3).

In both cases, i.e. for oxygen and CO, ligand rebinding follows a zero order time course (see Fig. 3), with an apparent rate constant which is 30 to 50 times slower than that measured for haemoglobin in solution (Antonini and Brunori, 1971).

The two characteristic features suggest that rebinding of the gas (O_2 or CO) to intracellular haemoglobin is rate limited by the ligand diffusion from extracellular space into the erythrocyte (Antonini et al., 1978). The very high intracellular haemoglobin concentration (20 mM heme, i.e. 20 times higher than that of

the extracellular ligand) accounts for the fact that the gaseous ligand is "sucked" from the bulk into the cell, and for a large part of the overall rebinding process the free ligand inside the cell is rapidly captured by the excess Hb molecules; therefore, its steady-state concentration is kept close to zero.

It is relevant to point out that in our experimental set-up, in contrast to previous studies by rapid mixing techniques (Coin et al., 1979), the erythrocytes were sitting in a stationary system, which enhanced the effect of "depletion" of the ligand from the unstirred layer of solvent around the cell. Moreover, the analysis beam is perpendicular to the thinner axis of the cell (Z-direction, see Fig. 4); therefore, inhomogeneous temporal evolution of the recombination process along the Z-axis cannot be resolved. In fact, experimental observations in different positions of the same red blood cell have shown that the same time course is obtained in different sections of the cell, even though, because of the discoid shape of the red blood cell, the diffusional contribution is larger in the Z-direction (Fig. 4).

The physics of the system has been described by a unidimensional model (Crank, 1975), in which the diffusional contribution takes place only in the Z-direction (Coletta et al., 1987).

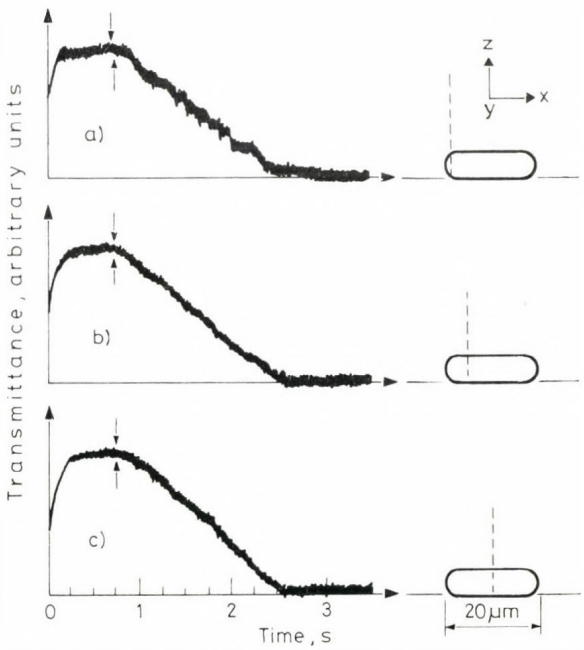


Fig. 4. Time course of transmittance changes at 434 nm after complete photolysis of CO equilibrated red cells from Scapharca inaequivalis. The photolytic beam was switched off after 1 sec irradiation (arrow). As depicted on the right of each trace, the three panels refer to setting of the observation light beam in different points of the cell: A: lateral border; B: intermediate position; C: center. Conditions: isotonic phosphate buffer pH 7.4, T = 27 C

According to a recent analysis based on this unidimensional model, and in agreement with the original observations by Antonini et al. (1978), the temporal evolution of the formation of CO haemoglobin is rate limited by the time necessary for the ligand molecules to diffuse from a region of space around the cell to the inside through the membranes, and thereafter within the cell. The role of ligand diffusional processes inside the erythrocyte has been tested by altering the intracellular total volume. Swollen camel red blood cells were used for this experiment, which are known to resist hyposmotic conditions (down to 50 mOsm/l) doubling their original volume without haemolysis (Perk, 1963). The result of Coletta et al. (1985) suggests that, besides the role of the extracellular unstirred layer, intracellular diffusion also appears to contribute – under some critical conditions – to the observed CO recombination process.

To test the possible effect of intracellular diffusion of haemoglobin on the rate process, we have studied human erythrocytes reacted with glutaraldehyde, which can be used to cross-link extensively and to fully polymerize intracellular haemoglobin, thereby abolishing altogether its translational motion. With these modified RBCells it was found that the kinetics of CO rebinding followed in single erythrocytes is essentially unchanged, as compared to non-modified human RBCells. This result is in complete agreement with the fact that the translational diffusion coefficient of CO (8×10^{-6}) is much larger than that of haemoglobin (7.5×10^{-8}: see Vandegriff and Olson 1984b), since cross-linking by glutaraldehyde freezes haemoglobin completely: the effect on the diffusion of small molecules, such as CO, is negligible.

It should be made clear that the unidimensional formalism described above (Crank, 1975) is an approximation and cannot reproduce, even qualitatively, the rate constant enhancement observed by lowering the osmotic pressure suggesting that in this case the X and Y component are not negligible. A more complex three dimensional model is required, and in fact in a fully developed three dimensional diffusion model for red blood cell oxygenation (Vandergriff and Olson, 1984a) intracellular diffusion of both haemoglobin and the gas, as well as the intrinsic association and dissociation kinetic constants, were all taken into account.

Conclusion

The brief review of results obtained by the application of single cell microspectroscopy to the dynamics of cellular reactions outlines the potentialities of this approach for a quantitative study of cell biophysics.

We have shown that quantitative spectrophotometry can be used to determine the distribution of different haemoglobin components and to estimate the functional homogeneity in a population of cells. This possibility is of interest in studies of cell aging and/or cell differentiation, for it may allow the detection of the distribution of microscopic states which may be crucial to the understanding of these complex processes.

Furthermore, observations on the dynamics of photoinducible reactions are possible down to the millisecond time range. In this area, the physiological significance of gas diffusion inside the red blood cell as a function of flow turbulence around the cell is of interest. Moreover, studies of molecular events involved in the pathological state characteristic of sickle cell anaemia (Eaton and Hofrichter, 1987) are important. Thus rapid photodissociation of bound ligand can mimick oxygen unloading in the capillaries, while recombination in the dark may mimick oxygen rebinding in the pulmonary district. These investigations are in progress and indicate that the microspectrophotometric technique can provide a unique insight into the molecular physiopathology of sickle cell anaemia.

Acknowledgements

A special grant from the University of Rome "La Sapienza" to M. B. for the construction of a fast scanning microspectrophotometer is gratefully acknowledged. This work was partially supported by a Grant from the Ministero della Publica Istruzione of Italy.

References

1. Antonini, E., and Brunori, M.: *Hemoglobin and myoglobin in their reactions with ligands.* Elsevier North-Holland Co., Amsterdam 1971.
2. Antonini, E., Brunori, M., Giardina, B., Benedetti, P. A., Bianchini, G., and Grassi, S.: Kinetics of the reaction with CO of human erythrocytes. *FEBS Lett. 86*, 209—212 (1978).
3. Antonini, E., Brunori, M., Giardina, B., Benedetti, P. A., and Pintus, N.: Kinetics of oxygen reactions within single erythrocytes: observations by microspectrophotometry. In: *Haemoglobin and Oxygen Binding*, Ho, C. (ed.), Elsevier, Amsterdam 1982, pp. 449—452.
4. Benedetti, P. A., Bianchini, G., and Chiti, G.: Fast scanning microspectroscopy: an electrodynamic moving condenser method. *Appl. Optics 15*, 2554—2558 (1976).
5. Brunori, M., Giardina, B., Antonini, E., Benedetti, P. A., and Bianchini, G.: Distribution of hemoglobin components of trout blood among the erythrocytes: observations by single cell spectroscopy. *J. Mol. Biol. 86*, 165—169 (1974).
6. Brunori, M.: Molecular adaptation to physiological requirements: the hemoglobin system of trout. *Curr. Top. Cell Reg. 9*, 1—39 (1975).
7. Coin, J. T., and Olson, J. S.: The rate of oxygen uptake by human red blood cells. *J. Biol. Chem. 254*, 1178—1190 (1979).
8. Coletta, M., Hofrichter, J., Ferrone, F. A., and Eaton, W. A.: Kinetics of sickle hemoglobin polymerization in single red cells. *Nature 300*, 194—197 (1982).
9. Coletta, M., Giardina, B., Amiconi, G., Gualtieri, P., Benedetti, P. A., and Brunori, M.: Kinetics of the reaction of intraerythrocytic hemoglobin by single red cell microspectroscopy: effect of shape and osmolarity. *FEBS Lett. 190*, 217—220 (1985).
10. Coletta, M., Brumen, M., Giardina, B., Benedetti, P. A., and Brunori, M.: A microspectroscopic analysis of ligand binding in single erythrocyte. *Biomed. Bioch. Acta 46*, 108—112 (1987).
11. Crank, J.: *The Mathematics of Diffusion*, Clarendon Press, Oxford 1975.
12. Eaton, W. A., and Hofrichter, J.: Hemoglobin S gelation and sickle cell disease. *Blood* 1987 (In press).

13. Hofrichter, J., and Eaton, W. A.: Linear dichroism of biological chromphores. *Ann. Rev. Biophys. Bioeng. 5*, 511—560 (1976).
14. Hofrichter, J.: Ligand binding and the gelation of sickle cell hemoglobin. *J. Mol. Biol. 128*, 335—369 (1979).
15. Perk, K.: The camel's erythrocyte. *Nature 200*, 272—273 (1963).
16. Vandegriff, K. D. and Olson, J. S.: A quantitative description in three dimensions of oxygen uptake by human red blood cells. *Biophys. J. 45*, 825—835 (1984).
17. Vandegriff, K. D. and Olson, J. S.: Morphological and physiological factors affecting oxygen uptake and release by red blood cells. *J. Biol. Chem. 259*, 12619—12627 (1984).

Haematologia 22 (2), pp. 79–87 (1989)

^{31}P NMR Studies on Rejuvenation of Outdated Red Blood Cells: Complete Regeneration of ATP Is Accompanied by Partial Mg-ATP Recomplexation

AREN VAN WAARDE[1,3], ARNOLD LOMBARTS[2], GUIDO VAN DEN THILLART[1], CEES ERKELENS[3], JOHAN LUGTENBURG[3]

Departments of [1]Animal Physiology and [3]Organic Chemistry, Gorlaeus Laboratories, University of Leiden, Einsteinweg 5, PO Box 9502, 2300 RA LEIDEN, The Netherlands; and [2]Department of Clinical Chemistry and Haematology, Leyenburg Hospital, THE HAGUE, The Netherlands

(Received 24 August, 1987; accepted 18 February, 1988)

We used ^{31}P Nuclear Magnetic Resonance (NMR) spectroscopy to investigate whether regeneration of ATP in outdated human blood is accompanied by Mg-ATP recomplexation. We found that the loss of 'total ATP' (i.e. the sum of ATP^{4-} + Mg-ATP^{2-}) which occurs during blood storage is completely reversible by treatment of RBC with precursors for purine nucleotide salvage. The magnesium-ATP complex, however, is only partially (70%) recovered due to a continuous decline of cytosolic free Mg^{2+} with increasing RBC age. Total RBC magnesium, as measured by flame absorption spectroscopy, did not show any significant change under these conditions.

Keywords: ^{31}P, NMR spectroscopy, ATP regeneration, blood storage, Mg-ATP recomplexation

Introduction

Storage of red blood cells (RBC) under blood bank conditions leads to a decrease in 2,3-diphosphoglycerate (DPG), ATP and intracellular pH [1–4]. Regeneration of DPG and ATP can be accomplished by incubation of outdated RBC with precursors for purine nucleotide salvage [4 5]. In comparative studies, Nakao et al. [5, 6] showed that optimal regeneration is achieved by the use of relatively large amounts of adenine and inosine in an isoosmotic sucrose medium. Although it has been established that regeneration of ATP has a beneficial effect on RBC viability and stability [5], there is evidence that other, unknown factors affect cell survival [7, 8]. The extent of Mg-ATP complexation might be one of these factors. Recently, Bock et al. [1] described a sizable decrease in the extent of Mg^{2+} complexation of ATP and a substantial decrease in free Mg^{2+} during the shelf-life of blood stored in Acid Citrate Dextrose (ACD) or Citrate Phosphate Dextrose Adenine-1 (CPDA-1).

The purpose of the present study is to investigate whether ATP-regeneration in a medium comparable to that used by Nakao et al. [5] is accompanied by Mg-ATP recomplexation. ^{31}P nuclear magnetic resonance (NMR) spectroscopy was used to measure the intracellular concentrations of phosphorus-containing metab-

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

olites in fresh blood, outdated blood from a blood bank and outdated blood during treatment with rejuvenation medium. Intracellular pH was estimated from the difference in chemical shift between the peaks of inorganic phosphate and an external standard of methylenediphosphonate in the NMR spectra. The amounts of MgATP and ionized Mg^{2+} were calculated from the distance (in Hz) between the α- and β-phosphate resonances of ATP, and the intracellular pH [1]. Whereas the concentration of total ATP, i.e. the sum of $ATP^{4-} + MgATP^{2-}$, could be returned to its original level by treatment of RBC with precursors for purine nucleotide salvage, the magnesium-ATP complex was only partially restored due to a continuous decline of intracellular free Mg^{2+} with increasing RBC age.

Materials and Methods

The following rejuvenation medium was prepared and sterilized at 120 °C for 20 min: 25 mM K_3PO_4, 30 mM inosine, 5.5 mM adenine, 5 mM EDTA-K_3, 3% mannitol, 1 g/l gentamicin, pH adjusted to 7.9 with 4 M HCl. Somewhat higher concentrations than stated were initially made by leaving out enough water to compensate for the 50 ml/l of 20% (human) albumin which was added aseptically after autoclaving and cooling. All chemicals, except gentamicin and the human albumin, were of biochemical grade. Due to the addition of mannitol, minimal haemolysis occurred during the 45 day incubation with precursors for purine salvage, as has been described previously [9, 10].

Outdated, 4 weeks old, buffy-coat depleted, CPDA-1 blood was centrifuged and filtered under sterile conditions through cotton wool to remove white blood cells and platelets as described [10, 11]. Part of this saline washed RBC served as 'untreated outdated blood'. Approximately equal volumes of another portion of the washed RBC concentrate (with a PCV of 70%) and the rejuvenation medium were mixed under sterile conditions in a blood bag. The final PCV was 35% and the final pH was 6.9 (20 °C). During occasional mixing, aliquots of about 6 ml were aseptically filtered through the connecting tube into sterile 6 ml screw-capped glass bottles in a laminar flow cabinet [10, 11]. All blood samples were stored at 4 °C until the time of analysis by NMR.

"Fresh" blood was drawn from an antecubital vein into an EDTA-K_3-containing Vacutainer (Becton Dickinson) tube and measured within 30 h (including a ca. 20 h postal delay).

For NMR-measurements, ca. 6 ml of blood were centrifuged for 5 min at 1000 g and 1.5 – 2 ml of the RBC concentrate of a PCV of ca. 80% were transferred to a 10 mm glass NMR tube. During acquisition of the spectra, the tube was spun at a rate of 10 revolutions per s. ^{31}P-NMR spectra were acquired on a Bruker WM-300 spectrometer operating at 121.5 MHz in the Fourier Transform mode. Two thousand transients were averaged using 8192 data points and a spectral width of 6000 Hz. A 60° pulse was used with a relaxation delay of 1 s after each transient. A co-axially placed capillary, containing 20 mM methylene diphosphonic acid in 5% D_2O, served both as an external reference and a field-frequency lock standard.

The intracellular pH was determined from the difference in chemical shift between the methylphosphonate and the inorganic phosphate peaks in the NMR spectra. Exponential noise filtering, causing a 10 Hz increase in line widths, and zero filling unto a size of 32678 data points were applied prior to Fourier transformation. Chemical shifts are quoted relative to external 85% orthophosphoric acid.

For determinations of total (bound+free) magnesium, RBC and rejuvenation medium were separated by short centrifugation (2.5 min at 12,000 g, Eppendorf centrifuge). Pellet and supernatant were separated and stored in a low-temperature freezer (−80 °C). On the day of measurement, 50 μl of supernatant or pellet were mixed with 100 μl concentrated HNO_3 and heated to 90 °C during a period of 2 h. The digested pellets were subsequently diluted with 5 mM EDTA to a volume of 10 ml. Magnesium was measured in the digests by atomic absorption spectrophotometry, using a calibration curve which ranged from 0 to 100 μM $MgSO_4$.

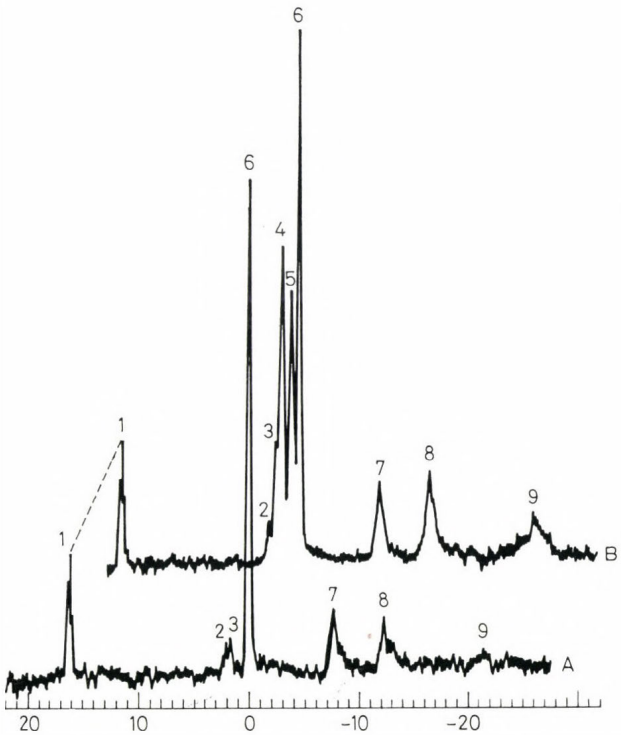


Fig. 1. ^{31}P-NMR spectra (121.5 MHz) of RBC from outdated human blood which had been resuspended in isotonic saline (A) or rejuvenation medium (B) during 7 days at 4 °C. Resonances are those of: 1. External standard (20 mM methylene diphosphonic acid in D_2O, co-axial capillary); 2. Sugar phosphates (position of dihydroxyacetone phosphate); 3. Sugar phosphates (position of fructose 1,6-diphosphate); 4. and 5. 2- and 3-phosphate groups of 2,3-diphosphoglycerate; 6. Inorganic phosphate; 7. γ-phosphate of ATP, with underlying β-phosphate of ADP; 8. α-phosphate of ATP, with underlying α-phosphate of ADP and phosphate group of NAD; 9. β-phosphate of ATP

Results

Figure 1 shows the ^{31}P-NMR spectra of RBC from outdated blood resuspended in isotonic saline (A) or in the adenine and inosine containing rejuvenation medium (B) for 7 days at 4 °C. It is evident that the rejuvenation medium causes substantial increases in the intensities of the resonances of DPG (nos 4 and 5) and the β-phosphate of ATP (no. 9).

The Table shows the levels of free Mg, total Mg, Mg-ATP, ATP, ADP, DPG, NAD and inorganic phosphate, besides the values of the intra- and extracellular pH in fresh blood, untreated outdated blood and outdated blood at various periods of cold (4 °C) rejuvenation. Differences between groups were tested by Wilcoxon's Q-test; a single-tail probability smaller than 5% was considered statistically significant.

Figure 2 depicts the time course changes in ATP, MgATP and DPG during a 45 day period of cold rejuvenation. It is clear that the loss of 'total ATP' during blood storage is completely reversible, albeit only temporarily, whereas Mg-ATP is partially recovered.

Figure 3 illustrates that the partial recovery of the magnesium-ATP complex is due to a continuous decline of intracellular free Mg^{2+} with increasing RBC age. The fraction of total ATP not complexed to magnesium (∅) thus shows a steady increase during the 45 day period. Total (i.e. bound + free) magnesium in the red blood cell is not significantly changed under these conditions. During the whole period of cold rejuvenation, the total magnesium concentration is maintained at the level observed in fresh blood (Table 1, [1]). Extracellular magnesium ranged from 40 to 90 μM and it did not correlate with age.

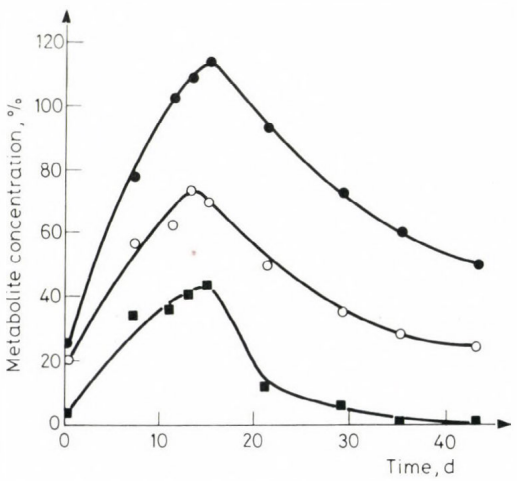


Fig. 2. Time course changes in ATP (●), MgATP (○) and DPG (■) during the 45 day-treatment of outdated blood with precursors fór purine salvage. Metabolite levels are expressed as percentages of the mean value of three fresh blood control samples

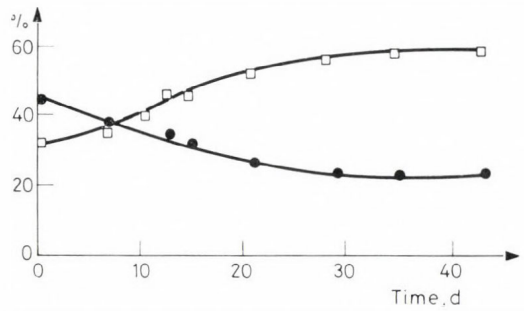


Fig. 3. Time course changes in the concentration of intracellular free magnesium (●) and the fraction of total ATP not complexed to magnesium (□) during the 45 day-treatment of outdated blood with precursors for purine salvage

Figure 2 also exhibits time course changes of DPG during the rejuvenation period. In contrast to ATP, DPG is only partially recovered, the maximum value amounting to ca. 45% of the concentration observed in fresh blood.

The time course of intra- and extracellular pH is illustrated in Fig. 4. Both pH's show a continuous decline due to production of lactate via anaerobic glycolysis. At the end of the rejuvenation period the pH-gradient over the cell membrane decreases, probably due to end product (lactate and H^+) inhibition of the glycolytic chain.

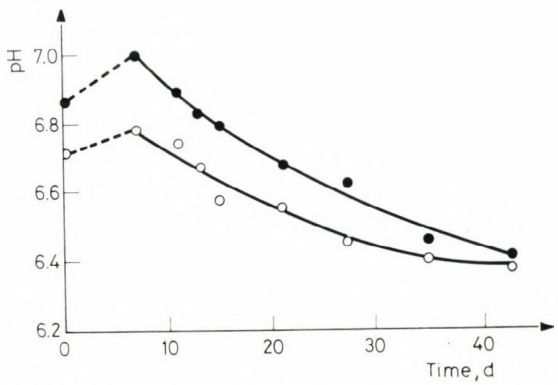


Fig. 4. Decline of the intra (○) and extracellular (●) pH's of outdated blood during the treatment with precursors for purine salvage. Extracellular pH was directly measured with a pH electrode and an electronic pH meter (Radiometer Copenhagen)

Table 1 finally indicates that NAD-levels are not raised by incubation of RBC with precursors for the salvage pathway.

Table 1

Intracellular magnesium and metabolite concentrations, as well as intra- and extracellular pH's in fresh blood and in outdated blood at various stages of cold (4 °C) rejuvenation. Values for ATP, MgATP, ADP, P_i, DPG and NAD are in μmoles/ml packed cells; those for Mg^{2+} and total Mg are in nmoles/ml packed cells; those for the distance between the α- and β-phosphate resonances for ATP are in Hz; those for $\varnothing$ (fraction of total ATP not complexed to magnesium) are without dimension

Parameter	Fresh blood	Untreated outdated blood		Outdated blood 10 d of rejuvenation			Outdated blood 36 d of rejuvenation		
	x±S.D.	x±S.D.	F	x±S.D.	F	U	x±S.D.	F	U
ATP	1.14±0.11	0.29±0.04	<0.005	1.24±0.05	NS	<0.005	0.70±0.10	<0 005	<0.005
Mg-ATP	0.96±0.19	0.15±0.04	<0.005	0.66±0.04	<0.025	<0.005	0.29±0.05	<0.005	<0.025
DPG	9.40±2.41	0.40±0.10	<0.005	3.68±0.38	<0.01	<0.005	0.22±0.22	<0.005	NS
ADP	0.31±0.21	0.46±0.11	NS	0.16±0.12	NS	<0.05	0.82±0.15	<0.025	<0.025
NAD	0.39±0.15	0.14±0.06	NS	0.16±0.03	NS	NS	0.08±0.01	<0.025	NS
P_i	4.05±2.19	6.04±0.45	NS	6.36±0.91	NS	NS	13.29±1.76	<0.005	<0.005
α, β-ATP	1072±11	1161±63	NS	1154±12	<0.005	NS	1201±2	<0.005	NS
Mg^{2+}	276±73	122±96	NS	94±9	<0.025	NS	63±3	<0.01	NS
$\varnothing$	.157±.037	.324±.124	NS	.430±.041	<0.005	NS	.587±.007	<0.005	<0.005
total Mg	n.d.	2014±205	—	2056±123	—	NS	1974±82	—	NS
pH_i	7.09±0.01	6.72±0.05	<0.005	6.74±0.05	<0.005	NS	6.43±0.03	<0.005	<0.005
pH_e	n.d.	6.87±0.01	n.d.	6.91±0.07	n.d.	NS	6.53±0.09	<0.005	<0.005

F = statistical significance of the difference between an experimental group and the value for fresh blood, tested by Wilcoxon's Q-test. U = statistical significance of the difference between an experimental group and the value for untreated outdated blood, tested by Wilcoxon's Q-test. NS = Not significant ($p > 0.05$); ND = Not determined

Discussion

Measurements of total ATP in RBC may be misleading as a measure of metabolic integrity. It is the MgATP complex, rather than ATP^{4-}, that is involved in most energy-requiring metabolic processes. Measurements of the MgATP complex and of free magnesium in intact cells are only possible by the use of ^{31}P-NMR. Recently, Bock et al. [1] demonstrated a substantial decrease in free Mg^{2+} during storage of blood. The decline occurs in all common storage media; it is not due to the presence of extracellular citrate [12]. Total (free + bound) magnesium in the erythrocytes, as measured by atomic absorption spectrometry, remained constant under these conditions. Therefore, the lowering of the free magnesium concentration seems to be due to increased intracellular binding of Mg^{2+} [1]. Accumulation of low-molecular weight chelators (like pyrophosphate) does occur, but the binding capacity of this compound is much too low to explain the phenomenon [12].

Although the decline of intracellular free magnesium during the shelf-life of blood has been well established [1], no information was available about the behavior of this parameter during erythrocyte rejuvenation. The present study shows that during incubation of RBC with precursors for purine nucleotide salvage, a similar decline of free magnesium is observed as during normal storage [1]. Apparently, the increases of the ATP and DPG concentrations which are induced by the rejuvenation medium do not reverse the process which leads to increased binding of Mg^{2+}. The disappearance of ATP and DPG during blood storage can therefore not be the cause of the decline of the free magnesium concentration.

It is known that magnesium binds to macromolecules like proteins. In erythrocytes, the ion is associated with the inside of the plasma membrane. Lowering of the pH from 7.5 to 6.5 results in a twofold increase of magnesium retention by erythrocyte ghosts [13]. The decline of the intracellular pH which occurs during storage could therefore be the factor responsible for increased binding of Mg^{2+}.

However, this explanation can be ruled out on the following grounds. When the intracellular pH of intact erythrocytes is lowered from 7.16 to 6.65 by the addition of extracellular HCl or lactic acid, the intracellular free magnesium concentration increases rather than showing a decline [12]. In the present study, we performed the opposite experiment by adding rejuvenation medium to outdated erythrocytes. This results in an initial increase of the intracellular pH from 6.74 to 7.15. Intracellular free magnesium was decreased rather than increased. Therefore, the drop of the intracellular pH cannot be the direct cause of increased magnesium retention.

During storage, the erythrocytes enter an unfavorable energetic condition, causing potassium efflux and sodium influx. It is therefore theoretically possible that an increase of the intracellular Na, K-ratio causes increased magnesium retention. This hypothesis can also be ruled out on two grounds. Bock et al. [12] have shown that an experimentally induced increase of the intracellular Na,K-ratio by treatment of RBC with gramicidin does not decrease the intracellular free mag-

nesium concentration. We have added a large amount of extracellular potassium to outdated RBC in order to decrease the intracellular Na, K-ratio. Nevertheless, the intracellular free magnesium concentration was not increased, but showed a similar behavior as during normal storage.

While some possible explanations are refuted by of the experimental evidence discussed above, the reason for the decline of free Mg in stored blood remains unclear. Fresh plasma contains unknown factor(s) which induce a reversal of the process [12]. Identification of these factors might lead to an improvement in storage techniques.

Every effort should be made towards a better understanding of blood storage lesions with the ultimate aim of better utilization of stored blood. In blood banks a substantial number of blood bags inevitably get outdated, ca. 2% in The Hague's blood bank, yielding several hundreds of bags per year [11].

Although our experiments are mainly aimed at possible *in vitro* applications [11], they might eventually be of significant importance for *in vivo* use [5]. The drawbacks of pretransfusional washing of the RBC to remove toxic (e.g. K^+, adenine, inosine) or poorly soluble (e.g. hypoxanthine, uric acid) substances might be offset by the advantages of substantially longer stability of RBC [5].

Acknowledgement

We thank Ms. M. I. Kammenga for excellent secretarial assistance.

References

1. Bock, J. L., Wenz, A. B., Gupta, R. K.: Changes in intracellular Mg adenosine triphosphate and ionized Mg^{2+} during blood storage: Detection by ^{31}P Nuclear Magnetic Resonance spectroscopy. *Blood 65*, 1526–1530 (1985).
2. Chapman, B. E., Kuchel, P. W., Lovric, V. A., Raftos, J. E., Stewart, I. M.: Regeneration of phosphorylated metabolites in stored erythrocytes in an open perfusion system: Studies using ^{31}P NMR spectroscopy. *Brit. J. Haemat. 61*, 385–392 (1985).
3. Ambruso, D. R., Hawkins, B., Johnson, D. L., Fritzberg, A. R., Klingensmith, W. C., McCabe, E. R. B.: Measurement of adenosine triphosphate and 2,3-diphosphoglycerate in stored blood with ^{31}P Nuclear Magnetic Resonance spectroscopy. *Biochem. Med. Metab. Biol. 35*, 376–383 (1986).
4. Valeri, C. R.: Use of rejuvenation solutions in blood preservation. *CRC Crit. Rev. Clin. Lab. Sci. 17*, 299–374 (1982).
5. Nakao, T., Nagai, F., Nakao, M.: Posttransfusion viability of rabbit erythrocytes preserved in a medium containing inosine, adenine and isoosmotic sucrose. *Vox Sang. 42*, 217–222 (1982).
6. Nakao, M., Nakao, T., Komatsu, Y., Sano, K., Sasakawa, S.: Isoosmotic sucrose, adenine, inosine media for preservation of blood. *Biomed. Biochim. Acta 42*, 527–535 (1983).
7. Högman, C. F., Arro, E., Hedlund, K.: Red blood cell preservation in protein-poor media 2. Studies of changes in red cell shape during storage. *Haematologia 13*, 135–144 (1980).
8. Laczko, J., Szabolcs, M., Jona, I.: Vesicle release from erythrocytes during storage and failure of rejuvenation to restore cell morphology. *Haematologia 18*, 233–247 (1985).

9. Nakao, M., Nakao, T., Nakayama, T., Nagai, F., Dogen, M.: Further investigation on ATP metabolism and red cell membrane integrity. *Acta Biol. Med. Germ. 40*, 1003—1008 (1981)
10. Lombarts, A. J. P. F., Leijnse, B.: Preparation and evaluation of a 7-parameter intra-laboratory control blood of 4-month stability. *Ann. Clin. Biochem. 20*, 302—307 (1983)
11. Lombarts, A. J. P. F., Leijnse, B.: Outdated blood and redundant buffy-coats as sources for the preparation of multiparameter controls for Coulter-type (resistive-particle) hemocytometry. *Clin. Chim. Acta 143*, 7—15 (1984)
12. Bock, J. L., Wenz, B., Gupta, R. K.: Studies on the mechanism of decreased NMR-measured free magnesium in stored erythrocytes. *Biochim. Biophys. Acta 928*, 8—12 (1987)
13. Romero, P. J.: The role of membrane-bound magnesium in the permeability of ghosts to K^+. *Biochim. Biophys. Acta 339*, 116—125 (1974).

Haematologia 22 (2), pp. 89—96 (1989)

Room Temperature Storage of Pooled Platelet Concentrates in Gas-permeable Plastic Bags for Five Days

Z. RÁCZ, F. HASKÓ

National Institute of Haematology and Blood Transfusion, 1502 Budapest, P.O.Box 44, Hungary

(Received 16 November, 1987; accepted 1 March, 1988)

Pooled platelet concentrates of different platelet count and volume were stored in gas-permeable plastic bags. Adequate oxygen supply kept the glucose consumption and therefore the lactic acid production relatively low. The change of pH seemed to depend largely on the platelet count and on the volume of the concentrate. The *in vitro* functions showed a decrease together with the adenine nucleotide content. The reduced *in vivo* viability of stored platelets corresponded to decreased hypotonic stress response, as well as to lowered ATP content. The results suggest that storage of platelets in gas-permeable bags may extend the shelf-life of pooled platelet concentrates, but the actual amount of platelets present should be taken into consideration.

Keywords: platelet function, life-span, storage

Introduction

Freeze-storage permits an extended preservation of platelets in the frozen state, while room temperature storage in the conventional PVC plastic bags is adequate only for 2—3 days. With the use of gas-permeable plastic bags, shelf-life of liquid-preserved platelets may be lengthened, as in the presence of oxygen supply lactic acid accumulation is decreased. It is possible that the enhanced carbondioxyde diffusion properties of the new containers also help to prevent a drop of pH. As the pH of the medium remains relatively high, the deterioration of platelets due to the acidic environment can be abolished.

We studied several functions of pooled platelets after 120 hours of storage, and attempts were made to determine factors to be taken into consideration for storage of pooled platelet concentrates.

In previous studies [17, 19, 23, 24] platelet concentrates were made from single units of blood in relatively small volumes. In contrast, in this present study we used pooled platelet concentrates in a higher volume produced by the buffy coat technique [14].

Materials and Methods

Platelet concentrates were obtained from CPD blood (400 ml blood to 63 ml CPD) by the buffy coat technique [14]. Briefly, blood was centrifuged at 1800*g*

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

for 12 min at 20 °C. Then the buffy coat portions of 3—4 ABO and Rh identical blood units were pooled (the work with the bags was carried out in an aseptic laminar air-flow box). The pooled buffy coat was centrifuged at 180*g* for 12 min at 20 °C; conventional PVC bags were used. After the second centrifugation the platelet-containing suprnatant was transferred into a transfer pack for platelet harvesting (PL 1240 container, Fenwal, USA). The plasticizer in this bag is tri-(2-ethylhexyl)-trimellitate. For comparison of the different storage conditions, platelet concentrates of different platelet counts ($0.52-2.00\times10^6/\mu l$) and different volumes (55—400 ml) were made.

These platelet concentrates were stored on an elliptical platelet rotator (six rpm, Fenwal, USA). Platelet functions, pH and glucose content of the medium were measured before and after 120 hours (= 5 days) of storage at $+22\pm1$ °C.

A light microscope was used for platelet counting. The pH was measured with a pH meter (Radelkis, Hungary), while the glucose content was determined with an Eppendorf ACP 5040 analyzer (Eppendorf, Austria). Aggregation, response to hypotonic stress and clot retraction tests were performed with platelet suspensions adjusted to a concentration of 300,000 per μl. As platelet aggregation is slightly inhibited at the physiological pH value, it was adjusted to pH 7.6 to achieve an increased aggregation response.

ADP-induced aggregation was studied at 37 °C (HU Aggregometer, H. Upchurch and Co. Ltd., Leicester, England) by using a final concentration of 50 μM adenosine-diphosphate and 2.5 mM of calcium chloride added. After the reaction 125 μM of epinephrine was added to induce a maximal aggregation response [4]. The maximum change in light transmission was expressed as a percent of the original span between upper and lower limits set with platelet-poor plasma and the platelet suspension.

As aggregometer oscillations correlated well with platelet shape [1], the platelet shape was characterized by the amplitude of oscillations on the aggregometer (before addition of ADP).

The response to hypotonic stress was measured by the aggregometer at 37 °C, 0.5 ml of distilled water (test) or physiological saline (blank) was added to 1 ml of platelet suspension. Reversal determined 2 min after the addition of distilled water was expressed as the percent of the original maximal change in light transmission.

Clot retraction was determined according to Breddin [3].

Intracellular and extracellular acid phosphatase activities were measured by using p-nitrophenylphosphate substrate [2], incubation time was 10 min. Results were given as $A_{4/0}^{1cm}/10^8$ platelets and $A_{4/0}^{1cm}/0.20$ ml supernatant plasma, respectively.

Malondialdehyde production was studied as described by Stuart et al. [25] with the following modifications: 2×10^9 platelets suspended in 1.9 ml of phosphate buffered saline medium, pH 7.6, were reacted with 50 IU of thrombin in 0.1 ml volume (Topostasine, La Roche, Switzerland) for 10 min at 37 °C. After adding 2 ml of the thiobarbituric acid reagent, the mixture was placed into a boiling water bath for 15 min. This was followed by rapid cooling to 10 °C, centrifuging and measuring of the clear supernatant at 532 nm against a reagent blank.

The amount of adenine nucleotides (ATP and ADP) were tested with Boehringer kits (Boehringer Mannheim GmbH, FRG).

All results were expressed as mean ± SD.

In vivo recovery and survival were determined after ^{51}Cr labelling [13] of platelets and infusing them into healthy volunteers with their consent (homologous transfusions to simulate normal clinical practice). *In vivo* behaviour of fresh platelets was studied in four volunteers (one of them did not return on the day following the infusion of platelets and on the 7th day). Characteristics of stored platelets were studied in three volunteers. Results are given in percent recovery of radioactivity.

Results

Platelet concentrates with different cell counts and different volumes were used for the experiments. Mean values, the range of platelet count, volume, change of pH and glucose consumption are presented in Table 1.

Table 1

Experimental conditions used, glucose consumption, and pH after 120 hours of storage (n = 23)

Platelet count/μl × 10^6	1.10 ± 0.40	(0.52—2.00)
Volume of platelet concentrate, ml	162.61 ± 66.74	(55—400)
Total number of platelets in the bags × 10^{11}	1.59 ± 0.42	(0.45—2.20)
ml/10^{11} platelets (derived from data above)	105.78 ± 40.51	(50—190.5)
	before storage	120 hours storage
Glucose, mmol/l	25.73 ± 1.08	16.41 ± 5.35
Glucose consumed, mmol/l		9.53 ± 5.37
pH	7.21 ± 0.09	6.60 ± 0.45
Drop of pH		0.78 ± 0.36

Due to different platelet counts and fluid volumes, the resulting drop in pH was also different. The linear regression analyses (Fig. 1) indicate that the parameters studied (platelet concentration, total platelet count and the volume of plasma in which 10^{11} platelets are suspended) significantly influence the pH change. Glucose consumption and drop of pH are strongly correlated.

Decrease of platelet count was not observed during the storage experiments.

Decreased *in vitro* functions of platelets after 120 hours of storage are shown on Table 2. The amount of adenine nucleotides (ATP and ADP) clearly reveal that the platelets have lost a substantial part of their intracellular contents. This loss is

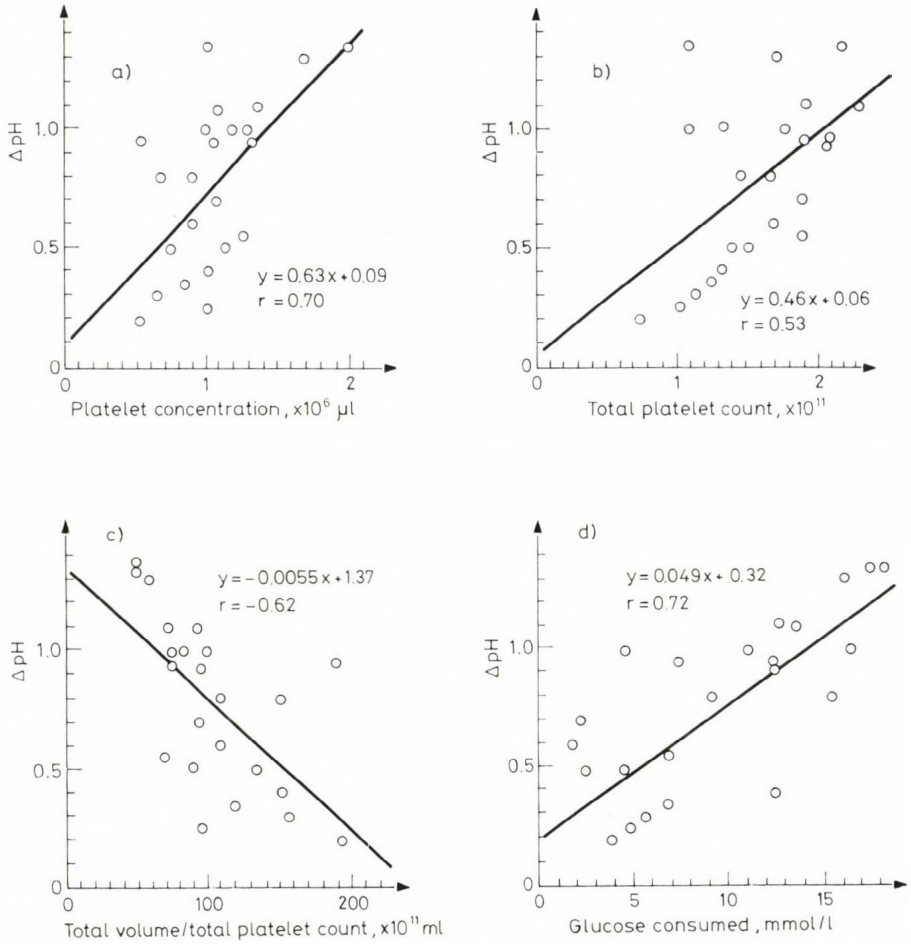


Fig. 1. Effect of platelet concentration (A), total platelet count (B), volume/10^{11} platelets (C), and glucose consumed (D) on drop of pH

not the result of a simple platelet lysis, for the number of platelets did not decrease during the storage period, nor is it reflected by the minor change of extracellular phosphatase activity.

The *in vivo* survival of platelets stored for 5 days seemed to be reduced when compared to fresh platelets (Fig. 2). This is not the result of platelet deterioration due to low pH values, as all the three platelet concentrates tested in *in vivo* studies had pH values well above 6.0 (7.25, 7.05 and 6.70, respectively).

The average viability index, compared to that of fresh platelets (ratio of areas under the survival curves) is 0.3, i.e. only 30 per cent. This figure is similar to the figure, which can be calculated from the hypotonic stress response [7].

Table 2

In vitro functions and biochemical characteristics

	Before storage	120 hours storage	
Platelet shape (oscillations), %	4.91±1.84 n=23	5.17±2.37 n=23	
Aggregation, % ADP	86.60±6.65 n=23	24.62±15.46 n=23	
+epinephrine		45.67±19.16 n=16	
Hypotonic stress, %	38.71±8.50 n=20	19.56±20.18 n=18	
Clot retraction, %	89.02±4.70 n=16	52.10±15.72 n=16	
MDA, nmol/10^9	5.25±2.23 n=16	0.91±1.15 n=16	
Intracellular phosphatase $A^{1\,cm}_{4/0}$/10^8 platelets	0.460±0.126 n=16	0.399±0.110 n=16	$p > 0.10$
Extracellular phosphatase $A^{1\,cm}_{4/0}$/0.20 ml supernatant	0.107±0.020 n=16	0.266±0.188 n=16	$0.005 > p$
ATP, μmol/10^{11}	4.91±0.64 n=16	2.09±1.22 n=15	$0.001 > p$
ADP, μmol/10^{11}	3.26±0.90 *n*=19	2.19±1.66 n=15	$0.05 > p$

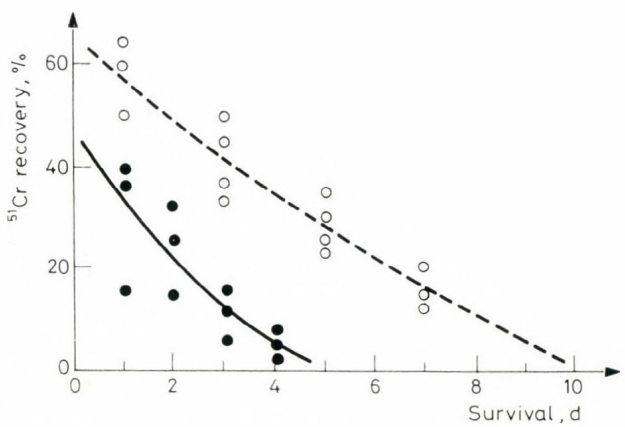


Fig. 2. In vivo recovery and survival of platelets labelled by ^{51}Cr. ------ and ○ fresh platelets, ——— and ● stored platelets. Viability index of stored platelets is 0.3.

Discussion

The essential problem of long-term platelet preservation in the liquid phase (at room temperature) is whether the life-span of platelets can be prolonged or not. The significance of pH drop during room temperature storage has been known for some time [13], while the improved gas permeability of storage bags has resulted in a better maintenance of pH at a relatively high value [8, 17, 19, 23, 26].

In our studies platelet concentrates prepared by the buffy coat technique were tested, thus our results may differ a little from those of other investigators using the platelet-rich plasma technique [18, 19, 23, 24, 26].

Moroff et al. [11] studied individual platelet concentrates prepared by the platelet-rich plasma technique and therefore the total platelet count in the bags was generally under 1×10^{11} platelets. In our experiments the pooled platelet concentrates of 55–400 ml volume contained a mean of 1.59×10^{11} (Table 1) with a maximum of 2.3×10^{11} platelets (Fig. 1). Moroff et al. [11] experienced a striking change of pH in specific platelet concentrations after storage. In our experiments the variation seems to be smaller, which is probably due to the use of pooled platelets.

Our results showed that not only platelet concentration and total platelet count influence pH, it is also affected by platelet concentrate volume. This role of volume was already stated by Rock et al. [15]. In contrast to their explanation, it is more likely that the volume of plasma has a role in the oxygen accumulation in the suspending medium, thus it provides improved oxygenation for the platelets. It can be calculated from the regression lines that a maximum of 1.0 unit of pH drop may be expected after storing for 120 hours, if the bag (R 9056, nominal capacity 400 ml, surface about 355 cm^2) contains about 2×10^{11} platelets in about 120 ml volume. In a higher volume, the drop of pH should be smaller, even at higher platelet count.

The aggregation response of stored platelets was weak – including pairs of aggregating agents [4] –, but it is a commonly experienced fact at room temperature storage.

The clot retraction activity and malondialdehyde formation show reduced platelet activity. The shape character of stored platelets is statistically not different from that of fresh ones (see oscillations), but the increase of the mean value of light scattering may be a result of the appearence of altered platelet morphology [6].

ATP and ADP content decreased to 40–70 per cent of original values, thus representing a substantial drop of adenine nucleotides of the metabolic pool [9]. Filip et al. [5] reported a 27% decrease of ATP+ADP after 72 hours of storage. In our experiments, the descrease is 48 percent after 120 hours of storage, which is perfectly proportional to time. Another group, Kotelba–Witdowska et al. [10] noted a 25–32 percent drop of platelet ATP content after 3 days of storage at high pH values maintained by ion-exchange resins.

Snyder [21] and Snyder et al. [22] experienced a substantial release of beta-thromboglobuline and lactate dehydrogenase during storage of platelet concentrates, while in our experiments there was no significant release of lysosomal acid

phosphatase. From these facts it is concluded that small stimuli during platelet storage may result in a dense-granule and alpha-granule release, but no lysosomal release.

Hypotonic stress response decreased by 50 per cent as compared to fresh platelets. This decrease corresponds to 30 percent *in vivo* viability according to Kim and Baldini [7], and this value was confirmed by our actual *in vivo* experiments (see Fig. 2). Actual viability index was reduced in accordance with other findings following 96 hours of storage [20].

From the work of Slichter and Harker [20] and Murphy and Gardner [13] it may be concluded that the pH of tested platelet concentrates probably has an influence on the *in vivo* behaviour of platelets, and there exists an optimal range of pH in regard to *in vivo* platelet viability. Rzad et al. [18] experienced a mean of 6.3 days life-span with a mean of 7.17 pH of transfused platelets after 7 days of storage. Snyder et al. [22, 24] reported 6.3—8.6 days of survival, mean pH of the concentrates was 7.16 after 5 days of storage. In our experiments the mean pH of the concentrates tested was 7.0 and the mean survival was 5 days (homologous infusions). The same survival was reported by Rock et al. [16] with platelets stored in CLX bags also for 5 days, but using autologous infusions.

Storage bags made from gas-permeable material seem to be beneficial for platelet storage [8, 16, 17, 18, 19, 22, 23, 24]. From the experimental facts we concluded that the gas-permeation capacity of the bags examined makes storage of about 1×10^{11} platelets possible for 5 days without substantial deterioration of functions. This amount of platelets can be harvested in the form of individual platelet concentrates from one unit of blood by the platelet-rich plasma technique. If the buffy coat technique is used [14] the pooled platelet concentrate contains about $1.9-2.5 \times 10^{11}$ platelets in 150—200 ml volume. This concentrate can also be stored in PL 1240 plastic bags, but because of the high amount of platelets present the storage should be limited to less than 5 days (3—4) to avoid harmful pH drop and to preserve biological functions. The lengthened shelf-life of platelet concentrates, however, seems to shorten in vivo survival of transfused platelets.

Actual keeping of quality is affected by the results of sterility tests; this aspect still needs to be cleared.

Acknowledgement

The skillful technical assistance of Mrs. Ilona Máté-Tóth and Mrs. Anikó Fejes is gratefully acknowledged.

References

1. Affolter, H., Pletcher, A.: Rheo-optical shape analysis of human blood platelets. *Thromb. Haemostas. (Stuttgart) 48*, 204 (1982).
2. Bramley, T. A., Ryan, R. J.: Interactions of gonadotropins with corpus luteum membranes. 1. Properties and distributions of some marker enzyme activities after subcellular fractionation of the superovulated rat ovary. *Endocrinology 103*, 778 (1978).
3. Breddin, K.: Die Thrombozytenfunktion. *Thrombos. Diathes. Haemorrh. (Suttgart) (Suppl. 27.)*, 20 (1968).

4. DiMinnio, G., Silver, M. J., Murphy, S.: Stored human platelets retain full aggregation potential in response to pairs of aggregating agents. *Blood 59*, 563 (1982).
5. Filip, D. J., Eckstein, J. D., Sibley, C. A.: The effect of platelet concentrate storage temperature on adenine nucleotide metabolism. .*Blood 45*, 749 (1975).
6. Fratantoni, J. C., Sturdivant, B., Pointdexter, B. J.: Aberrant morphology of platelets stored in five day containers. *Thromb. Res. 33*, 607 (1984).
7. Kim, B. K., Baldini, M. G.: The platelet reponse to hypotonic shock. Its value as an indicator of platelet viability after storage. *Transfusion 14*, 130 (1974).
8. Koerner, K.: Platelet function of room temperature platelet concentrates stored in a new plastic material with high gas permeability. *Vox Sang. 47*, 406 (1984).
9. Kotelba-Witkowska, B., Holmsen, H., Mürer, S. H.: Storage of human platelets: effects on metabolically active ATP and on the release reaction. *Brit. J. Haematol. 22*, 429 (1972).
10. Kotelba-Witkowska, B., Harmening-Pittiglio, D. M., Schiffer, A. A.: Storage of platelet concentrate using ion exchange resin charged with dibasic phosphate. *Blood 58*, 537 (1981).
11. Moroff, G., Friedman, A., Robkin-Kline, L.: Factors influencing changes in pH during storage of platelet concentrates at 20—24 °C. *Vox Sang. 42*, 33 (1982).
12. Murphy, S., Gardner, F. H.: Platelet preservation — effect of storage temperature on maintenance of platelet viability — deleterious effect of refrigerated storage. *N. Engl. J. Med. 280*, 1094 (1969).
13. Murphy, S., Gardner, F. H.: Platelet storage at 22 °C: role of gas transport across plastic container in maintenance of viability. *Blood 46*, 209 (1975).
14. Rácz, Z., Thék, M.: Buffy coat or platelet-rich plasma? Comparison of two platelet--processing techniques. *Vox. Sang. 47*, 108 (1984).
15. Rock, G., Tittley, P., Sherring, V., Culley, C., Wong, S. C.: Platelet storage: an assessment of the requirements for plasma and oxygen. *Transfusion 21*, 167 (1981).
16. Rock, G., Sherring, V. A., Tittley, P.: Five-day storage of platelet concentrates. *Transfusion 24*, 147 (1984).
17. Rolland, G. F., Van Essche, R.: Technical support document for PL 1240. The PL 1240 container for extended platelet storage. Prepared by medical and Scientific Depts. Travenol International Services, Inc. (1983).
18. Rzad, L., Murphy, S., Buchholz, D. H., Davisson, W., Gajewski, M., Aster, R. H.: Storage of platelet concentrates for seven days in PL-732™ plastic. *Transfusion 22*, 417 (1982).
19. Simon, T. L., Nelson, E. J., Carmen, R., Murphy, S.: Extension of platelet concentrate storage. *Transfusion 23*, 207 (1983).
20. Slichter, S. J., Harker, L. A.: Preparation and storage of platelet concentrates. II. Storage variables influencing platelet viability and function. *Br. J. Haematol. 34*, 403 (1976).
21. Snyder, E. L.: Release of β-thromboglobuline during storage of platelet concentrates. *Vox. Sang. 40. Suppl. 1.*, 115 (1981).
22. Snyder, E., Aster, R., Murphy, S., Davisson, W., Ferri, P., Pope, C., Smith, E., Kiraly, T., Kakaiya, R., Ezekowitz, M., Buchholz, D.: Clinical evaluation of platelets stored five days in PL 1240 plastic packs. *Transfusion 23*, 425 (1983).
23. Snyder, E. L., Bookbinder, M., Kakaiya, R., Ferri, P., Kiraly, T.: 5-day storage of platelet concentrates in CLX containers: effect of type of agitation. *Vox Sang. 45*, 432 (1983).
24. Snyder, E. L., Ezekowitz, W., Aster, R., Murphy, S., Ferri, P., Smith, E., Rzad, L., Davisson, W., Pope, C., Kakaiya, R., Buchholz, D. H.: Extended storage of platelets in a new container. II. In vivo response to infusion of platelets stored for 5 days. *Transfusion 25*, 209 (1985).
25. Stuart, M., Murphy, S., Oski, F. E.: A simple non-radioisotope technique for the determination of platelet life-span. *N. Engl. J. Med. 292*, 1310 (1975).
26. Wallvik, J., Åkerblom, O.: Platelet concentrates stored at 22 °C need oxygen. The significance of plastics in platelet preservation. *Vox. Sang. 45*, 303 (1983).

Haematologia 22 (2), pp. 97–100 (1989)

Rapid Determination of the Human Complement Factor B Phenotypes

J. KRAMER, E. THIRY, G. FÜST

National Institute of Haematology and Blood Transfusion H-1502 Budapest, Pf. 44, Hungary

(Received 1 February, 1988; accepted 28 March, 1988)

A rapid phenotyping method for human complement factor B (Bf) was worked out using high voltage acetate electrophoresis. The results were compared with those obtained by conventional agarose gel electrophoresis. Separation of the bands was performed in the same way, but only 2.5 hours were required for the evaluation of 24 samples. Furthermore, at least ten times less specific antibody was needed than for the conventional technique.

Keywords: rapid determination, complement factor B phenotypes

Introduction

Factor B (Bf) is a limiting component in the alternative pathway of complement activation. The Bf phenotype is inherited as an autosomal co-dominant trait which is encoded on the short arm of the sixth chromosome. Two other complement proteins (C2, C4) are encoded in this chromosome between the I and II class MHC genes [1] resulting in an extended haplotype. Both the mean level and the haemolytic capacity show strong correlation with the particular Bf phenotype in the order BfF (fast) > BfFS (fast-slow) > BfS (slow). Interestingly, however, the phenotype frequencies are 60.4% for BfS, 31.7% for BfFS and 4.4% for BfF. The residual 3.5% stands for the rare phenotypes[2]. The common allotypes F and S reside in the Ba, while most rare variants are located in the Bb fragment of factor B [3]. Evidence for autosomal co-dominant inheritence of at least four alleles was presented. There is an increased risk for insulin dependent diabetes mellitus in carriers of the extended haplotype HLA-B18, C2*C, Bf*F1, C4A*3, C4B*Q0, HLA-DR3 [4]. BfF1 may also play a role in the pathogenesis of idiopathic membranous nephropathy and the BfF allotype may be associated with the production of cytotoxic antibodies in multiparous women [5].

The Bf phenotyping was introduced by Alper et al. [3] using agarose gel electrophoresis followed by immunofixation. This method gives strong bands which can easily be evaluated. The electrophoresis takes about 3 hours and the immunofixation another hour. Then the gel is washed overnight, dried and stained. For the immunifixation of approximately 20 samples half a ml of specific antisera is required. By modifying the method of Martin and Ziegler [6] we worked out a rapid and more economical method for the determination of factor B phenotypes.

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Materials and Methods

Sera or EDTA-plasma samples of healthy individuals were collected and stored at −70 °C until used. Conventional agarose gel electrophoresis followed by immunofixation was performed according to Alper et al. [3]. Reference samples were run by Prof. C. A. Alper (The Center for Blood Research, Harvard Medical School, Boston). Cellulose acetate electrophoresis was carried out on a Shandon electrophoresis system using 60 × 150 mm cellulose diacetate membranes (Shandon Southern Product Limited, United Kingdom). Approximately 0.2 μl of each of the eight samples were applied onto one membrane. Electrophoresis was performed at 400 V (10 mA) for 70 minutes in a Tris-glycin buffer (21.95 g glycin, 4.55 g Tris and 0.369 g Ca-lactate to 1 liter), pH 8.6 at room temperature. The Shandon apparatus allows the simultaneous use of three membranes (24 samples). After electrophoresis 70 μl of five times dliuted specific anti-human properdin factor B IgG fraction (Atlantic Antibodies) was applied onto the middle of each membrane. After 10 minutes of immunofixation the membranes were washed for 2 × 15 minutes in saline, containing TWEEN 20 (one drop/1). The membranes were stained with Coomassie BBR250 and destained with 50% methanol plus 10% acetic acid. Densitograms were recorded using a Shandon Cello 3 densitometer.

Results and Discussion

In this study we developed a rapid method for the determination of Bf phenotypes.

Figure 1/A shows the mobility of bands obtained by cellulose acetate electrophoresis followed by immunofixation. The respective densitograms are depicted on Figure 1/B. Positions of Bf*F or Bf*S correspond to those of respective bands of Bf*FS. We compared the results obtained by agarose gel electrophoresis with those of cellulose acetate electrophoresis for 14 samples. Two of these samples were BfF, another two were BfFS and one was BfF1S. The remaining samples were BfS. Both methods gave identical results. Different dilutions of the anti-human properdin factor B were used. The best resolution could be obtained by five times diluted antisera, and the whole procedure took only 2.5 hours.

We performed population studies with this method, phenotyping 198 healthy individuals living in Budapest, the capital of the country. The allotype frequencies for Bf*F, Bf*S, Bf*F1 and Bf*S0.7 alleles were found to be 0.194, 0.795, 0.002 and 0.008, respectively. These data correlate well with those of Mauff [7]. In his study these frequencies belong and fit well into the Central-European average.

Phenotyping of some complement proteins (C2, C4 and Bf) together with HLA group typing is an indispensable tool in discovering correlation between certain diseases and extended haplotypes. Our rapid and economical procedure may facilitate a further extension of these experiments.

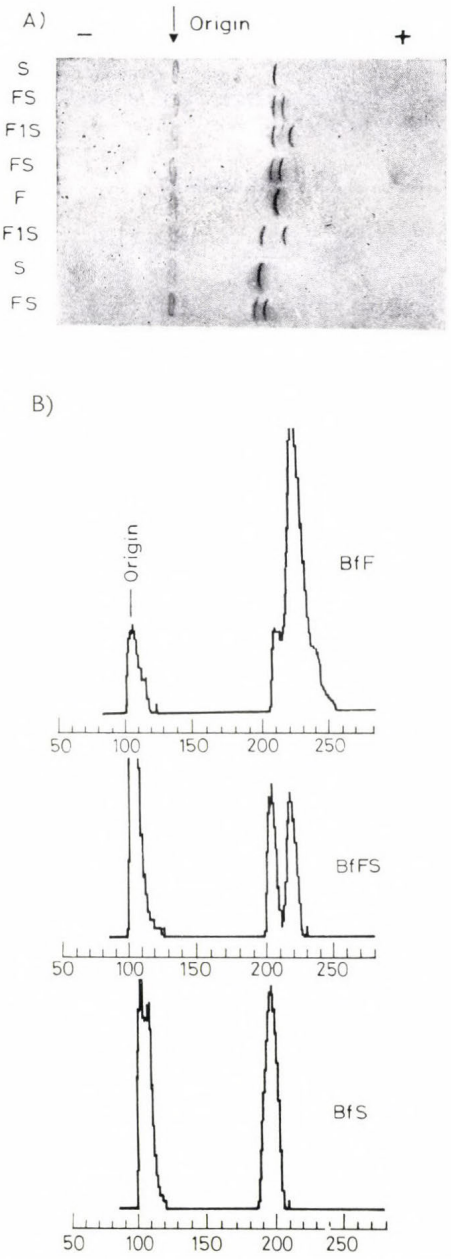


Fig. 1 A. Mobility of the common phenotypes on cellulose acetate membrane; B. Densitogram of the three most common Bf phenotypes using Shandon Cello 3 densitometer

Acknowledgement

We would like to thank Prof. C. A. Alper for the determination of Bf phenotypes by agarose gel electrophoresis from reference sera. This work was supported by 6/969/86 OTKA and by VEPEX Co. Ltd. (Hungary).

References

1. Carroll, M. C., Campbell, R. D., Bentley, D. R. and Porter, R. R.: A molecular map of human major histocompatibility complex class III region linking complement genes C4, C2 and factor B. *Nature 307*, 237 (1984).
2. Mortensen, J. P., and Lamm, L. U.: Quantitative differences between complement factor B phenotypes. *Immunology 42*, 505 (1981).
3. Alper, C. A., Boenish, R. and Watson, L.: Genetic polymorphism in human glycine-rich beta-glycoprotein. *J. Exp. Med. 135*, 68 (1972).
4. Alper, C. A., Awdeh, Z. L. and Yunis, E. J.: Complotypes, extended haplotypes, male segregation distortion, and disease markers. *Human Immunol. 15*, 366 (1986).
5. Dyer, P. A., Davidson, I. A.: Allotypes of properdin factor B (Bf) and lymphocytoxic antibody production. *Immunbiol. 164*, 234 (1983).
6. Martin, W. und Ziegler, Ch.: Bf-immunfixation auf cellogel-folien. *Blut 42*, 23 (1981).
7. Mauff, G.: Application of the MHC-Class III complement markers to population genetics. *Colloque INSERM, 142*, 143 (1986).

Haematologia 22 (2), pp. 101–108 (1989)

Cytochemical Profile of Lymphocytes in Haemophilia Patients at Risk of AIDS

G. GAMBA, G. IPPOLITI, R. INVERNIZZI, V. GATTI, M. T. ZOCCHI, O. PERUGINI

Department of Internal Medicine, Section of 2nd Clinical Medicine, University of Pavia and IRCCS Policlinico San Matteo, 27100 Pavia, Italy

(Received 2 September, 1987; accepted 25 March, 1988)

We report the cytochemical and immunologic features of lymphocytes of 31 patients (26 with haemophilia A and 5 with haemophilia B) chronically treated with commercial clotting factor concentrates. 6 patients, asymptomatic for AIDS were anti-HIV positive. In comparison to aged cross-matched male controls, significant increases of absolute number of lymphocytes and of T and B cell subpopulations with significant reduction of the percentages of CD_3, CD_4, CD_2, CD_5, sIg, and CD_{16} cells was found in haemophiliacs. PAS reaction showed a lower grading score in haemophiliacs and the staining for NBE, AP, NABG revealed a significant reduction of the percentage of the lymphocytes with dot-like positivity in the same patients. The percentage number of AP reactive lymphocytes with dot-like positivity was directly related to the percentage of CD_3, and CD_4 cells. The anti-HIV positive patients presented lower percentages of CD_4 and of dot-like reactive lymphocytes.

Keywords: AIDS, cytochemistry, haemophilia, lymphocytes

Introduction

Haemophilia patients, chronically treated with clotting factor concentrates obtained from large pool of donors, present abnormalities of immunologic status related to AIDS virus exposure [1, 2] or to some undefined antigenic stimulation induced by frequent transfusions [3–5].

Changes of T cell subsets characterized by low CD_4/CD_8 ratios and polyclonal activation of the B cells are the main features described by several authors in these patients [3–9].

On the other hand, cytochemistry may represent an alternative method of investigating lymphocyte patterns; in fact, the expression of hydrolase such as acid phosphatase (AP), N-acetyl-β-glucosaminidase (NABG), acid α-naphtyl acetate esterase (ANAE) and α-naphtyl butyrate esterase (NBE) have been shown to discriminate T/non T cells [10, 11]. Furthermore, PAS reaction may be considered a useful marker of the glycogen content in lymphocytes exposed to viral or to other antigenic stimuli [12].

The aim of investigation was to characterize the lymphocytes of haemophilic patients receiving commercial clotting factor concentrates in relation to the presence of antibodies to HIV with immunologic and cytochemical methods.

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Materials and Methods

We studied 31 patients (26 with haemophilia A and 5 with haemophilia B), aged between 10 and 44 years. All the patients were being treated with commercial clotting factor concentrates ranging in amounts from 5000 to 50.000 U/year/patient.

Each patient was carefully followed for symptoms or signs indicating LAS, ARC or AIDS according to the criteria suggested by the NIH AIDS Working Group [13].

After the patient had fasted overnight, venous blood samples were collected to perform the tests as described below.

Haematologic and cytochemical methods

Leukocyte counts were performed on EDTA anticoagulated blood using an automated blood cell analyzer. The following specific cytochemical staining tests were performed on peripheral blood smears: PAS [14], AP [15], NABG [16], ANAE [17] and NBE [18]. PAS positivity was scored in 100 lymphocytes. The following criteria were used: 0 = no PAS positive granules, 1+ = only few and scattered granules, 2+ = larger granules with an incomplete ring distribution, 3+ = many granules forming one or more complete perinuclear rings. The intensity of AP staining in 100 consecutive lymphocytes was scored as follows: 0 = negative, 1+ = 1 to 5 scattered granules, 2+ = 5 to 10 granules or 1 larger plus 2 to 5 small granules, 3+ = more than 10 granules. NABG activity was graded as follows: 0 = no activity, 1+ = 1 large or 1 to 5 small granules, 2+ = 5 to 10 small granules or 1 large plus 2 to 5 small granules, 3+ = 10 to 15 granules, 4+ = 15 or more granules. The total activity score was the sum obtained by multiplying the number of cells by the observed grade. Moreover, in each case, the percentage of cells with at least 1 large granule was evaluated.

For the ANAE and NBE reactions only lymphocytes with a coarse block of localized reaction were considered to be positive; in each case the percentage of positive lymphocytes was evaluated.

Immunologic Methods

Peripheral blood mononuclear cells were isolated from heparinised blood by using the Ficoll-Hypaque density centrifugation. Lymphocyte surface markers were assessed by an indirect immunofluorescent method using an anti-mouse Ig-FITC in the second step. We used the following MoAb reactants:

a) with T cells (19): CD_3 (T_3), CD_4 (T_4), CD_8 (T_8), CD_2 (T_{11}), CD_5 (T_1), (Coulter Ltd., USA).

b) with B cells (20): I_2 (I_{a1}, DR), CD_{20} (B_1), sIg (Coulter Ltd., USA).

c) with NK cells: CD_{16} (Leu_{11}) (Becton-Dickinson, USA).

Virological studies

Anti-HIV Ig antibodies were determined by ELISA method according to Vezinet-Brun [21] with a slight modification. Virus grown on a T-lymphoblastoid cell line (CEM) was used as antigen to coat the plates at a protein concentration of 50 ng/wall.

Sera were considered positive when the differential optical density obtained on viral and control antigens (a cytoplasmic extract of uninfected cells) was over 0.3. The positivity of the sample was confirmed by Western blot technique.

Normal range values for all parameters tested were obtained from 30 healthy male subjects cross-matched for age.

Statistical analysis was performed using Student's *t* test and simple linear regression when appropriate.

Results

All patients involved had a good clinical status. Six patients were anti-HIV positive, three of them presented LAS. No patient showed symptoms or signs indicating AIDS or ARC.

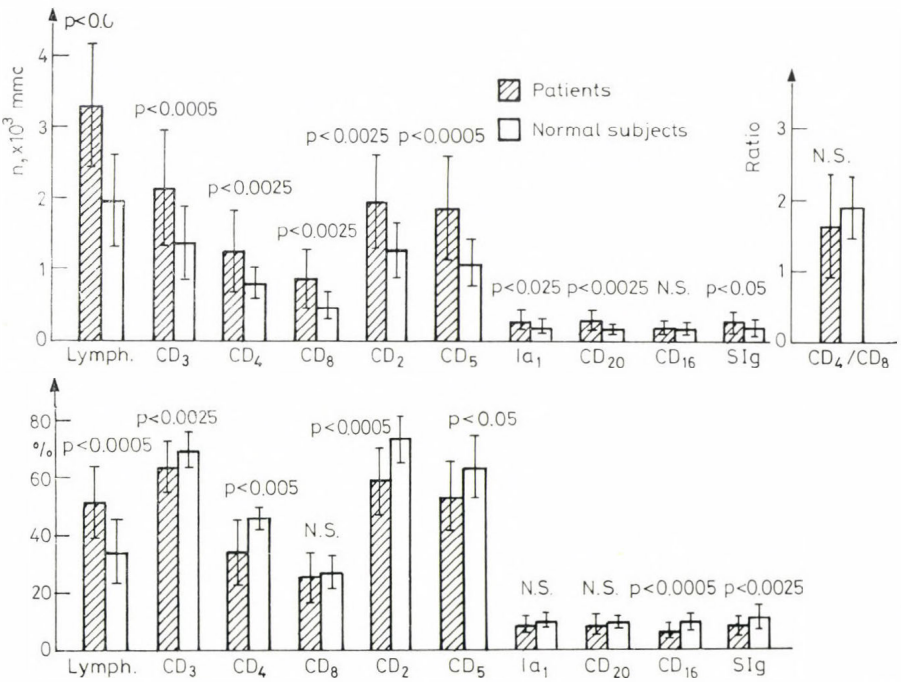


Fig. 1. Immunologic characterization of lymphocyte subpopulations in haemophilic patients and in normal subjects. (Mean values ± SD)

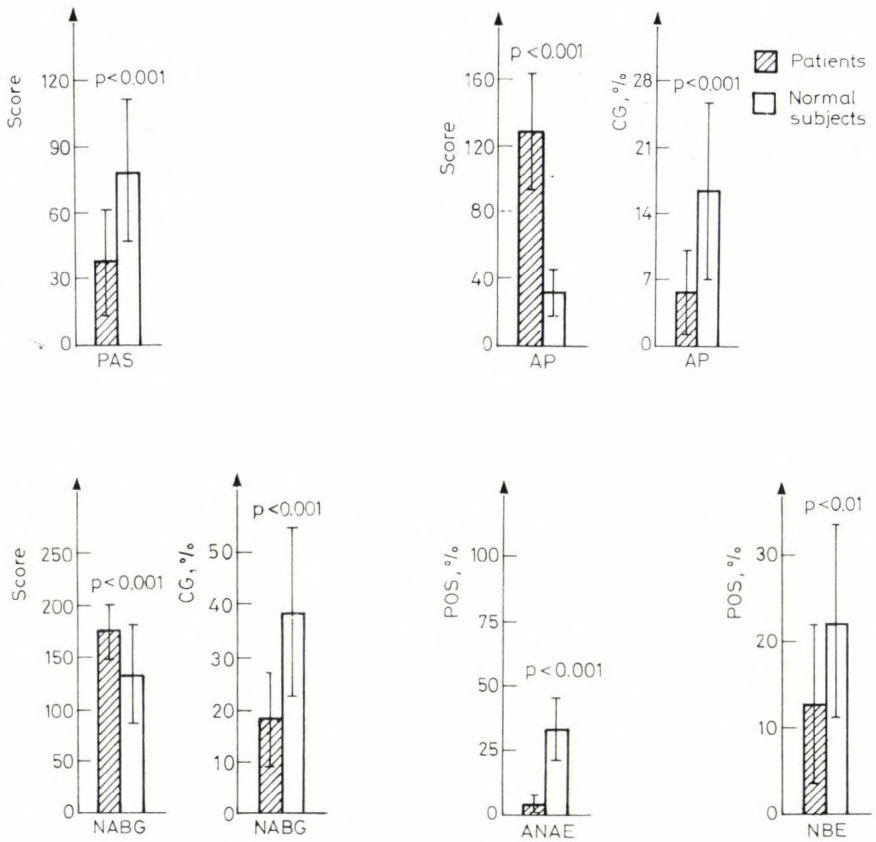


Fig. 2. Cytochemical behaviour of lymphocytes in haemophilia and in controls. (Mean values $\pm$SD)

In haemophiliacs we observed a significant increase in the absolute number of lymphocytes and in T and B cell subpopulations, and a significant reduction in percentage of CD_3, CD_4, CD_2, CD_5, sIg and CD_{16} (Fig. 1). All the six patients with anti-HIV antibodies had CD_4 below the normal range values. Furthermore, the percentages of other types of leukocytes were not increased. Figure 2 shows cytochemical staining in the patients and controls. PAS positivity of patient lymphocytes was significantly lower than in control lymphocytes. The AP score was significantly increased in the lymphocytes of the patients, but the percentage of dot-like reactive cells was sharply reduced in the same subjects. NBBG reaction was similar. The proportion of NBE and of ANAE reactive lymphocytes was significantly lower in the haemophiliacs. No differences were found in the anti-HIV positive and negative patients, nevertheless the proportion of dot-like AP reactive cells was lower in patients with antibodies to HIV (Fig. 3). The percentage of AP dot-like reactive

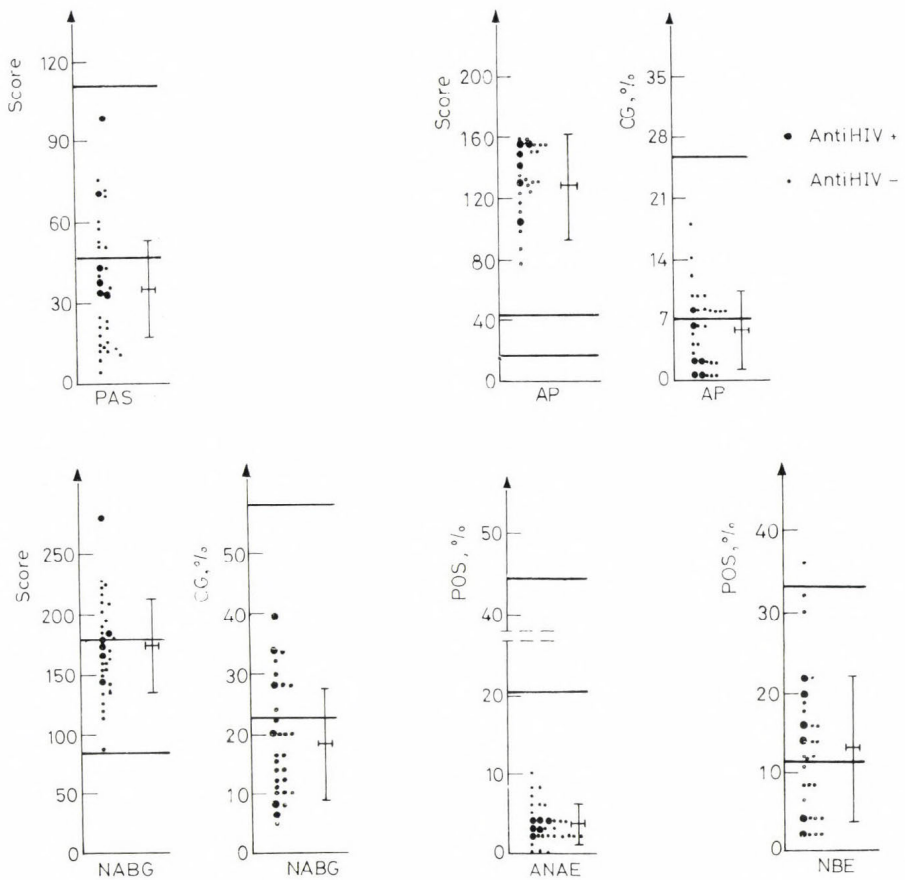


Fig. 3. Cytochemical features of lymphocytes in patients with and without antibodies to HIV. (Mean values $\pm$SD). The area between the horizontal lines represent the range of normal values

lymphocytes was directly related to the CD_3 ($p < 0.001$) and CD_4 cells ($p < 0.05$) (Fig. 4).

No relationships were observed between immunologic and cytochemical features for the other reactions studied.

Discussion

It is well known that in normal lymphocytes there is a close relationship between the expression of some acid hydrolyses and membrane phenotype [22–24]. Our data show that also in haemophilia the cytochemical features are in

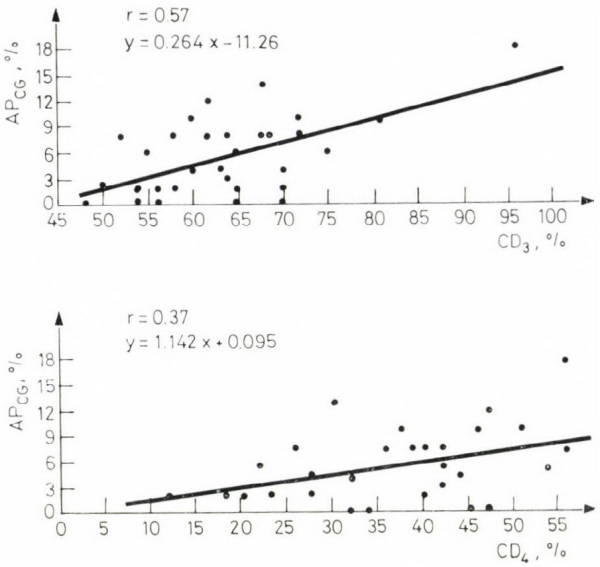


Fig. 4. Significant relationships between the AP dot-like reactive lymphocytes and lymphocyte subsets immunologically detected in haemophilic patients

agreement with the immunologic characterization of lymphocytes. In fact, the lower number of lymphocytes with dot-like NABG, AP and NBE reactions corresponds to the reduction of CD_4 observed in haemophilia patients. A significant positive linear correlation between the percentages of AP dot-like reactive lymphocytes and CD_4 cells was found. The specific tropism of HIV virus for CD_4 cells [25, 26] may explain the low percentage of CD_4 cells in patients with antibodies to HIV. Therefore, in anti-HIV positive patients the AP reaction showed a particularly low number of dot-like reactive lymphocytes.

On the other hand, the increase of the absolute number of T and B cells as well as the higher score of AP and NABG, and the reduction of PAS score may be attributed to chronic antigenic stimulation from repeated infusion of lyophilised concentrates containing allogenic proteins [27] and several viruses [28, 29, 30], rather than to HIV infection.

In conclusion, acid hydrolase cytochemistry, especially the dot-like AP reaction may represent a useful, simple and unexpensive method for following modification of CD_4 subset in patients with HIV infection at risk of developing AIDS.

References

1. Goedert, J. J., Sarngadharan, M. G., Eyster, M. E., Weiss, S. H., Bodner, A. J., Gallo, R. C., Blattner, W. A.: Antibodies reactive with human T cell leukemia viruses in the serum of haemophiliacs receiving factor VIII concentrate. *Blood 65*, 492 (1985).

2. Rouzioux, Ch., Brun-Vezinet, P., Couroucé, A. M., Gazengel, C., Vergoz, D., Desmyter, D. Vermylen, J., Vermylen, C. Klatzmann, D., Geroldi, D., Barreau, C., Barré-Sinoussi, F., Chermann, J. C., Christol, D., Montagnier, L.: Immunoglobulin G antibodies to lymphoadenopathy associated virus in differently treated French and Belgian hemophiliacs, *Ann Intern. Med. 102*, 476 (1985).
3. Lechner, K., Niessner, H., Bettelheim, P., Deutsch, E., Fasching, I., Fuhrmann, M., Hinterberger, W., Korninger, E., Liszka, K., Knapp, W., Mayr, W. R., Strugl, G., Zeitlhuber, U.: T cell alterations in hemophiliacs treated with commercial clotting factor concentrates. *Thromb. Haemostas. 50*, 552 (1983).
4. Goldsmith, J. M., Kalish, S. B., Green, D., Chmiel, J. S., Wallemark, C. B., Phair, J. P.: Sequential clinical and immunologic abnormalities in haemophiliacs. *Arch Intern. Med. 145*, 431 (1985).
5. Madhok, R., Gracie, A., Follett, E., Lowe, G. D. O., Forbes, C. D.: Impaired in vivo cell-mediated immunity in hemophilia in the absence of HTLVIII/LAV infection. *La Ricerca Clin. Lab. 16*, 173 (1986).
6. Ledermann, M. H., Ratnoff, O. D., Scillian, J. J., Jones, P. K., Schachter, B.: Impaired cell-mediated immunity in patients with classic haemophilia. *New Engl. J. Med. 308*, 79 (1983).
7. Clauvel, J. P., Brouet, J. C., Maissoneuve, P., Perol, Y., Sultan, Y.: Etude des sous-populations lymphocytaires chez l'hémophile. *Presse Med. 13*, 19 (1984).
8. Gjerset, G. P., Martin, P. J., Counts, R. B., Fast, L. D., Hausen, J. A.: Immunologic status of haemophilia patients treated with cryo-precipitate or lyophilized concentrate. *Blood 64*, 715 (1984).
9. Stein, S .F., Evett, B. L., McDougal, J. S., Lawrence, D. N., Holman, R. C., Ransey, R. B., Spiro, T. J.: A longitudinal study of patients with hemophilia; immunologic correlates of infection with HTLVIII/LAV and other viruses. *Blood 66*, 973 (1985).
10. Kulenkampff, J., Janossy, G., Greaves, M.: Acid esterase in human lymphoid cells and leukaemic blasts: a marker for T lymphocytes. *Br. J. Haemat. 36*, 231 (1977).
11. Crockard, A. D.: Cytochemistry of lymphoid cells: a review of findings in the normal and leukaemic state. *Histochem. J. 16*, 1027 (1984).
12. Hayhoe, F. G. J., Quaglino, D.: *Haematological cytochemistry*. Churchill Livingstone, Edinburgh 1980.
13. NIH AIDS Working Group. Surveillance and definition of AIDS. In: *AIDS: a basic guide for clinicians*. Ebbesen, P., Biggar, R. J. Melbye, M., (eds), Munksgaard, Copenhagen 1984, p 234.
14. Hotchkiss, R. D.: A microchemical reaction resulting in the staining of polysaccaride structures in fixed tissue preparation. *Arch. Biochem. 16*, 131 (1948).
15. Li, C. Y., Yam, L. T., Lam, K. W.: Acid phosphatase isoenzyme in human leucocytes in normal and pathologic conditions. J. *Histochem. Cytochem. 18*, 473 (1970).
16. Hayashi, H.: Histochemical demonstration of N-acetyl-betaglucosaminidase employing naphtol AS-BI-N-acetyl-glucosaminidase as substrate. *J. Hist. Cytochem. 13*, 355 (1965).
17. Yam, L. T., Li, C. Y., Crosby, M. H.: Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Path. 55*, 283 (1971).
18. Higgy, K. E., Burns, C. F., Hayhoe, F. G. J.: Discrimination of B, T and null lymphocytes by esterase cytochemistry. *Scand. J. Haemat. 18*, 437 (1977).
19. Clark, E. A., Yokochi, T.: Human B cell and B cell blast associated surface molecules defined with monoclonal antibodies. In: *Leucocyte Typing*. Bernard, A., Boumsel, L., Dausset, G., Milstein, G., Schlossmann, S. F. (eds), Springer-Verlag, Berlin: 1984, p. 339.
20. Harsen, J. A., Martin, P. J., Beatty, P. G., Clark, E. A., Ledbetter, J. A.: Human T lymphocyte cell surface defined by the workshop monoclonal antibodies (T cell protocol). In: *Leucocyte Typing. Bernard*, A., Boumsel, L., Dauset, G., Milstein, C. C., Schlossmann, S. F., (eds). Springer-Verlag, Berlin: 1984, p. 15.

21. Vezinet-Brun, F., Barré-Sinoussi, F., Samot, A. G., Christal, D., Montagnier, L., Rouzioux, C., Klatzmann, D., Rozenbaum, Gluckmann, J. C., Chermann, J. C.: Detection of IgG antibodies to lymphoadenopathy syndrome. *Lancet* 1, 1253 (1984).
22. Basso, G., Cocito, M. G., Semenzato, C., Pezzuto, A., Zanesco, L.: Cytochemical study of thymocytes and T lymphocytes. *Br. J. Haemat. 44*, 577 (1980).
23. Armitage, R. J., Linch, D. C., Worman, C. P., Cawley, J. C.: The morphology and cytochemistry of human T-cell subpopulations defined by monoclonal antibodies and Fc receptors. *Br. J. Haemat. 51*, 605 (1982).
24. Bernard, J., Durer, J.: Cytochemical analysis of human peripheral blood lymphocyte subsets defined by monoclonal antibodies. *Scand. J. Immunol. 17*, 89 (1983).
25. Dagleish, A. G., Beverley, P. L., Clapham, P., Crawford, D., Greaves, M. P., Weiss, R. A.: The CD_4 (T_4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature 312*, 763 (1984)
26. Klazmann, D., Champagne, E., Chamaret, S. et al: T-lymphocyte T_4 molecule behaves as the receptor of human retrovirus LAV. *Nature 312*, 767 (1984).
27. Kaplan, J., Sarnaik, S., Levy, J.: Transfusion induced immunologic abnormalities not related to AIDS virus. *N. Engl. J. Med. 313*, 1227 (1985).
28. Bach, M., Bah, J.: Imbalance in T-cell subsets in human disease. *Int. J. Immunopharmacol. 3*, 269 (1981).
29. Rubin, R. H., Carneg, W. P., Schooley, R. J.: The effect of infection on T lymphocyte subpopulations: a preliminary report. *Int. J. Immunopharmacol. 3*, 307 (1981).
30. Thomas, H. C.: T-cell subsets in patients with acute and chronic HBV infection, primary biliary cirrhosis and alcohol induced liver disease. *Int. J. Immunopharmacol. 3*, 301 (1981).

Haematologia 22 (2), pp. 109–113 (1989)

A Second Case of Neonatal Alloimmune Thrombocytopenia Associated with Anti-Pl^{A2} (Zw^b) Antibodies

K. MASLANKA[1], G. F. LUCAS[2]*, A. GRONKOWSKA[1], J. G. DAVIS[3], B. ZUPANSKA[1]

[1] Institute of Haematology, Warsaw, Poland; [2] Blood Group Reference Laboratory, Harkness Building, Radcliffe Infirmary, Woodstock Road, Oxford OX2, 6HE, England; [3] Tissue Typing Laboratory, Regional Transfusion Centre, Oxford, England

(Received 19 January, 1988; accepted 28 March, 1988)

A case of neonatal alloimmune thrombocytopenia due to fetomaternal incompatibility against the platelet specific antigen Pl^{A2} (Zw^b) is described. Anti-Pl^{A2} antibodies were present in the maternal serum together with anti-HLA antibodies. Initial identification of the platelet-specific antibodies was achieved by using chloroquine-treated $Pl^{A1}(+)$ and $Pl^{A1}(-)$ platelets. Antibody specificity was later confirmed using a panel of platelets of known phenotype.

Keywords: Anti-Pl^{A2} (Zw^b) antibodies, neonatal alloimmune thrombocytopenia

Introduction

Neonatal alloimmune thrombocytopenia (NAITP) is a rare but clinically significant disorder caused by placental transfer of maternal alloantibodies against platelet specific antigens inherited from the father. The specificity of the alloantibodies in NAITP is frequently found to be anti-Pl^{A1} (Zw^a) [1–4]. Occasionally other antibody specificites have been implicated in this disorder. namely anti-DUZO [5], anti-Pl^{E2} [4], anti-Bak^a [6], anti-Yuk^a [7], anti-Yuk^b [8] and anti-Pl^{A2} (Zw^b) [9]. Recently, we have observed a second case of NAITP due to anti-Pl^{A2} antibodies.

Case History

The neonate was the first child of a 21 year old healthy mother (M. Dan.) with no history of haematological disorders or blood transfusions; all haematological indices at the time of delivery were within the normal range. At delivery, the child appeared normal although a subcutaneous haematoma in the regionis occipitale was observed. Two days later the child developed purpura (platelet count: $28 \times 10^9/l$), became anaemic (Hb: 12g/dl) and icteric (bilirubin: 325 μmoles/l). The

* To whom correspondence should be addressed.

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

spleen was not palpable and there were no signs of septicemia. Plasma coagulation studies revealed no abnormalities and the direct antiglobulin test was negative.

The neonate was transfused with 50 ml of fresh blood on the 5th and 6th days after birth and the platelet count increased to $60 \times 10^9/1$. Subsequently, the platelet count decreased to $30 \times 10^9/1$ (day 13) but thereafter steadily increased to normal levels without further treatment. Full recovery was achieved after four weeks.

Materials and Methods

ABO and Rh antigens and red cell-bound immunoglobulins were detected by conventional serological techniques. HLA-A, B, C typing was performed using the standard two-stage NIH lymphocytotoxicity test (LCT). HLA-DR typing was performed using B lymphocyte preparations in the LCT with prolonged incubation. Platelet antigens were detected by the platelet suspension immunofluorescence test (PSIFT) [10].

Serum samples were screened for platelet reactive antibodies using the platelet agglutination test (PAT) [11] and the PSIFT (utilising both untreated and chloroquine-treated target platelets [12]). The functional activity of the anti-platelet antibodies in the maternal serum was assessed by measuring the interaction between opsonised platelets and normal human monocytes in a chemiluminescence assay (CLT). Results of the CLT were expressed as an opsonic index by dividing the response of monocytes to platelets opsonised by the test serum by the mean response of monocytes to platelets incubated with five normal controls [13]. Anti-HLA antibodies were detected using the LCT. Identification of antibodies was performed against a panel of lymphocytes of known HLA phenotype and against a panel of platelets of known ABO, HLA and Pl^{A1}, Pl^{A2}, Ko^a, Ko^b and Bak^a phenotype.

Results

Preliminary studies

Serum obtained from the mother 11 days after delivery contained IgG antibodies which reacted strongly in the PSIFT with the father's platelets. Anti-platelet antibodies were not detectable in the child's serum.

Anti-Pl^{A1} typing sera revealed that the mother's platelets were $Pl^{A1}(+)$ while those of the father were $Pl^{A1}(-)$. (Table 1 shows the ABO, Rh(D), HLA, Pl^A and Bak^a phenotypes of the mother, father and child.) The maternal serum did not react with platelets from a patient with Glanzmann's thrombasthenia. These findings suggested that the anti-platelet antibodies in the maternal serum might be specific for Pl^{A2}. However, further investigation was complicated by the presence of polyspecific (but predominantly anti-B14) anti-HLA antibodies in the serum. Chloroquine-treated platelets (which no longer express HLA antigens [12]) were

Table 1

Blood groups, HLA and platelet phenotypes of family Dan

	ABO	Rh(D)	HLA	Platelet antigens	
Mother	A	+	A25, A32 B35, Bw57 Cw4, Cw7 DRw6, DR7	Pl(A1+, A2−)	Bak(a+)
Father	A	+	A29, − B14, B27 Cw5, Cw7 DRw3, DRw6	Pl(A1−, A2+)	Bak(a−)
Child	A	+	n. d.	n. d.	n. d.

n. d. = not determined

therefore used as target cells in the PSIFT. Positive reactions were observed when maternal serum was incubated with untreated $Pl^{A1}(-)$ platelets from four donors (including the father). Chloroquine-treated platelets from these donors also gave positive reactions although the intensity of the reaction was reduced. In contrast, reactions with $Pl^{A1}(+)$ platelets became negative after chloroquine treatment. Only IgG anti-platelet antibodies were detected in the PSIFT.

The maternal serum also opsonised $Pl^{A1}(-)$ platelets for recognition by human monocytes in the chemiluminescence test [13] (opsonic index: 2.5; normal range: 1.0 ± 0.5)

Table 2

Reactivity of maternal serum (M. Dan) against a panel of platelets of known phenotype

Methods	Platelets						
	Father	Panel Donors					
	Pl(A1−, A2+) Bak(a−) n. d.	Pl(A1−, A2+) Bak(a+) Ko(a+, b+)	Pl(A1−, A2+) Bak(a+) Ko(a−, b+)	Pl(A1+, A2+) Bak(a+) Ko(a−, b+)	Pl(A1+, A2−) Bak(a+) Ko(a+, b+)	Pl(A1+, A2−) Bak(a−) Ko(a−, b+)	Pl(A1+, A2−) Bak(a−) Ko(a−, b+)
PSIFT	+++	+++	+++	+	−	+	+
PSIFT-CQ	+	+	+	−	−	−	−
PAT	n. d.	+	+	−	−	−	−

PSIFT − utilising untreated platelets; PSIFT-CQ − utilising chloroquine-treated platelets; − negative reaction; +, +++ : graded positive reactions; n. d. − not determined

Antibody identification using a platelet panel

Strong positive reactions were observed in the PSIFT when the maternal serum was incubated with both untreated and chloroquine-treated platelets from individuals homozygous for the PI^{A2} antigen. Positive reactions were also observed in the PAT after maternal serum was incubated with homozygous PI^{A2} platelets. The pattern of reactivity against the platelet panel excluded the presence of anti-PI^{A1}, anti-Bak^a, anti-Ko^a and anti-Ko^b antibodies in this serum. The presence of anti-Bak^b antibody specificity in the maternal serum could not be excluded by the lack of reaction with chloroquine-treated Bak^a negative platelets (Table 2).

The anti-PI^{A2} antibodies could only be detected in undiluted maternal serum samples taken in the two months after delivery; serum taken after six months contained no detectable anti-PI^{A2} antibodies.

Discussion

Clinically, this case presented as a typical example of NAITP. However, the thrombocytopenia appears to have been caused by anti-PI^{A2} antibodies. The data presented suggests that during her first pregnancy, the Pl(A1 +, A2 −) mother was immunized by fetal PI^{A2} antigen inherited from the Pl(A1 −, A2 +) father. The maternal serum contained anti-PI^{A2} IgG antibodies, although the weak positive reactions in the PAT suggest that IgM antibodies were also present. The anti-PI^{A2} antibodies opsonised $PI^{A1}(-)$ platelets for recognition by human monocytes in the CLT and this may reflect one mechanism by which these antibodies initiate platelet destruction in vivo [13].

In the only previously reported case of immunization against PI^{A2} causing NAITP [9], only platelet-specific anti-PI^{A2} antibodies were detected. In contrast, the present case was complicated by the presence of anti-HLA antibodies and required the use of chloroquine-treated platelets to determine the specificity of the anti-platelet antibodies involved. In both cases, the anti-PI^{A2} antibodies were present only transiently in the maternal serum and disappeared after six months. This report confirms that anti-PI^{A2} antibodies can mediate NAITP and illustrates the value of using chloroquine-treated platelets to identify platelet-specific antibodies in sera also containing anti-HLA antibodies.

Acknowledgements

We are grateful to Mrs. C. van der Plas-van Dalen for her co-operation and for access to the platelet panel and anti-PI^{A2} typing sera at the Central Laboratory of the Netherlands Red Cross Transfusion Service, Amsterdam. We also wish to thank Dr. M. Kicinska and Dr. I. Kobierska (Neonatal Department, The Medical School, Lodz) for refering this sample for investigation.

References

1. Von Dem Borne, A. E. G. Kr., Van Leeuwen, E. F., Von Riesz, L. E., Van Boxtel, C. J., Engelfriet, C. P.: Neonatal allo-immune thrombocytopenia; detection and characterization of the responsible antibodies by the platelet immunfluorescence test. *Blood 57*, 649 (1981).
2. Maslanka, K. Zupanska, B.: Clinical significance of platelet alloantibodies detected in immunofluorescence test (Neonatal alloimmune thrombocytopenia and post-transfusion purpura). *Folia Haematol, 112*, 760 (1985).
3. Muller, J. Y., Reznikoff-Etievant, M. F., Patereau, C., Dangu, C., Chesnel, N.: Thrombopenies neonatales alloimmunes. Etude clinique et biologique de 84 cas. *Presse Med. 14*, 83 (1985).
4. Shulman, N. R., Marder, V. J., Hiller, M. C., Collier, E. M.: Platelet and leucocyte isoantigens and their antibodies: serologic, physiologic and clinical studies. In: *Progress in Hematology*. C. V. Moore, E. B. Brown (eds). Grune and Stratton, New York 1964, p222.
5. Moulinier, J.: Iso-immunization maternalle anti-plaquettaire et purpura neonatal. Le système de groupe plaquettaire 'DUZO'. In: *Proc. 6th Congr. Eur. Soc. Haematol.*, Karger, Basel 1957, p817.
6. Von Dem Borne, A. E. G. Kr., Von Riesz, E., Verheugt, F. W. A., Ten Cate, J. W., Koppe, J. G., Engelfriet, C. P., Nijenhuis, L. E.: Bak^a, a new platelet-specific antigen involved in neonatal allo-immune thrombocytopenia. *Vox Sang. 39*, 113 (1980).
7. Shibata, Y., Matsuda, I., Miyaji, T., Ichikawa, I.: Yuk^a, a new platelet antigen involved in two cases of neonatal alloimmune thrombocytopenia. *Vox Sang. 50*, 177 (1985).
8. Shibata, Y., Miyaji, T., Ichikawa, Y., Matsuda, I.: A new platelet antigen system, Yuk^a/Yuk^b. *Vox Sang. 51*, 334 (1986).
9. Mueller-Eckhardt, C., Becker, T., Weisheit, M., Witz, C., Santoso, S.: Neonatal alloimune thrombocytopenia due to fetomaternal Zw^b incompatability. *Vox Sang. 50*, 94 (1986).
10. Von Dem Borne, A. E. G. Kr., Verheught, F. W. A., Oosterhof, F., Von Riesz, E., Brutel de la Riviere, A., Engelfriet, C. P.: A simple immuno-fluorescence test for the detection of platelet antibodies. *Br. J. Haematol. 39*, 195 (1978).
11. Van der Weerdt, Ch. M., Veenhoven-von Riesz, L. E., Nijenhuis, L. E., Van Loghem, J. J.: The Zw blood group system in platelets. *Vox Sang. 8*, 513 (1963).
12. Nordhagen, R., Flaathen, S. T.: Chloroquine removal of HLA antigens from platelets for the platelet immunofluorescence test. *Vox Sang. 48*, 156 (1985).
13. Lucas, G. F., Hadley, A. G., Holburn, A. M.: Anti-platelet opsonic activity in alloimmune and autoimmune thrombocytopenia. *Clin. Lab. Haematol. 9*, 59 (1987).

Haematologia 22 (2), pp. 115–123 (1989)

Effect of Plasma Fibronectin on the Adhesive Properties of Human Peripheral Lymphocytes

G. ALTANKOV, L. SMILENOV

Central Laboratory of Biophysics, Bulgarian Academy of Sciences, Acad. G. Gonchev Str. Block 21, 1113 Sofia, Bulgaria

(Received 18 March, 1988; accepted 3 June, 1988)

The adhesive properties of human peripheral lymphocytes (HPL) towards immobilized substrate in vitro have been investigated. The role of plasma fibronectin was studied in two different systems: a) immobilized fibronectin as a model of cell adhesion in the tissues; b) soluble fibronectin as a model for cell adhesion in blood circulation. Collagen (Type I) or collagen-fibronectin-coated surfaces were used as substrate for lymphocyte adhesion in system b. A very small amount of HPL ($5.1 \pm 1.2\%$) was attached to the collagen surface. The adhesion to fibronectin or to collagen-fibronectin surfaces, however, was substantially higher, reaching a value of $34.1 \pm 3.8\%$ and $32.7 \pm 5.8\%$, respectively. The addition of soluble fibronectin to system a leads to a competitive inhibition of adhesion. On the contrary, in the presence of soluble fibronectin, the augmentation of the lymphocyte adhesion to collagen was found to be dose-dependent and nearly 4 times higher as compared to the control level. This effect was found to be maximal at fibronectin concentrations of 250–300 μg/ml.

The data demonstrate the important role of plasma fibronectin in the adhesive interactions of lymphocytes.

Keywords: adhesion, collagen and fibronectin-coated surfaces, peripheral lymphocytes

Introduction

Fibronectin is a large glycoprotein whose major functional properties are related mainly to its adhesion capacity [1, 2]. It is widely accepted that fibronectin participates in numerous biological phenomena including mediation of cell adhesion and migration, embryonic differentiation, malignant transformation, wound healing etc. [3].

Fibronectin is a normal component of blood plasma [3, 4] and is identical with the cold insoluble globulin [4] or CB-glycoprotein [5]. The physiological role of plasma fibronectin is not fully understood [3]. The circulating fibronectin is supposed to facilitate phagocytosis in the reticuloendothelial system (RES) [4, 6] and takes part as a co-factor in coagulation [3], but similarly to the structurally slightly different cellular fibronectin, it can also mediate cell adhesion and spreading [3]. It has been shown that plasma fibronectin can increase the adhesive properties of some blood cells, such as monocytes [7], neutrophil granulocytes [8], platelets [9] and haemopoietic cells [10], which suggests another functional role of this protein. There are few data [11], however, concerning the role of fibronectin on

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

the adhesive properties of lymphocytes. It has been demonstrated in vitro that these cells show an ability to adhere to glass [12, 13], other synthetic materials [14] or to foreign (target) cells [15]. In spite of the fact that lymphocytes continuously recirculate between the blood and lymph organs, a major part of them were found in immobilized state, closely adhered to definite regions of the lymph nodes phollilculous [16] or other tissue structures. This is why specific interactions between these cells and the extracellular matrix components, such as fibronectin and collagens may be expected [3, 17].

In the present paper the solid surface-immobilized fibronectin and the soluble fibronectin were tested in systems modelling lymphocyte adhesion in tissues and blood circulation conditions, respectively.

Materials and Methods

Preparation of lymphocyte suspension

The experiments were performed with human peripheral lymphocytes (HPL) obtained from 4 healthy volunteers (drawn repeatedly). Sodium citrate (3.8%) in 9 : 1 ratio was used as an anticoagulant. The lymphocytes were prepared from blood diluted 1 : 1 with PBS by centrifugation on Limphoprep (TM, Nyegard & Co. A/S. Oslo, Norway) at 800*g* for 20 min, according to the method of Boüm (1974). The lymphocytes were washed three times with PBS and were resuspended in TC medium 199 (Difco) to a final concentration of 5×10^6 cells/ml. The purity of the lymphocyte suspension (measured microscopically) was: 94–98% lymphocytes, 1–5% monocytes and 1–3% red blood cells. Viability was determined by trypan blue exclusion test and was usually higher than 95%.

Purification of fibronectin

Acid citrate dextrose plasma (ACD-plasma) was collected from healthy donors. Aliquots of the plasma were stored at −40 °C and were used only after 1 hour preincubation at 37 °C. The fibronectin was prepared by affinity chromatography on gelatin-sepharose 4B according to the method of Engvall and Rouslahti [18], and stored in 4 M urea. Before use the fibronectin was transferred to 50 mM Tris pH 7.3 by gel filtration with Sephadex G-25. The purity of the fibronectin was assayed by SDS polyacrylamide electrophoresis (10% gel). A single band at 220 kDa was observed.

Measurement of the lymphocyte adhesion

The adhesion assay was carried out in 96 wells tissue culture polystirol plates (Linbro), which were coated with various proteins as described below. 5×10^6 cells in 0.1 ml were pipetted into each well and the plates were incubated at 37 °C

for 60 min in a humidified 5% CO_2-air incubator. At the end of incubation the nonadherent cells were removed by washing twice with PBS. The cells adhered to the bottom of the wells were fixed in 3% formaldehyde and stained with 1% toluidine blue for 15 min. In some cases the supernatant from each well was resuspended gently and collected, in order to estimate the concentration of unattached cells.

The degree of adherence was assayed in two ways: (a) by counting the unattached cells and calculation of % adhesion, and (b) photometrically, by measuring the optical density of the bound to the attached cells.

In assay (a) the supernatant from each well was gently collected and counted in a haemocytometer. A trypan blue exclusion method was used to determine the number of viable unattached cells. The percent of adhesion was calculated from the following formula:

$$\text{ADHESION}\ (\%) = \frac{\text{NUMBER OF VIABLE CELLS AFTER INCUBATION}}{\text{NUMBER OF VIABLE CELLS BEFORE INCUBATION}} \times 100$$

In assay (b) a method similar to those published in reference [19] was used, applying another dye (toluidine blue). After staining the attached cells (see above) 10 µl 1% SDS were pipetted to each well (for extraction of the dye) and absorbance was estimated after 30 min at 650 nm in an ELISA processor (Sumal, Zeiss, DDR). The results obtained using this method gave a satisfactory correlation compared to method (a). These data are suitable only for comparative experiments. The main advantages of this technique are higher sensitivity, quickness and possibility for a multiplication of the experiment.

Preparation of fibronectin and collagen coated surfaces

The collagen coated surfaces were prepared by adding to the wells 50 µl acid soluble collagen type 1 in conc. 1 mg/ml and drying them overnight at 37 °C. The collagen was prepared according to reference [20] by extraction from rat tail tendons using 0.1% acetic acid. Before use the wells were rinsed 5 times with PBS.

Fibronectin adsorption to the surface was performed in two ways: (a) directly on polystirene by preincubation of the wells with various concentrations of protein in 50 mM Tris, pH 7.3, at room temperature for 2 hours, and (b) adsorption to collagen-coated surfaces in the same buffer at 37 °C for 30 minutes.

Any uncoated sites were further filled by coating the wells with 2 mg/ml BSA (Sigma) for 30 min. For control the wells were coated with BSA only.

Results

Adhesion of HPL to immobilised fibronectin

The solid surface-immobilised fibronectin was accepted as a model of lymphocyte adhesion in the tissues. In the course of our experiments the fibronectin

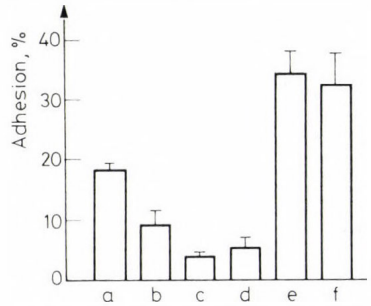


Fig. 1. Adhesion of HPL to various surfaces: (a) polystyrol, (b) polystyrol in the presence of 5% BSA, (c) albumin-coated surface, (d) collagen-coated surface, (e) fibronectin-coated surface and (f) collagen-fibronectin-coated surface. The calculation of the adhesion in percentage is described under Materials and Methods. Each value is mean ± Confidence Limit from five different experiments. The measurements were performed in triplicates

was immobilised to the surface either by direct binding to the polistyrol or by adsorption to the surface precoated with collagen.

Our initial aim was to achieve a maximal diminution of the nonspecific cell attachment to the surface and to specify the optimal conditions for fibronectin, collagen or fibronectin-collagen coating. As shown in Fig. 1 the HPL displayed a high tendency of nonspecific adherence to polistyrol. Under our experimental conditions nearly one fifth of the settled lymphocytes (18.3±1.4%) was attached to the bottom of the wells. Addition of 5% BSA to the medium resulted in a nearly two-fold but not complete inhibition of adhesion (8.9±2.1). Substantial blocking

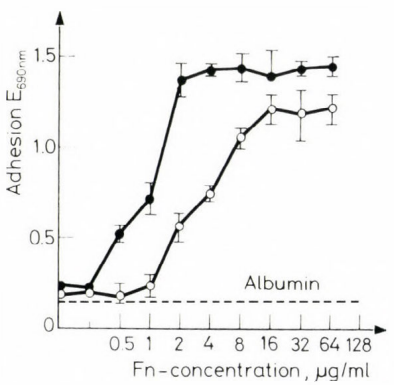


Fig. 2. Adhesion of HPL to surfaces coated with different concentrations of fibronectin. Different concentrations of fibronectin in 50 mM Tris buffer, pH 7.4, were used to coat the polystyrol (—●—) or collagen (—○—) surfaces. The degree of adherence was assayed photometrically as described under Materials and Methods. Each point is mean ± S. E. M. from four experiments

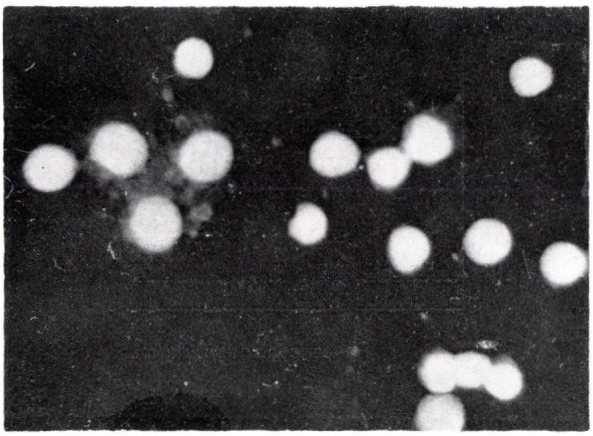


Fig. 3. Morphology of HPL adhered to fibronectin-coated surface. HPL were incubated for 1 hour at 37 °C in tissue culture polystyrol plates coated with 20 μg/ml fibronectin (see Materials and Methods)

of the nonspecific lymphocyte adhesion to polystyrol ($4.2 \pm 0.2\%$) was also attained after pretreatment of the wells with 2 mg/ml BSA.

Coating with collagen alone did not support significant adhesion of lymphocytes (5.1 ± 1.2) compared to the albumin coated surface (Fig. 1). Coating of the substrate with fibronectin, however, dramatically increased HPL adhesion in both types of protein immobilisation: the adhesion for fibronectin-coated-alone surfaces was $34.1 \pm 3.8\%$ and for collagen-fibronectin surfaces $-32.7 \pm 5.8\%$.

Various concentrations of fibronectin were tested in order to establish the optimal protein coating. In general, the extent of adhesion was proportional to the fibronectin concentration used to precoat the wells. Although the maximal adhesion in both systems was similar (Fig. 1 and Fig. 3), we observed different kinetics of the adhesion processes depending on the method of protein immobilisation (Fig. 2). It was estimated that the maximal adhesion for fibronectin-coated surfaces was reached at about $2-4$ μg/ml, whereas it was nearly $15-30$ μg/ml for collagen-fibronectin surfaces (Fig. 2).

The morphology of the adherent cells was studied by phasecontrast microscopy and we found that most of the lymphocytes showed a typical round shape (Fig. 3). None of them was observed to spread on surfaces coated with fibronectin.

Effect of soluble fibronectin on the HPL adhesion

The effect of various concentrations of soluble fibronectin on the adhesive properties of HPL to immobilized fibronectin and collagen was tested, in order to modelling blood circulation conditions. The results indicate that lymphocyte adhe-

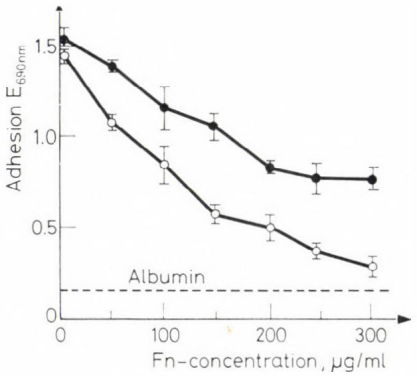


Fig. 4. Inhibition of HPL adhesion to fibronectin (—●—) or collagen-fibronectin-coated surface (—o—) in the presence of various concentrations of soluble fibronectin. The degree of adherence was assayed photometrically as described under Materials and Methods. Each point is mean ± S. E. M. of from four experiments

sion to solid phase immobilised fibronectin could be inhibited by addition of soluble fibronectin to the system (Fig. 4). This effect was concentration dependent and substantially higher for collagen-fibronectin surfaces.

In contrast, HPL adhesion to collagen-coated surfaces increased in the presence of soluble fibronectin (Fig. 5). This effect was also dose dependent and maximal adhesion was reached at fibronectin concentration of 250–300 μg/ml. Further increase of fibronectin concentration was found to be uneffective.

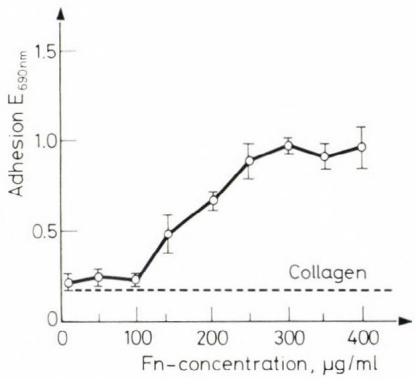


Fig. 5. Adhesion of HPL to collagen-coated surface in the presence of different concentrations of soluble fibronectin. The degree of adherence was assayed photometrically as described under Materials and Methods. Each point is mean ± S. E. M. from four different experiments

Discussion

Our results suggest the presence of a lymphocyte subpopulation which shows an ability to interact with both immobilized and soluble fibronectin. In experiments with solid phase-immobilized fibronectin (a model for tissue interactions) approximately one third of HPL adhered to fibronectin-coated surfaces. The process is specifically connected with this protein, since it is competitively inhibited by addition of soluble fibronectin. Our preliminary data indicate that both T and B lymphocytes are equally engaged in the adhesion process (results not shown). HPL do not interact directly with collagen (type I), but preadsorbtion of fibronectin enhanced lymphocyte adhesion in the same manner as it does on the fibronectin coated surface. In the latter system the adhesion was inhibited even more by addition of soluble fibronectin, this effect being more pronounced compared to fibronectin-coated surfaces. This is probably due to differences in the amount of absorbed fibronectin molecules (see below). Some conformational differences should not be eliminated, however, which undoubtedly require more profound investigations.

The results could be explained with the existence of specific fibronectin receptors on the surface of the adherent lymphocytes. In our concept, the interaction of these receptors with solid phase-immobilized fibronectin leads to the process of cell adhesion (Fig. 6a), while preliminary saturation of the receptors with soluble fibronectin induces a competitive inhibition of the process, similarly to other cell systems [3] (Fig. 6b). The latter fact is of particular importance as it could explain the "nonadhesive" behaviour of lymphocytes in a circulation where a great amount of soluble fibronectin is present (approx. 300 μg/ml). In a recent paper of Liao et al. (1987) concerning the adhesion of different lymphoid cell lines to fibro-

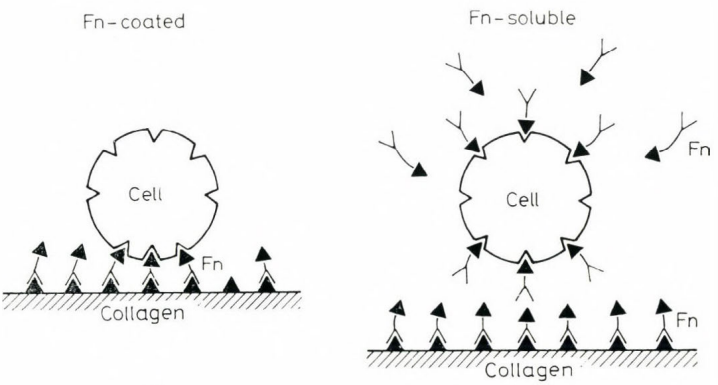


Fig. 6. Model for dualistic effect of fibronectin on the lymphocyte adhesion. (a) Model for lymphocyte adhesion in tissue conditions. The interaction of solid phase immobilized fibronectin with cell receptors leads to the process of lymphocyte adhesion. (b) Model for lymphocyte adhesion in blood circulation conditions. An excess of soluble fibronectin leads to a competitive inhibition of lymphocyte adhesion to fibronectin-coated surface

nectin-coated surfaces, a similar fibronectin receptor has been identified on MOPS--315 lymphoma cells [21]. It has been shown that the receptor was identical with the 140 kDa fibronectin receptor of CHO cells [11]. Nevertheless, it is obviously necessary to confirm these results with normal lymphocytes.

Our data showed that preincubation of polystyrol substrate with 2—4 μg/ml fibronectin is sufficient to saturate the surface, whereas 15—20 μg/ml (a nearly 10 fold higher) concentration is necessary to saturate the collagen substrate. Data suggest a difference in the fibronectin binding capacity of these two surfaces. This is in a good accordance with previously reported results [21].

The effect of soluble fibronectin was tested, since this protein is a normal component of the plasma and is known to mediate adhesion of a wide variety of cells [1, 2, 3, 7, 8, 9, 10]. We found that the soluble fibronectin is able to promote HPL adhesion to collagen coated surface. The effect was dose dependent and reached a plateau between 250—300 μg/ml. It is noteworthy, that the observed saturation concentration of fibronectin coincides well with the physiological plasma concentration of this protein (approx. 300 μg/ml) [3, 4]. As far as the saturation effect for collagen surface was observed at relatively low fibronectin concentration 15—20 μg/ml (as discussed above), evidently the observed effect could be due to saturation of the cell receptors. One can presume that there is a close dependence between the lymphocyte fibronectin receptor synthesis and the local concentration of the ligand. The changes of this concentration-saturation ratio could be a reason for alteration of the lymphocyte adhesive behaviour.

In conclusion, the present study established the ability of plasma fibronectin to promote adhesion of certain part of HPL. Since fibronectin is a major component of ECM [3], this observation is important for elucidation of the adhesive interactions of lymphocytes in tissues during their continuous recirculation in the organism. On the other hand, the results obtained with soluble fibronectin, made us suggest, that the concentration of this protein in the blood (or other biological fluids) could be an important factor for modulation of the lymphocyte adhesive properties.

References

1. Hynes, R. O., Vaheri, A.: Fibronectins, multifunctional modular glycoproteins. *J. Cell. Biol. 95*, 369 (1982).
2. Mosher, D.: Physiology of fibronectin. *Ann. Rev. Med. 35*, 561 (1984).
3. Yamada, K. M., Akyama, S. K., Nesegawa, T. D., Hasegawa, E., Humphries, M. D., Kennedy, D. W., Nagata, K., Orushara, H., Olden, K., Chen, W. T.: Recent advances in research on fibronectin and other cell attachment proteins. *J. Cellular Biochem. 28*, 79 (1985).
4. Saba, T. M., Jaffe, E.: Plasma fibronectin (opsonic glycoprotein): its synthesis by vascular endothelial cells and role in cardiopulmonary integrity after trauma as related to reticuloendothelial function. *Am. J. Med. 68*, 5577 (1980).
5. Blumenstock, F. A., Saba, T. M., Weber, P., Laffin, R.: Biochemical and immunological characterisation of human opsonic a2CB glycoprotein: its identity with cold insoluble globulin. *J. Biol. Chem. 253*, 4287 (1978).

6. Van De Water, L.: Phagocytosis. In: *Plasms fibronectin, structure and function.* McDonath, J. (ed.): Marcel Dekker, Inc., New York, Basel, 1986, p. 175.
7. Bevilacque, M. P., Amzani, D., Mosseson, M. W., Bianco, C.: Receptors for cold insoluble globulin (plasma fibronectin) on human monocytes. *J. Exp. Med. 153*, 42 (1981).
8. Wall, R. T., Cooper, S. L., Kosek, J. C.: The influence of exogenous fibronectin on blood granulocyte adherence to vascular endothelium in vitro. *Exp. Cell. Res. 140*, 205 (1982).
9. Chazov, E. V., Alexeev, A. V., Antonov, A. S., Koteliansky, V. E., Leytin, V. L., Lyubimova, E. V., Repin, S. V., Sviridov, D. D., Torchilin, V. P., Smirnov, V. N.: Endothelial cell culture on fibrillar collagen: Model to study platelet adhesion and liposome targeting to intracellular collagen matrix. *Proc. Natl. Acad. Sci. USA 78*, 5603 (1981).
10. Patel, V. P., Cichanover, A., Plat, O., Lodish, H. F.: Mammalian reticulocyte lose their adhesion to fibronectin during maturation to erythrocytes. *Proc. Natl. Acad. Sci. USA 82*, 440 (1985).
11 Liao, M. L., John, J. S., Du, Z. S., Cheng, H. T.: Adhesion of lymphoid cell lines to fibronectin coated substratum: biochemical and physiological characterisation and identification of a 140 kDa fibronectin receptor. *Exp. Cell Res. 171*, 306 (1987).
12. Altankov, G., Georgieva, M.: Adhesive properties of lymphocytes — a possible mechanism for their selective distribution in the organism. *Scri. Sci. Med. 20*, 132 (1983).
13. Wigzell, H.: Specific affinity fractionation of lymphocytes using glass or plastic bead columns. *Scand. J. Immunol. 3*, Suppl. 3 (1976).
14. Medea, M., Kimura, M., Inoue, S., Kataoka, K., Okano, T., Sakurai, Y.: Adhesion behavior of rat lymphocyte subpopulations (B cells and T cells) on the surface of polystirene/polypeptide graft copolymer. *J. Biomed. Mather. Res. 20*, 25 (1986).
15. Hollander, N., Ginsburg, H.: Specific adherence of in vitro differentiated lymphocytes to target cells. *J. Exp. Med. 136*, 1344 (1972).
16. Curtis, A. S. G., De Sousa, A.: Factors influencing adhesion of lymphoid cells. *Nature New Biol. 45*, 244 (1973).
17. Kleinman, H. K., Klebe, R. J., Maetin, G. R.: Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol. 88*, 473 (1981).
18. Engvall, E., Ruoslahti, E.: Binding of soluble form of fibroblast surface protein, fibronectin to collagen. *Int. J. Cancer 20*, 1 (1977).
19. Laughton, C.: Quantification of attached cells in microtitre plates based on comassie brilliant blue G-25 staining of total cellular protein. *Anals of Biochem. 140*, 417 (1984).
20. Storm, S. C., Michalopopoulos, G.: Collagen as a substrate for cell growth and differentiation. *Methods of Enzymol. 82*, 544 (1982).
21. Hanski, C., Zimmer, T., Heidemann, H., Reutter, W.: Quantitative study of cell interaction with collagen and fibronectin. *Biol. Chem. Hoppe-Seyler 367*, 913 (1986).

Abstracts

DNA repair in lymphocytes from humans and rats with chronic iron overload. A. Pietrangelo, A. Cossarizza, D. Monti, E. Ventura and C. Franceschi (Institutes of Medicine and General Pathology, University of Modena, Modena, Italy). *Biochem. Biophys. Res. Comm. 154*, 698 (1988).

A marked reduction of the proliferative capability after a mitogenic stimulus and a dramatic decrease of the capacity to repair DNA damages were found in lymphocytes from iron overloaded rats. These immunological parameters were not significantly different from controls in peripheral blood lymphocytes from patients with primary iron overload: hereditary hemochromatosis and porphyria cutanea tarda. This discrepancy could be due to the accelerated modality of iron overload in the rat model and to the fact that rat lymphocytes were obtained from an highly iron repleted microenvironment (i.e. spleen). These data indicate that iron overload can affect the structure and/or the function of cellular DNA thus offering new insights on the close association of iron overload conditions and cancer.

A. Egyed

Membrane transport of non-transferrin-bound iron by reticulocytes. E. H. Morgan (Department of Physiology, University of Western Australia, Nedlands, Western Australia, Australia). *Biochim. Biophys. Acta 943*, 428 (1988).

The transport of non-transferrin-bound iron into rabbit reticulocytes was investigated by incubating the cells in 0.27 M sucrose with iron labelled with ^{59}Fe. In most experiments the iron was maintained in the reduced state, Fe(II), with mercaptoethanol. The iron was taken up by cytosolic, haem and stromal fractions of the cells in greater amounts than transferrin-iron. The uptake was saturable, with a K_m value of approx. 0.2 μM and was competitively inhibited by Co^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}. It ceased when the reticulocytes matured into erythrocytes. The uptake was pH and temperature sensitive, the pH optimum being 6.5 and the activation energy for iron transport into the cytosol being approx. 80 kJ/mol. Ferric iron and Fe(II) prepared in the absence of reducing agents could also be transported into the cytosol. Sodium chloride inhibited Fe(II) uptake in a non-competitive manner. Similar degrees of inhibition was found with other salts, suggesting that this effect was due to the ionic strength of the solution. Iron chelators inhibited Fe(II) uptake by the reticulocytes, but varied in their ability to release ^{59}Fe from the cells after it had been taken up. Several lines of evidence showed that the uptake of Fe(II) was not being mediated by transferrin. It is concluded that the reticulocyte can transport non-transferrin-bound iron into the cytosol by a carrier-mediated process and the question is raised whether the same carrier is utilized by transferrin-iron after its release from the protein.

A. Egyed

Effect of phenylhydrazine on red blood cell metabolism. M. Magnani, L. Rossi, L. Cucchiarini, V. Stocchi and G. Fornaini (Istituto di Chimica Biologica, Università degli Studi, Urbino, Italy). *Cell Biochem. and Function 6*, 175 (1988).

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

In addition to the well known effect of phenylhydrazine on red blood cells (methaemoglobin and Heinz body formation, autologous IgG binding, lipid peroxidation, etc.) an increased glucose utilization was observed. Measurement of $^{14}CO_2$ formation from [1—^{14}C]-glucose showed a maximum value at 2 mM phenylhydrazine followed by a progressive inhibition on increasing the drug concentration to 16 mM. Concomitantly it was found a reduction in the reduced glutathione concentration but not a corresponding increase in the level of oxidized glutathione. Phenylhydrazine also causes ATP depletion. The ATP is in part dephosphorylated to ADP and AMP and in part converted to inosine monophosphate and hypoxanthine. Measurement of the cell content of reduced and oxidized pyridine nucleotides was also performed and showed a progressive increase in the reduced forms of these coenzymes. Thus phenylhydrazine promotes cellular ATP depletion followed by adenine nucleotide catabolism that is not effciently counteracted by an increase in glucose utilization. The relevance of these data to the mechanism of phenylhydrazine-induced anemia is discussed.

G. Gárdos

The use of cis-parinaric acid to determine lipid peroxidation in human erythrocyte membranes. Comparison of normal and sickle erythrocyte membranes. J. J. M. Van den Berg, F. A. Kuypers, J. H. Qju, D. Chiu, B. Lubin, B. Roelofsen and J. A. F. Op den Kamp (Department of Biochemistry, State University of Utrecht, Utrecht, The Netherlands). *Biochim. Biophys. Acta 944*, 29 (1988).

The recently developed parinaric acid assay is shown to offer possibilities for studying peroxidation processes in biological membrane systems. Taking the human erythrocyte membrane as a model, several initiating systems were investigated, as well as the effect of residual hemoglobin in ghost membrane preparations. The effectivity of a radical generating system appeared to be strongly dependent upon whether radicals are generated at the membrane level or in the water phase. Thus, cumene hydroperoxide at concentrations of 1.0–1.5 mM was found to be a very efficient initiator of peroxidation in combination with submicromolar levels of hemin-Fe^{3+} as membrane-bound cofactor. In combination with cumene hydroperoxide, membrane-bound hemoglobin appeared to be about 6-times more effective in promoting peroxidation than hemoglobin in the water phase. Results comparing the behaviour of normal and sickle erythrocyte ghost suspensions in the peroxidation assay suggest that the increased oxidative stress on sickle erythrocyte membranes could be due to enhanced membrane binding of sickle hemoglobin, but also partly to a characteristically higher capability of sickle hemoglobin to promote peroxidation. The order of peroxidation-promoting capabilities that could be derived from the experiments was hemin > sickle hemoglobin > normal hemoglobin.

G. Gárdos

Deficiency of protein 4.2 in erythrocytes from a patient with a Coombs negative hemolytic anemia. Evidence for a role of protein 4.2 in stabilizing ankyrin on the membrane. A. C. Rybicki, R. Heath, J. L. Wolf, B. Lubin and R. S. Schwartz (Childrens Hospital-Oakland Research Institute, Oakland, California, USA). *J. Clin. Invest. 81*, 893 (1988).

A patient with a mild hemolytic anemia and osmotically fragile, spherocytic erythrocytes was studied. Analysis of the erythrocyte membrane proteins by SDS-PAGE revealed a deficiency of protein 4.2 ($< 0.10\%$ of normal). The protein 4.2-deficient erythrocytes contained normal amounts of all other membrane proteins, although the amount of band 3 was slightly reduced and the amount of band 6 (G3PG) was slightly elevated. The spectrin content of these cells was normal, as measured by both SDS-PAGE and radioimmunoassay. Erythrocytes from the patient's biologic parents were hematologically normal and contained normal amounts of protein 4.2. Immunological analysis using affnity purified antibodies revealed that the patient's protein 4.2 was composed of equal amounts of a 74-kD and 72-kD protein doublet, whereas the normal protein was composed primarily of a 72-kD monomer. Proteolytic digestion studies using trypsin,

α-chymotrypsin and papain demonstrated that the patient's protein 4.2 was similar but not identical to the normal protein. Binding studies showed that the protein 4.2-deficient membranes bound purified protein 4.2 to the same extent as normal membranes, suggesting that the membrane binding site(s) for the protein were normal. Depleting the protein 4.2-deficient membranes of spectrin and actin resulted in a loss of nearly two-thirds of the membrane ankyrin, whereas similar depletion of normal membranes resulted in no loss of ankyrin. Repletion of the protein 4.2-deficient membranes with purified protein 4.2 before spectrin-actin extraction partially prevented the loss of ankyrin. These results suggest that protein 4.2 may function to stabilize ankyrin on the erythrocyte membrane.

Ilma Szász

Kinetic independence between red cell anion exchange and urea transport. O. Fröhlich and S. C. Jones (Emory University School of Medicine, Atlanta, GA, USA). *Biochim. Biophys. Acta 943*, 531 (1988).

Urea equilibrium exchange fluxes were measured in human red cells under conditions which recruit the anion transporter into an outward-facing or an inward-facing state (with respect to the anion transport site). Regardless of these conditions, urea transport always occurred at the same rate: 41 ± 2 mol. (kg cell solids · min)$^{-1}$ with 1.5 M urea at 0 °C. These data suggest that the pathway on the band-3 protein which mediates anion transport is kinetically uncoupled from urea transport and is probably not involved in the transport of urea across the red cell membrane.

B. Sarkadi

Preparative separation of human B and T lymphocytes by free flow electrophoresis. T. G. Baier, G. Weber, K. Hartmann, U. Heinrich, and D. Schönberg (Division of Pediatric Endocrinology, Department of Pediatrics, University of Heidelberg, F. R. G.). *Anal. Biochem. 171*, 91 (1988).

An electrophoretic method for the quantitative separation of human B and T lymphocytes in a carrier-free system is presented. The method is based on the fact that B and T lymphocytes show marked overlap in their size and density characteristics, but differ suffciently in surface charge to be separable by electrophoresis. The technique is performed in phosphate-buffered saline and appears to be especially suitable for the enrichment of nonstimulated, functionally intact lymphocytes which can be directly used for further immunological or biochemical studies.

B. Sarkadi

Human monoclonal antibodies produced by primary in vitro immunizaton of peripheral blood lymphocytes. C. A. K. Borrebaeck, L. Danielsson, and S. A. Möller (Department of Biotechnology, University of Lund, Lund, Sweden). *Proc. Nat. Acad. Sci. 85*, 3995 (1988).

A general procedure is described for the production of human monoclonal antibodies from peripheral blood lymphocytes immunized *in vitro* against T-cell-dependent antigens. These lymphocytes immunized in culture were used to produce human-human or human-mouse hybridomas secreting monoclonal antibodies specific for digoxin, hemocyanin, a recombinant fragment of the gp120 envelope glycoprotein of human immunodeficiency virus (PB1), or a melanoma-associated antigen (p97). Depletion of a lysosome-rich cell population, containing large granular lymphocytes, monocytes, cytotoxin T cells, and a subset of CD8-positive T cells, was shown to be crucial before the cells could be immunized *in vitro*. This depletion was accomplished by treating the peripheral blood lymphocytes with the lysosomotropic agent L-leucine methyl ester. In addition, the *in vitro* immunization had to be supported by interleukin 2, γ-interferon, and B-cell growth and differentiation factors, derived from irradiated poke-weed-mitogen-stimulated human T cells. The production of human monoclonal antibodies from primary, antigen-specifically activated peripheral lymphocytes might obviate the need to immunize volunteers or patients.

B. Sarkadi

Molecular cloning and expression of the major protein kinase C substrate of platelets. M. Tyers, R. A. Rachubinski, M. I. Stewart, A. M. Varrichio, R. G. L. Shorr, R. J. Haslam and C. B. Harley (Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada). *Nature 333*, 470 (1988).

In platelets, agonists that stimulate phosphoinositide turnover cause the rapid phosphorylation of a protein of apparent relative molecular mass (M_r) 40–47.000, called P47, by protein kinase C (PKC). Diverse identities have been ascribed to P47 including lipocortin, inositol 1,4,5-trisphosphate 5-phosphomonoesterase, pyruvate dehydrogenase α subunit and an actin regulatory protein. It have been isolated human P47 clones by immunological screening of a λ gtll complementary DNA library from HL-60 cells, a human promyelocytic leukaemia cell line. P47 recombinants thus identified hybridized to a 3.0 kilobase (kb) messenger RNA in mature white blood cell lines; the same mRNA was induced in HL-60 cells during differentiation. A 1.050 base pair (bp) open reading frame that could encode a protein of M_r 40.087 was confirmed by comparison with peptide sequences from platelet P47, and by expression of the putative recombinant P47 in *E. coli* and *in vitro*. The P47 sequence appears to have been conserved throughout vertebrate evolution, and is not similar to any other known sequence including human lipocortin and the α subunit of pyruvate dehydrogenase. The P47 protein contains a potential Ca^{2+}-binding, "EF-hand" structure and a region that strongly resembles known PKC phosphorylation sites. *B. Papp*

Fluoride-dependent calcium-induced platelet procoagulant activity shows that calpain is involved in increased phospholipid transbilayer movement. P. F. J. Verhallen, E. M. Bevers, P. Comfurius and R. F. A. Zwaal (Department of Biochemistry, University of Limburg, Maastricht, The Netherlands). *Biochim. Biophys. Acta 942*, 150 (1988).

Treatment of platelets with fluoride (10 mM) was found to result in a transient increase in Ca^{2+}-permeability of the platelet plasma membrane. This phenomenon was used to provide supplementary evidence for the suggestions made earlier, that cytoskeletal disrupture by calpain is involved in the process leading to transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity. This was achieved by relating both calpain activity and exposure of phosphatidylserine with platelet procoagulant activity. It was found that only upon addition of extracellular Ca^{2+} to fluoride-treated platelets, procoagulant activity, expressed as prothrombinase activity, and calpain activity, estimated from protein patterns after gel electrophoresis, were generated. Both Ca^{2+}-inducible prothrombinase activity and calpain activity followed an identical time-course during incubation with fluoride: after a time-lag of about 10 min they sharply increased towards a peak level. Upon further incubation with fluoride, both activities decreased towards a final plateau, still above basal level. The presence of leupeptin during incubation with fluoride was found to inhibit Ca^{2+}-inducible calpain activity and prothrombinase activity in an identical way. Ca^{2+}-inducible exposure of phosphatidylserine, as determined with extracellular phospholipase A_2, showed a similar pattern as Ca^{2+}-inducible calpain activity and prothrombinase activity. From the strict parallelism between prothrombinase activity, calpain activity and exposure of phosphatidylserine, it is concluded that calpain plays an important role in the activation-dependent transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity. It is suggested that degradation of the platelet membrane-skeleton by calpain disturbs the structural organization of the lipid bilayer of the platelet plasma membrane leading to enhanced transbilayer movement of phospholipids and appearance of phosphatidylserine at the platelet outer surface.

B. Papp

Effects of two methylxanthines, pentoxifylline and propentofylline, on arachidonic acid metabolism in platelets stimulated by thrombin. L. Rossignol, M. Plantavid, H. Chap and L. Douste-Blazy (Inserm Unité 101,

Biochimie des Lipides, Hôpital Purpan, Toulouse, France). *Biochem. Pharmacology 37*, 3229 (1988).

[^{3}H] Pentoxifylline and [^{3}H] propentofylline were taken up by human platelets in a dose-dependent manner probably involving a passive diffusion through the plasma membrane. *In vitro*, the two drugs were able to inhibit platelet activation induced by thrombin. Serotonin secretion was reduced from 57% to 38% and 28% in the presence of 1 mM pentoxifylline and 1 mM propentofylline, respectively. Platelet aggregation was inhibited in the same way Modifications of [^{14}C] arachidonic acid metabolism in human platelets stimulated by thrombin were then measured in the presence of drugs. Preincubation of platelets with 1 mM pentoxifylline or propentofylline inhibited the production of [^{14}C] arachidonic acid metabolites, without any accumulation of free arachidonic acid, suggesting an action at a step preceding its conversion. Phosphatidylinositol and phosphatidylcholine hydrolysis measured upon thrombin treatment as well as phosphatidic acid production were reduced or suppressed in the presence of the drugs. A dose-dependence study showed that phosphatidylcholine hydrolysis was totally inhibited at 5.10^{-4}M propentofylline, while phosphatidic acid formation was reduced by only 40%. Propentofylline was in general more efficient than pentoxifylline in inhibiting events occuring upon thrombin stimulation. The results suggest that the two methylxanthines inhibit both phospholipase A_2 and phospholipase C, the former displaying a greater sensitivity to the two drugs.

B. Papp

GUIDELINES ON SELECTION OF LABORATORY TESTS FOR MONITORING THE ACUTE-PHASE RESPONSE

Quantitative measurement of acute-phase proteins in blood is a valuable indicator of the presence of an inflammatory response to tissue damage. An expert panel on blood rheology of the International Committee for Standardization in Haematology (ICSH) has prepared guidelines on the selection of laboratory tests for detecting and subsequently monitoring the protein component of the acute-phase response. The guidelines recommend quantitative assay of C-reactive protein when short-term changes (less than 24 hours) occur. Appropriate tests for monitoring longer-term changes (more than 24 hours) include the erythrocyte sedimentation rate and plasma viscosity – the latter having several advantages. The guidelines, which have now been published in the Journal of Clinical Pathology (1988; *41*, 1203–1212), include recommendations on standardisation of these methods and their quality control.

Adoption of the ICSH guidelines should improve comparability between diagnostic laboratories and lead to more selective use by clinicians of tests such as the erythrocyte sedimentation rate.

Copies of the guidelines are available from Professor J. Stuart, Chairman, ICSH Expert Panel on Blood Rheology, Department of Haematology, Medical School, University of Birmingham, Birmingham B15 2TJ, UK.

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

THE EUROPEAN SCHOOL OF HAEMATOLOGY

PROGRAMME 1989

3–5 April

Growth and Differentiating Factors in Haematology

P. Reizenstein, L. Sachs

1–5 May

BMT in Lymphomas

(In collaboration with European School of Oncology)

F. Cavalli

22–26 May

Allogeneic Bone Marrow Transplantation

E. Gluckman, T. Gordon-Smith, B. Speck

28–29 June

Acute and Late Effects of Irradiation on the Haemopoietic System

(Satellite Symposium of the 17th International Congress of Radiology)

M. Tubiana, C. Parmentier

2–6 October

Fibrinolysis

F. Bachmann M. Samama

6–10 November

Drepanocytose et Beta Thalassemie

(Special session in French)

Y. Beuzard

Registration and Information:

ESH Hôpital Saint Louis — Centre HAYEM

1, avenue Claude Vellefaux, 75475 Paris Cedex 10, France

Tel.: (1) 42 06 32 60 • Telex: IRM 212 309 • Telefax: 42 41 14 70

Printed in Hungary

Akadémiai Kiadó és Nyomda Vállalat, Budapest

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 brackets. 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.

Authors are entitled to 50 reprints free of charge.

HAEMATOLOGIA
VOLUME 22 · NUMBER 2 19

Contents

Weitberg, A. B.: The effect of alkaline phosphatase on platelet aggregation 65

Brunori, M., Coletta, M., Bellelli, A., Evangelista, P. A. Benedetti, P. A., Brumen, M.: Microspectroscopy of red blood cells 69

Van Waarde, A., Lombarts, A., van den Thillart, G., Erkelens, C., Lugtenburg, J.: ^{31}P NMR studies on rejuvenation of outdated red blood cells: complete regeneration of ATP is accompanied by partial Mg-ATP recomplexation 79

Rácz, Z., Haskó, F.: Room temperature storage of pooled platelet concentrates in gas-permeable plastic bags for five days 89

Kramer, J., Thiry, E., Füst, G.: Rapid determination of the human complement factor B phenotypes 97

Gamba, G., Ippoliti, G., Invernizzi, R., Gatti, V., Zocchi, M. T., Perugini, O.: Cytochemical profile of lymphocytes in haemophilia patients at risk of AIDS 101

Maslanka, K., Lucas, G. F., Gronkowska, A., Davis, J. G., Zupanska, B.: A second case of neonatal alloimmune thrombocytopenia associated with anti-PI^{A2} antibodies 109

Altankov, G., Smilenov, L.: Effect of plasma fibronectin on the adhesive properties of human peripheral lymphocytes 115

Abstracts 125

Announcements 131

Index: 26.861

VOLUME 22 • NUMBER 3 • 1989

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

///VSP///

UTRECHT, THE NETHERLANDS,
TOKYO, JAPAN

AKADÉMIAI KIADÓ, BUDAPEST

HAEMATOLOGIA

Haematologia is an international journal publishing original papers on all clinical and theoretical aspects of haematology. In addition to regular papers, book reviews and abstracts of selected papers from other periodicals also are a special feature of the journal.

Haematologia, published at quarterly intervals, provides the reader with complex and up-to-date information.

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

Copy Editor:
D. Keszthelyi

Distributor for

Albania, Bulgaria, China, Cuba, Czechoslovakia, German Democratic Republic, Korean People's Republic, Mongolia, Poland, Romania, U.S.S.R., Viet-Nam, and Yugoslavia:

KULTURA
Hungarian Foreign Trading Company
P.O.B. 149, H-1389 Budapest 62, Hungary

For all other countries subscriptions should be sent to

VSP
P.O.B. 346
3700 AH ZEIST, The Netherlands

Subscription price for volume 22:

DM 330,— (including postage)

Haematologia is covered in Current Contents/Clinical Practice, ISI/Biomed and the SCI.

VOLUME 22 · NUMBER 3

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

VSP UTRECHT, THE NETHERLANDS, TOKYO, JAPAN

AKADÉMIAI KIADÓ, BUDAPEST

1989

Haematologia 22 (3), pp. 135–141 (1989)

Dynamism of the Development of "Fc Receptor-blocking Antibodies" after Immunization with Buffy Coat

ILDIKÓ B. PETRI[1], ÉVA GYÓDI[2], G. KAISER[1], M. KASSAI[3], G. GY. PETRÁNYI[2]

[1] Blood Transfusion Centre, H-6701 Szeged, P.O.Box 464, Hungary [2] National Institute of Haematology and Blood Transfusion, H-1502 Budapest, P.O.Box 44, Hungary [3] County Hospital, H-6800, Hódmezővásárhely, Hungary

(Recieved 28 January, 1988; accepted 1 June 1988)

Eight healthy male blood donors who had not recieved transfusion previously, were immunized 5 times at 6-week intervals. The immunization was carried out with "buffy coat" separated from 400 ml freshly obtained donor blood. Donor–recipient pairs showed a mismatching for 1 to 4 HLA antigens. The production of HLA antibodies could be demonstrated in 4 patients during the repeated immunizations.

Fc receptor-blocking antibody activity was determined with the erythrocyte antibody inhibition (EAI) method in serum samples for the recipients. An increase in the Fc receptor-blocking antibody serum level could be detected already on the second day after the first immune stimulus. Fc receptor-blocking activity was maximum on the 10th day after immunization. After repeated immune stimuli, the increased non-specific Fc receptor-blocking antibody activity was demonstrated independently of the appearance of specific HLA cytotoxic antibodies.

Keywords: immunization, Fc receptor-blocking antibody, immunosuppression

Introduction

Fc receptor-blocking antibodies have been demonstrated with the EAI method in anti-Rh(D) gammaglobulin preparations [1], the effect is independent of the anti-D activity. Similar antibodies have been detected in serum samples after blood transfusions, promoting the in vivo survival of transplants [3]. These antibodies were shown to be absent in patients after spontaneous abortion [4]. Different authors reported prevention of recurrent spontaneous abortion with leukocyte transfusions [5] or with a purified lymphocyte preparation from the husband [6], but anti-paternal antibodies or cytotoxic antibodies could not be measured in all of them.

The present work sets out no explain the period of formation of Fc receptor--blocking antibodies following repeated immunization with buffy coat and to establish whether they may be detected in the serum with the EAI method.

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Materials and Methods

Eight healthy males, previously unstimulated volunteers were immunized with "buffy coat" separated freshly from 400 ml donor blood. The interval between two stimuli was 6 weeks. The donor-recipient pairs were incompatible for 1 to 4 HLA-ABC and 1 HLA-HLA-Dr antigen (Table 1).

For in vitro testing, blood was taken before the start of immunization and 2, 4, 7, 10, 15 and 21 days following a stimulus. Recipient serum samples were inactivated for 30 min at 56 °C; after ultracentrifugation ($10^5 g$ for 90 min), samples were stored at −20 °C before testing. Pooled serum samples of 3 healthy, non-immunized blood donors were used as controls.

EAI (erythrocyte antibody inhibition) was carried out as described previously [2]. Blood samples of donors, recipients and outsider donors (third-party donors) were treated with colloidal iron powder (GAF, USA), and the lymphocytes were separated on a Ficoll-Uromiro gradient [7]. T cells depleted by rosetting fractions [8], referred to as B cells, were incubated with ultracentrifugated ($10^5 g$, 90 min) test serum samples from recipients in 20% concentration for 30 min at 4 °C. After washing, the cells were incubated overnight at 4 °C with 2% human Rh(D) erythrocytes previously incubated with anti-D antibodies. A rosette was

Table 1

HLA types of donor-recipient pairs

Pairs	HLA types				
	A	B	C	DR	
1. Donor	24.32	5,w41	w2	3	after 2. immunisation
Recipient	3	35,51	—	4.w8	anti-HLA A9 antibody (1:8)
2. Donor	11,w33	18,w55	w1	5	
Recipient	11	18.21	—	3w8	
3. Donor	1.32	18,w52	—	2.5	after 3. immunisation
Recipient	1.32	18.27	w1	5	anti-HLA Bw52 antibody (1:8)
4. Donor	1.29	12,w52	—	5.7	
Recipient	1.23	w52,w60	—	w6.7	
5. Donor	2.24	13.35	—	5.7	after 5. immunisation
Recipient	2.24	27	w2	w6.7	anti-HLA B35 antibody (1:1)
6. Donor	2.3	21.35	w4	4.5	
Recipient	2.3	12.35	w4	3.4	
7. Donor	31.32	35.40	—	w6.w8	
Recipient	1.31	7.35	w4	4.w6	
8. Donor	2.11	15.35	w3.4	5.w6	after 5. immunisation
Recipient	1.31	39	—	w6.w8	anti-HLA A2 antibody (1:2)

defined as 3 or more RBC lymphocytes, and 200 lymphocytes were counted. The percentage EAI was calculated:

$$\text{EAI}\,(\%) = 100 \times \left\{1 - \frac{\text{EA rosettes in the test}}{\text{EA rosettes in control pooled serum)}}\right\}$$

The separated B cell suspension contained 45–70% rosetting cells. The cell suspension with control pooled serum contained 40–65% rosetting cells.

The sera of the recipients were tested for anti-HLA antibodies on the 10th day after each immunization, by the standard NIH lymphocyte cytotoxicity microtest.

Results

The sera of eight immunized healthy male blood donors were examined for antibody production. The circumstances of immunization (antigen input and time) were identical, and anti-HL antibodies were detected independently of the number of immunizing stimuli (Table 1).

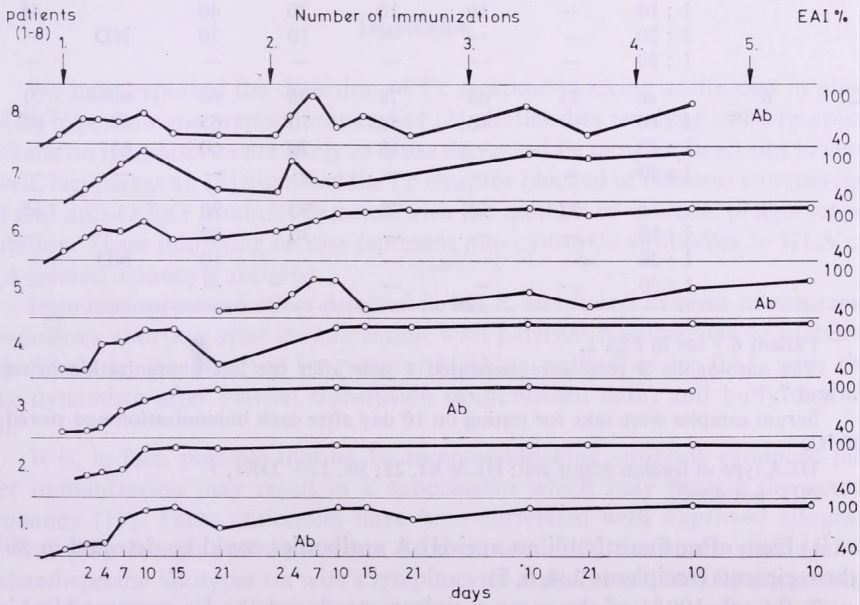


Fig. 1. Dynamism of production of Fc receptor-blocking antibodies following repeated immunization

●: Detection of activity of Fc receptor-blocking antibody with EA inhibition of a foreign (third-party) indicator B lymphocyte.
HLA type: HLA-A 2,3: B 8,40; DR 2,4
Ab: Appearance of complement dependent cytotoxic HLA antibody

Table 2

Serum titration on EAI with autologous cells (A), foreign donor cells (B) and the immunizing donor cells (C)

Patients		Serum dilution in RPMI	Number of immunization						After 1 year
			0	1	2	3	4	5	
A.	6	cc	—	—	8	10	10	12	—
		1 : 10	—	—	—	—	—	—	—
		1 : 20	—	—	—	—	—	—	—
		1 : 50	—	—	—	—	—	—	—
	7	cc	8	5	10	11	15		10
		1 : 10	—	—	—	—			—
		1 : 20	—	—	—	—	—	ND	—
		1 : 50	—	—	—	—	—		—
B.	6	cc	15	65	85	87	90	90	60
		1 : 10	—	—	40	40	50	50	50
		1 : 20	—	—	—	—	41	50	19
		1 : 50	—	—	—	—	—	—	—
	7	cc	30	56	60	70	78		50
		1 : 10	—	10	10	20	40		45
		1 : 20	—	—	—	10	10	ND	—
		1 : 50	—	—	—	—	—		—
C.	6	cc	21	60	78	90	90	90	71
		1 : 10	—	40	50	70	65	67	45
		1 : 20	—	—	10	70	60	65	—
		1 : 50	—	—	—	—	—	—	—
	7	cc	27	50	78	90	90		65
		1 : 10	—	—	—	30	60		—
		1 : 20	—	—	—	—	10	ND	—
		1 : 50	—	—	—	—	—		—

Patient 6.7 see in Fig. 1.

The autologous B cells were separated 1 year after the last immunization in cases N°.6 and 7.

Serum samples were take for testing on 10 day after each immunization and stored at −20 °C.

HLA type of foreign donor cell: HLA-A1, 25; B8, 12+ DR1, 7

ND: not done

1) Even after five stimuli no anti-HLA antibodies could be detected in 50% of the recipients (recipients 2, 4, 6, 7)

2) though 100% of the same recipients produced the Fc receptor-blocking antibodies.

Fc receptor-blocking antibodies were demonstrated already on the second day after the first injection. Maximum EA blocking was attained on the 10th day of the immunization (Fig. 1) (see a representative experiment tested on third-party indicator B cells). Values lower than 40% EA blocking before immunization were

found in recipients 1, 6, 7, 8. Recipients 2, 3, 4 and 5 hand starting values around or higher than 40% EA inhibition. After repeated immunizations under similar antigen conditions, an increased EAI activity was observed in the sera of recipients tested on third-party indicator cells.

The Fc receptor-blocking exerted on the autologous cell was below 15% (Table 2a) in each case, in serum dilutions the above mentioned effect could not be detected. The same activity reached 90% third-party indicator cells (Table 2b). Following the first immune stimulus the level of Fc receptor-blocking antibodies increased after repeated immunization. This activity decreases after a year but can still be demonstrated.

Testing the Fc receptor-blocking antibodies on the immunizing donor lymphocytes, the effect was almost similar to results found in third party indicator cells (Table 2c).

Cytotoxic HLA antibodies appeared in 50% of the cases independently of the number of immune stimuli. The stimulator of anti-HLA-A 9 was the HLA-A 24 antigen in the case of recipient No. 1, following repeated immunization not only the split (A 24 antibody) was produced, but a specificity appeared against the broad antigen.

Discussion

We have reported the detection of Fc receptor-blocking antibodies in anti-Rh(D) hyperimmune preparations earlier [2]. Antibodies resulting in Fc receptor blockade on lymphocytes are likely to cause decreased Fc receptor functions in vivo as well. Neppert et al. [9] discussed the Fc receptor blocked of different commercial IgG and anti-D IgG products detected with the method of immune phagocytosis inhibition. These inhibiting factors represent non-cytotoxic antibodies to HLA or HLA-related monocyte antigens.

Immunosuppressive effect detected in MLR was found in cases of recurrent spontaneous abortion after immunization with paternal lymphocytes or platelets [10]. Similarly, we observed Fc receptor-blocking antibodies by EAI, with the same dynamism after platelet transfusion (unpublished data) and buffy coat as well.

It is, in fact, possible that an Fc receptor-blocking antibody produced just after immunization may result in a suppression which may protect the actual pregnancy [11]. These antibodies have been correlated with improved allograft survival [12]. Joseph and co-workers demonstrated [13] that antibodies against husband-specific idiotypes on wife's lymphocytes could be induced by pregnancy. The MLC-inhibitory activity in the sera of parous women was on the IgG fraction, but absent in women who had had abortion(s).

The distribution of Fc receptors and the variety of functions suggested that they comprise a heterogeneous group of proteins. For example, human T and B lymphocytes and monocytes possess Fc receptors of different molecular weights [14], and have been shown to possess Fc receptors with different avidities for IgG

[15]. Moreover, two types of Fc receptors could be distinguished: membrane-labile (FcRI) and membrane stabile (FcRII) forms [16]. The two types of receptors coexist on the same cell type [17], but they appear to be antigenically distinct. According to the latest results the Fc receptor-blocking IgG antibody may not react primarily with the Fc receptor but with an alloantigen in close relation with the Fc receptor [18]. This structure is expressed on T and B lymphocytes, polymorphs and platelets.

The present work has proved the appearance of the Fc receptor-blocking antibodies in the serum following the immune stimulus. These antibodies can be detected already on the second day after the immune stimulus and the maximal effect can be demonstrated on the 10th day. One year after the repeated immune stimuli a low EAI activity can still be demonstrated. Our findings have some important clinical implications. High-dose intravenous immunoglobulin (IVIG) has been shown to be an effective method for treating immune thrombocytopenic purpura (ITP) [19]. One possible explanation of this therapeutic effect is the Fc receptor-blocking of immune competent cells [20] through antibody mediated inhibition of antigen presentation. The direct B cell inhibitory effect was proven by an other author as the target cell of polyspecific monomeric IgG [21].

Panzer et al. reported an increased number of platelets in ITP patients during both anti-D IgG and high-dose IVIG treatment. The therapeutic response to high-dose IVIG and anti-D IgG is independent of the degree of induced haemolysis [22] and thus the Fc receptor-blocking antibodies present in anti-D preparations are likely to result in a good therapeutic effect independently of the anti-D activity. Other authors [23] describe the suppression of maternal antibody synthesis when Rhesus haemolytic disease was treated with high-dose IVIG. A further, possibly similar situation may be the protective effect of small transfusions before kidney transplantations [24].

In our testing system we detected specific (HLA) antibodies and non-specific (Fc receptor-blocking) antibodies as well. Fc receptor-blocking antibodies appeared very quickly after immunization, much earlier than the HLA-related ones.

Fc receptor-blocking activity in anti-D IgG preparations are well producible by active immunization. This opinion is supported by the fact that in the sera of Rh immunized subjects this non-specific blocking activity is often maintained many years after stimuli which have not been repeated.

Fc receptor blockade, as part of the immune regulation, may be helpful in several clinical conditions, like autoimmune diseases, cases of recurrent spontaneous abortion and possibly also in the course of immunosuppressive therapy after organ transplantation.

References

1. Petri, I. B., Lőrincz, A., Berek, I.: Detection of Fc receptor-blocking antibodies in anti-Rh(D) hyperimmune gammaglobulin. *Lancet ii*, 1478 (1984).
2. Petri, I. B., Lőrincz, A., Berek, I.: Further investigation of nonspecific biological substance in anti-Rh/D preparations. *Vox Sang. 51*, 287 (1986).

3. MacLeod, A. M., Mason, R. J., Stewart, K. N., Power, D. S., Shewan, W. G., Edward, N., Catto, G. R. D.: Fc receptor-blocking antibodies develop after blood transfusions and correlate with good graft outcome. *Transplant. Proc. 15*, 1019 (1983).
4. Fizet, D., Bousquet, J., Piquet, Y., Cabantous, F.: Identification of a factor blocking a cellular cytotoxicity reaction in pregnant serum. *Clin. Exp. Immunol.* 52, 648 (1983).
5. Taylor, C., Faulk, W. P.: Prevention of recurrent abortion with leucocyte transfusions. *Lancet ii*, 68 (1981).
6. Mowbray, J. F., Liddeli, H., Underwood, J. I., Gibbins, C., Reginald, P. W., Beard, R. W.: Controlled trial of immunisation with paternal cells. *Lancet ii*, 941 (1985).
7. Böyum, A.: Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Invest (Suppl. 97) 21*, 77 (1968).
8. Madsen, H., Johnsen, H. E.: A methodological study of E-rosette formation using AET--treated sheep red blood cells. *J. Immunol. Methods, 27*, 61 (1979).
9. Neppert, J., Clemens, M., Mueller-Eckhardt, C.: Immune pathogocytosis inhibition by commercial immunoglobulins. *Blut 52*, 67 (1986).
10. Mowbray, J. F.: Effect of immunisation with paternal cells on recurent spontaneous abortion. In: Clark D. A., B. A. (eds), *Reproductive Immunology*, 1986.
11. Power, D. A., Mason, R. J., Stewart, K. N., Noble, D. N., MacLeod, A., Shewan, G., Catto, G. R. D.: B-lymphocyte antibodies associated with succesful pregnancy: evidence for MHC linkage. *Transplant. Proc.* 15, 890 (1983).
12. MacLeod, A. M., Mather, A., Hillis, A. M., Bone, J. M., Catto, G. R. D.: Effect of cyclosporin, previous third-party transfusion, and pregnancy on antibody development after donor-specific transfusion before renal transplantation. *Lancet i*, 416 (1987).
13. Joseph, S., Butler, L., Liao, S. K., Singal, D. P.: Antiidiotypic antibodies associated with successful pregnancy. *Transplant. Proc. 17*, 909 (1985).
14. Cohen, L., Sharp, S., Kulczycki, A.: Human monocytes, B-lymphocytes and non-B lymphocytes each have structurally unique Fc-receptors. *J. Immunol. 131*, 378 (1983).
15. Sandilands, G. P., Gray, K. G., Reid, F. M., Anderson, J. R.: Demonstration of Fc receptors on the surface of B lymphocytes. *Int. Arch. Allergy Appl. Immunol. 57*, 411 (1978).
16. Sándor, M., Füst, G., Medgyesi, A., Erdei, A., Gergely, J.: The hetergenity of Fc receptors on human pripheral mononuclear blood cells. *Immunology 38*, 553 (1979).
17. Sandilands, G. P., Moira, G. Peel, M. G., Froebel, K. S., Belch, J. F., Macsween, R. N. M.: Isolation of labile Fc-receptors from human peripheral blood lymphocytes and production of an antiserum. *Immunol. 55*, 7 (1985).
18. Forwel, M. A., Peel, M. G., Froebel, K. S., Belch, JJF., MacSween, RNM., Sandilands G. P.: Transfusion-induced Fc-receptor-blocking antibodies: Spectrum of cellular reactivity. *J. Clin. Lab. Immunol. 20*, 63 (1986).
19. Imbach, P., Barandun, S., d'Appuzo, V., Baumgartner, C., Hirt, A., Morell, A., Rossi, E., Schöni, M., Vest, M., Wagner, H. P.: High-dose intravenous gammaglobulin for idiopathic thrombopenic purpura in childhood. *Lancet ii*, 1128 (1981).
20. Jungi, T. W., Imbach, P., Barandun, S.: Specific and non-specific mechanisms of action of immunoglobulin G in therapy of ITP. *Blut 48*, 345 (1984).
21. Stohl, W.: Cellular mechanisms in the in vitro inhibition of pokeweed mitogen-induced B cell differentiation, *J. Immunol. 136*, 4407 (1986).
22. Panzer, S., Grümayer, E. R., Haas, O. A., Niessner, H., Graninger, W.: Efficacy of Rhesus antibodies (anti-Rh/D) in Autoimmune Thrombocytopenia: correlation with response to high-dose IgG and the degree of haemolysis. *Blut 52*, 117 (1986).
23. Berlin, G., Selbing, A., Ryden, G.: Rhesus haemolytic disease treated with high-dose intravenous immunoglobulin. *Lancet ii*, 1153 (1985).
24. Suzuki, S., Mizuochi, I., Sada, M., Amemiya, H.: Transplantation tolerance mediated by suppressor T cells and suppressive antibody in recipient of a renal transplant. *Transplantation 40*, 357 (1985).

Haematologia 22 (3), pp. 143–149 (1989)

Cytochemistry of Dipeptidylaminopeptidase IV in T Lymphoblastic Leukaemia — Lymphoma

R. INVERNIZZI*, O. PERUGINI, P. DE FAZIO, F. BONETTI, L. NESPOLI

Department of Internal Medicine, Section of Second Clinical Medicine and Department of Pediatrics, University of Pavia, Italy

(Received 2 December, 1987; accepted 15 June, 1988)

The cytochemical distribution of dipeptidylaminopeptidase IV (DAP IV) was studied in 5 cases of T lymphoblastic leukaemic lymphoma and 12 cases of acute T lymphoblastic leukaemia, in order to ascertain differences between the enzyme positivity patterns of T cells at different stages of differentiation. Early thymic phenotype cases were almost completely negative; those of intermediate and mature thymic phenotype showed positivity in various percentages of blasts: either a single coarse granule or many coarse and small granules were detected. In mature phenotype cells a particularly intense DAP IV reaction was observed. In conclusion, our findings suggest that DAP IV reaction could be a useful tool for the cytochemical characterization of T acute leukaemia subtypes.

Keywords: cytochemistry, dipeptidylaminopeptidase IV, T lymphoblasic leukaemia, T lymphoblastic lymphoma

Introduction

Several authors have suggested that, among lymphoid subpopulations, DAP IV (dipeptidylaminopeptidase IV, glycylproline-napthylamidase, E.C 3.4.14.4) is expressed mainly by the helper subset of T cells in a manner similar to that of alpha-naphthyl-acetate esterase [1 – 8].

However, only a few cytochemical studies have been done on DAP IV distribution in malignant T lymphoblasts.

In the present paper we report a cytochemical study of DAP IV activity in T lymphoblastic leukaemia-lymphoma cells, designed to discover relationships between cytochemical reactivity and membrane phenotype.

Materials and Methods

Seventeen patients, 5 females and 12 males, aged 4 to 49 years, with acute T lymphoid malignancies were studied. Five cases of T lymphoblastic leukaemic

* To whom correspondence should be addressed: Clinica Medica II, Policlinico S. Matteo, Pz. le Golgi, 27100 Pavia, Italy

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

lymphoma (T-LbLy) and 12 cases of acute T lymphoblastic leukaemia (T-ALL) were included.

We also studied 27 cases of common (c) ALL and 10 cases of acute non-lymphoid leukaemia (ANLL). Diagnosis was made on the basis of morphological, cytochemical and immunological criteria.

Immunologic studies were carried out on cell suspensions from bone marrow aspirates. The following tests were performed: E rosette test [9], detection of surface immunoglobulins [10], analysis by immunofluorescence using OKB-cALLa (CD10), OKT11 (CD2), OKT6 (CD1), OKT4 (CD4), OKT8 (CD8), OKT3 (CD3) (Ortho) and Leu 9 (CD7) (Becton-Dickinson) [11] monoclonal antibodies.

DAP IV activity was examined in bone marrow smears of leukaemic patients according to Lojda's method [12]. Preparations were fixed in formaldehyde vapours for 4 minutes and then incubated in the following medium: 4 mg glycyl-prolyl-4--methoxy-2-naphtyl-amide (Sigma) dissolved in 0.5 ml N,N-dimethylformamide and 10 mg Fast Garnet GBC Salt (Sigma) dissolved in 10 ml 0.1 M phosphate buffer pH 7.2. Incubation was carried out for 90 minutes at 37 °C. After incubation

Table 1

T lymphoblastic leukaemia-lymphoma patients: clinical and immunologic features

Case	Sex	Age	Diagnosis	WBC x10⁹/l	Blasts %	Medi-astinal mass	Immunologic Phenotype*						
							cALLa CD10	T11 CD2	T6 CD1	T4 CD4	T8 CD8	T3 CD3	LEU9 CD7
1	M	24	T-LbLy	7	35	−	+	+		+	−	−	
2	M	40	T-LbLy	3	48	+	−	+	−	+	−	+	
3	M	49	T-LbLy	9.9	24	+		+	−	+	+	+	
4	M	14	T-ALL	22	80	+		+		+	+	−	
5	M	39	T-ALL	18	60	−	−	+	−	−	−	−	+
6	F	7	T-ALL	225	100	+	−	+	−	−	−	−	+
7	F	25	T-ALL	15	28	−	−	+	+	+	+	−	+
8	M	13	T-ALL	5.5	41	−	−	+		−	+	−	+
9	M	22	T-ALL	67	100	+	−	+	−	+	−	+	+
10	M	4	T-LbLy	30	42	+	+	+	+	+	−	+	+
11	F	9	T-ALL	15.8	36	+	−	+	−	−	−	−	+
12	F	5	T-ALL	234	100	+	−	+	+	+	+	−	+
13	M	7	T-ALL	7.4	35	+	−	+	−	−	−	−	+
14	M	5	T-ALL	161	98	−	−	+		−	−	−	
15	F	7	T-ALL	21	76	+	−	+		+	+	−	+
16	M	8	T-LbLy	66	98	+	−	+		+	+	−	+
17	M	10	T-ALL	295	100	+	−	+	−	−	−	−	+

* + = over 25% of leukaemic cells reactive

the smears were washed in water and counterstained with Mayer's haematoxylin for 2 min.

Besides DAP IV reactivity, the following cytochemical reactions were studied in all bone marrow smears: peroxidase [13], acide phosphatase (AP) [14], alpha--naphtyl-acetate esterase (ANAE) at a pH 6.1 [15], N-acetyl-beta-glucosaminidase (NABG) [16].

Each enzyme activity was evaluated by determining the percentage of positive blasts.

Results

The results of clinical, immunological and cytochemical studies in T LbLy and ALL cases are reported in Tables 1 and 2. Six cases (numbers 5, 6, 11, 13, 14 and 17) showed an early thymic phenotype; 9 cases (1, 3, 4, 7, 8, 10, 12, 15 and 16) an intermediate thymic phenotype and 2 cases (2, 9) a mature thymic phenotype (Table 1). DAP IV reaction was completely negative in almost all the cases of early thymic phenotype, while the cases with intermediate and mature thymic phenotype showed DAP IV positivity in various percentage of blasts (m 44.09 ± 31.9): either a single coarse granule or many coarse and small granules, generally focally distributed were detected (Fig. 1). The highest percentages of positive blasts were observed in the $T4^+$ $T8^-$ cases, where a significant positive linear correlation was found between the percentage of OKT4 and DAP IV positive cells, while the blasts of the only $T4^-$ $T8^+$ case were completely DAP IV negative.

In T lymphoblasts no significant linear correlation between DAP IV positivity and the reactivity of acid phosphatase or N-A-β-Glucosaminidase was found (Table 2). The only significant linear correlation found was between DAP IV activity and the focal distribution of α-NAE (Fig. 2).

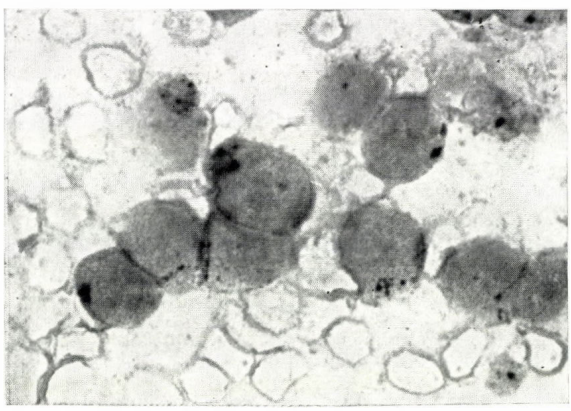


Fig. 1. DAP IV reactivity in T lymphoblasts: cells of case 15 (Table 1) are positive with a single coarse granule or many small granules, focally distributed

Table 2

T lymphoblastic leukaemia-lymphoma patients: results of cytochemical reactions

Case	Percentage of positive blasts			
	DAP IV	AP focal	ANAE focal	NABG focal
1	90	50	45	100
2	40	0	44	68
3	15	48	10	77
4	40	88	90	
5	0	73	0	64
6	0	46	18	89
7	25	60	30	
8	0	90	15	95
9	48	84	78	100
10	85	80	100	75
11	0	10	36	69
12	4	80	66	80
13	24	77	56	100
14	2	20	38	40
15	55	73	76	93
16	83	77	65	84
17	5	95	50	90
mean	30.3	48.0	61.8	81.6
±SD	32.1	28.6	28.8	16.7

All non-T cases of ALL and also the ANLL cases were almost completely DAP IV negative.

AP and ANAE reactions in non-T cases of ALL were either completely negative or showed just a weak fine granular positivity. Only very few blasts dis-

Table 3

Results of the cytochemical reactions in non T-ALL cases

	Percentage of positive blasts			
	DAP IV	AP	ANAE	NABG
mean	0.3	4.6	8.2	31.5
SD	1.1	12.7	13.8	23.6
range	0–5	0–65	0–53	6–73

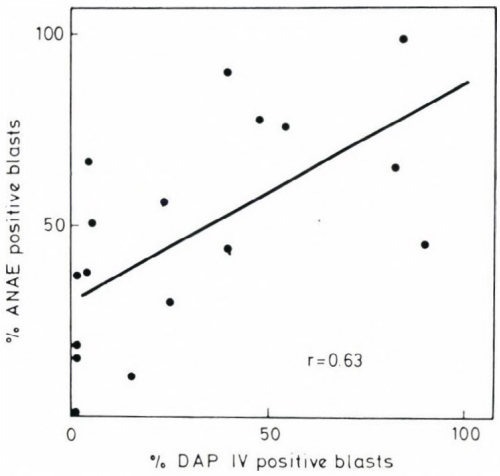


Fig. 2. Correlation between DAP IV positivity and focal reactivity of ANAE in T lympho blastic leukaemia lymphoma patients

played focal positivity. NABG was generally very strong, expressed by either a single coarse drop-like granule or multiple fine and coarse granules scattered within the cytoplasm (Table 3).

Discussion

Previous cytochemical studies reported by several authors led to the opinion that a strong DAP IV positivity is characteristic for both normal and neoplastic T helper lymphocytes [1 – 8]. Peripheral T helper cell malignancies, such as T-chronic lymphocytic leukaemia and T-prolymphocytic leukaemia were found to be strongly positive, whereas B-lymphoproliferative disorders were completely DAP IV negative [4, 5, 6, 8, 17].

Controversial results were found in the studied T-ALL cases (1, 6, 17, 18, 19). In fact, whereas non-T ALL cases were always almost completely negative, DAP IV reactivity was absent or expressed in a minority of T blasts in the few cases described by Wertmüller et al. [5], Crockard et al. [6] and Srivastava et al. [19], but present in many blasts from some T-ALL patients reported by Feller [18]. Furthermore, in leukaemic human cell lines no correlation was found between DAP IV activity and OKT4 positivity or maturation stage in T cells and DAP IV was seen also in B cells [19].

The results of our study have shown variable reactivity in lymphoblasts of T cell malignancies, too. We observed DAP IV activity only in lymphoblasts displaying either intermediate or mature thymic phenotype. Positivity was stronger at the more advanced stages of cell maturation. It is worth noting that 8 out of 10 $T4^+$ cases presented more than 25% DAP IV positive blasts, while the only

$T4^-$ $T8^+$ case was DAP IV negative. No distinguishing clinical features of DAP IV positive versus DAP IV negative T cases were observed.

Our data, in partial disagreement with the results of Crockard et al. [6] and Srivastava et al. [19] who, however, studied very few patients, indicate that DAP IV reaction could be a useful tool for the cytochemical characterization of a particular T acute leukaemia-lymphoma subtype. This reaction is therefore much less apt than AP reaction to recognize T cell malignancies, in particular the more immature cases, and its expression, peculiar to $T4^+$ cells only, seems to parallel that of aspecific esterase. It is, however, much more specific than other hydrolases reactions: in fact, with very few exceptions, this enzyme activity has only been found in T cells.

References

1. Chilosi, M., Pizzolo, G., Menestrina, F., Iannucci, A. M., Bonetti, F., Fiore-Donati, L.: Dipeptidyl(amino) peptidase IV(DAP IV) histochemistry on normal and pathologic lymphoid tissues. *Am. J. Clin. Pathol. 77*, 714 (1982).
2. Feller, A. C., Heijnen, C. J., Ballieux, R. E., Parwaresch, M. R.: Enzymehistochemical staining of T lymphocytes for glycyl-proline 4-Methoxy-betanaphthylamide-peptidase (DAP IV). *Br. J. Haematol. 51*, 227 (1982).
3. Feller, A. C., Parwaresch, M. R., Bartels, H., Lennert, K.: Enzymecytochemical heterogeneity of human chronic T-lymphocytic leukemia as demonstrated by reactivity to dipeptidylaminopeptidase IV (DAP IV; EC 3.4.14.4). *Leuk. Res. 6*, 801 (1982).
4. Feller, A. C., Parwaresch, M. R., Lennert, K.: Subtyping of chronic lymphocytic leukemia of T-type by dipeptidylaminopeptidase IV (DAP IV), monoclonal antibodies and Fc-receptors. *Cancer 52*, 1609 (1983).
5. Wirthmuller, R., Dennig, D., Oertel, J., Gerhartz, H.: Dipeptidylaminopeptidase IV (DAP IV) activity in normal and malignant T-cell subsets as defined by monoclonal antibodies. *Scand. J. Haematol. 31*, 197 (1983).
6. Crockard, A., McFarlane, E., Andrews, C., Bridges, J. M., Catovsky, D.: Dipeptidylaminopeptidase IV activity in normal and leukemic T-cell subpopulations. *Am. J. Clin. Pathol. 82*, 294 (1984).
7. Hirt, A., Auner, H. L., Wagner, H. P.: Cytochemistry of human T lymphocyte subpopulations in peripheral blood. *Scand. J. Haematol. 32*, 190 (1984).
8. Invernizzi, R., Bertolino, C., Girino, M., Perseghin, P., Michienzi, M., Nano, R.: Cytochemistry of dipeptidylaminopeptidase IV and II in normal and neoplastic lymphoid cells. *Blut 50*, 277 (1985).
9. Jondal, M., Holm, G., Wigzell, H.: Surface markers on human T and B lymphocytes. *J. Exp. Med. 136*, 207 (1972).
10. Seligmann, M., Preud'Homme, J. L., Brouet, J. C.: B and T cell markers in human proliferative blood diseases and primary immunodeficiencies, with special reference to membrane bound immunoglobulins. *Transplant. Rev. 16*, 85 (1973).
11. Reinherz, E. L., Kung, P. C., Goldstein, G., Schlossman, S. F.: Separation of a functional subset of human T cell by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA 76*, 4061 (1979).
12. Lojda, Z.: Studies on Glycyl-proline naphthylamidase. I Lymphocytes. *Histochemistry 54*, 299 (1977).
13. Graham, R. C., Karnovsky, J. M.: The early stages of absorption of injected horse-radish peroxidase in the proximal tubules of mouse kidney; ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem. 14*, 2191 (1966).

14. Li, C. I., Yam, L. T., Lam, K. W.: Acid phosphatase isoenzyme in human leukocytes in normal and pathologic conditions. *J. Histochem. Cytochem. 18*, 473 (1970).
15. Yam, L. T., Li, C. I., Crosby, W. H.: Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Pathol. 55*, 283 (1971).
16. Hayashi, H.: Histochemical demonstration of of N-acetyl-beta-glucosaminidase employing naphthol-AS-BI-N-acetyl-beta-glucosaminide as substrate. *J. Histochem. Cytochem. 13*, 355 (1965).
17. Khalaf, M. R., Bevan, P. C., Hayhoe, F. G. J.: Comparative cytochemical study of dipeptidyl aminopeptidase (DAP) II and IV in normal and malignant haemic cells. *J. Clin. Pathol. 39*, 891 (1986).
18. Feller, A. C., Parwaresch M. R.: Zytochemische Darstellung der Blasten bei akuter T-lymphoblastischer Leukämie mit Hilfe der Glycyl-prolyl-4-methoxy-beta-naphthylamid-Peptidase (DAP IV). *Klin. Pädiatr. 192*, 150 (1980).
19. Srivastava, M. D., Bhargava, A. K.: Expression of dipeptidylaminopeptidase IV in some human T and B cell lines. *Acta Haemat. 76*, 25 (1986).

Haematologia 22 (3), pp. 151–159 (1989)

IgE Levels in Normal Human Sera and IgG Preparations

K. Merétey[1], Á. Jakab[2], K. Szilassy[3], G. A. Medgyesi[3]

[1] National Institute of Rheumatology and Physiotherapy Budapest, Frankel Leó út 17–19, H-1027 Budapest, Hungary
[2] National Institute of Pulmonology, Budapest, Hungary
[3] National Institute of Haematology and Blood Transfusion, Budapest, Hungary

(Received 18 March, 1988; accepted 28 June, 1988)

Serum IgE levels were determined in 318 Hungarian blood donors by PRIST technique. Age-dependent decrease of IgE and higher values in males than in females were found. Commercial IgG preparations contained variable amounts of IgE related to the procedures for purification. The normal IgE level in healthy Hungarian adults were compared to the reported normal values of other countries.

Keywords: blood donors, IgE, IgG, normal level, serum

Introduction

Since the discovery of IgE, many studies have been performed to investigate the IgE serum levels in human populations with different genetic and environmental backgrounds [1, 2, 3]. When comparing different populations, the problems of methodology also need to be considered. Early data obtained by RIA are not comparable with the more recent ones, which have been found with the PRIST or ELISA methods. [4, 5, 6].

In our study we measured the IgE levels in Hungarian blood donors of different ages, which means that we tested a sample of the adult Hungarian urban population. Our results appear to support earlier data on a higher average IgE level in the Central European area compared to North America or Scandinavia. The question is whether high IgE levels in donor blood or plasma play a role in the safety of haemotherapy.

Materials and Methods

Blood samples were taken from 318 Hungarian blood donors living and working in Budapest, in different districts. The sera were stored frozen until use. To achieve an even sex and age distribution, the sera of 164 males and 154 females between the age of 18–60 years were collected. Eight age groups were formed and in each 20–20 samples were tested. The blood donors were found healthy according to the medical examination and tests performed before blood donation. The sampling period lasted from November till March to avoid the influence of the pollen season.

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

IgG preparations were collected from different sources. The available intravenous gamma globulin preparations were obtained from the respective manufacturers (Venoglobulin, Merieux, Lyon; Intraglobin and Pentaglobin, Biotest, Frankfurt; Endobulin, Immuno, Vienna; Sandoglobulin, Sandoz, Bern; Institute HUMAN, Budapest, National Institute of Haematology and Blood Transfusion (NIHBT), Budapest) an intramuscular gamma globulin of Institute HUMAN, Budapest and an instramuscular IgG of the NIHBT prepared for anti-D-prophylaxis were also included in the study.

The IgE levels were determined by the IgE PRIST test (Pharmacia), and the values were expressed as kU/l (1kU = 2,4 μg). The values obtained were transformed into logarithms and statistical analysis was performed afterwards.

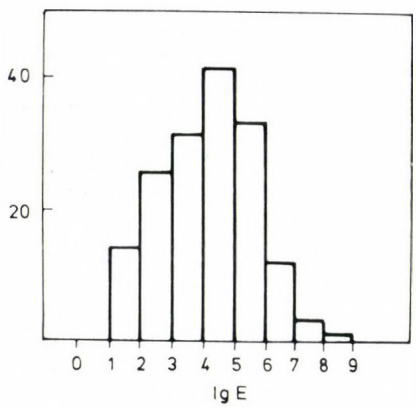


Fig. 1. Distribution of the IgE serum levels in adult males. The IgE values were transformed into logarithms and the frequency distribution of the different categories is shown. Categories: 0–1 = below 2 kU/l; 1–2 = 3–5 kU/l; 2–3 = 6–15 kU/l; 3–4 = 16–40 kU/l; 4–5 = 41–100 kU/l; 5–6 = 101–250 kU/l; 6–7 = 251–700 kU/l; 7–8 = 701–1800 kU/l; 8–9 = 1801–4000 kU/l

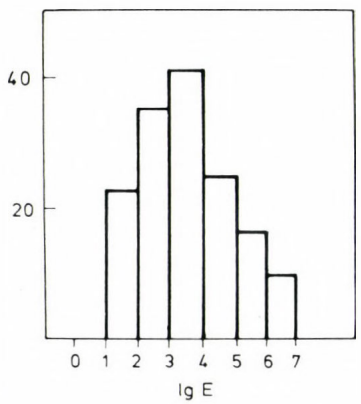


Fig. 2. IgE levels in female blood donors. For other explanations see Fig. 1

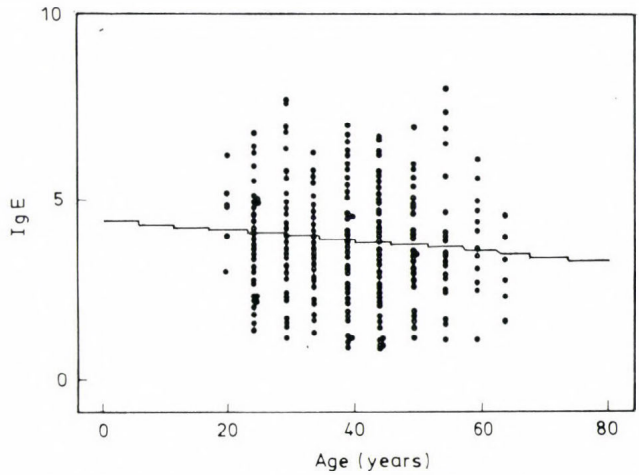


Fig. 3. Age dependent changes of IgE serum values. The logarithms of the IgE values (ordinate) are plotted against the age of the donors (abscisse: ages are grouped by 5 years). The numbers show the cases of identical values. The slope showed a statistical significance $p = 0.05$

After establishing the individual IgE values a questionnaire on family- and personal history of possible allergic problems and smoking habits was sent to those with high IgE levels.

Results

IgE levels in males and females

The IgE levels were evaluated separately in males and females. Figure 1 and 2 demonstrate that in males the IgE levels are higher than in females. The maximum was shifted to the right by one category and the highest values were found in males.

Age related changes of IgE levels

The IgE levels were plotted against the age of the persons studied. Figure 3 shows that the IgE levels decrease with aging. The slope of the regression line is at the borderline of significance.

The background of the high IgE levels

A questionnaire was sent to the 23 persons with IgE levels higher than 499 kU/l (Table 1). The answers were evaluated according to the personal features possibly related to IgE production and the allergic disease within the first degree relatives. Four women had some allergic problems while among the male patients there were 4 without any possibly "allergic" complaints. Twelve of the persons with high IgE were regular smokers. However, there were 2 males without any of the known causes or predisposing circumstances of high IgE (SP, DI).

Table 1

Background of high IgE levels

Name	Sex	Allergy	Family history of allergy	Smoking	IgE kU/l
CsK	F	urticaria	+	+	900
NL	F	drug allergy	?	+	900
RA	F	pollen sens.	?	+	900
DP	F	pruritus	−	+	500
SP	M	−	−	−	3000
DI	M	−	−	−	1000
PM	M	−	−	+	1500
GyL.	M	−	−	++	890
BI	M	−	−	+	540
MS	M	drug allergy	−	++	1100
ZT	M	urticaria	−	++	800
PI	M	rhinitis	−	++	800
LGy	M	penicilline	+	−	660
KL	M	fuel oil	+	+	2300
KE	M	pollen sens.	+	++	600

Table 2

IgE content of commercial IgG preparations

Source	Batch No	Method of purification	Protein Content (%)	IgE kU/l	IgE/IgG kU/g
Venoglobulin	V		5	120	2.4
Venoglobulin	V		5	76	1.5
Intraglobin			5	200	4.0
Endobulin			5	160	3.2
Pentaglobin	462086		5	10	0.2
Sandoglobulin	S2	pH4 + trypsine	3	80	0.2
Sandoglobulin	S4	pH4 + trypsine	3	74	2.5
HUMAN					
vénagamma	107	ethanol fract.	6	400	6.7
vénagamma	109	ethanol fract.	6	430	7.2
HUMAN gamma globulin		ethanol fract.	16	1600	10.0
NIHBT D IgG	018701	ethanol fract.	4.8	100	2.1
	028301	ethanol fract.	5.4	150	2.8
	038701	ethanol fract.	5.4	115	2.2
NIHBT i.v. gamma globulin		Chromatography	7.5	11	0.2
NIHBT i.v. gamma globulin		Chromatography	7.5	20	0.4

IgE levels in the IgG preparations

We have measured the IgE amount in the commercially available IgG preparations (Table 2). The IgE levels varied with the source of IgG and the batches, too. The preparations isolated by different techniques showed marked differences. The highest value was found in the preparation of HUMAN (Budapest) prepared for intramuscular use. The lowest values were measured in the Pentaglobin of Biotest, declared a specially IgM rich preparation. The chromatographically purified IgG seems to be free of the IgE.

Discussion

The normal serum IgE level is subject to several influences. Several studies have confirmed that the serum IgE levels show a bimodal distribution suggesting that the population is not homogenous for the IgE producing capacity [2, 7, 8]. Most of the studies are concordant with the two allelic genetic background of IgE levels, where the gene responsible for the low IgE level is dominant [9].

Apparently differing IgE levels may stem from variations in the method used for IgE determination [4, 6, 10, 11]. Higher values have been obtained with the Phadebas IgE test widely used in the last decade than with the PRIST technique. Therefore, in earlier publications, the normal limits of IgE levels were higher than in the recent ones (Table 3).

Indeed, in some studies using older techniques the normal values are much higher than ours. In some of the published results the sex ratio was not taken into consideration and this might cause further variations.

The different environmental influences can also modify the mean values of normal IgE levels. Smokers have higher IgE levels than nonsmokers [12, 13]. The exhaust dust from Diesel oil increases the IgE levels in animals [14]. We have no data on human studies. The pollution of the living area could increase the IgE levels in children [15]. The difference in IgE levels between NorthAmerica, Western Europe on the one hand and Hungary (and probably the neighbouring countries) on the other hand may result either from a different genetic background, from certain environmental circumstances, or both. Similarly, higher normal IgE serum levels were reported from Italy than from Sweden [16]. The Budapest area, where the donor samples were taken, can be suspected to provide stimuli for the enhancement of IgE. The finding of relatively high IgE content in gamma globulin preparations manufactured in Hungary, however, may suggest that the incidence of high IgE levels in blood donors is not restricted to the capital as the plasma source is collected in the whole country.

We attempted to elucidate the background of the high IgE level in the respective individuals by the use of questionnaires. Nobody appeared to be infected by parazites, but some donors were possibly atopic. None of them was taking antihistamines showing that among the blood donors involved there was no severe case.

Table 3

Comparison of data from different countries, measured by different techniques

Country	Reference no.	No. of cases	Range of age	Geometric. mean
Africa	23	39	adults	149*
USA	22	73	adults	105^{+}
USA	23	95	adults	179^{+}
USA	24	102	adults	103***
USA	25	106	2—55	55
USA	27	2743	6—80	32.1*
		249	6—14	79
		472	15—24	53.1
		393	25—34	36.4
		236	35—44	34.1
		333	45—54	28.2
		400	55—64	21.6
USA	26	5	19—24	19—49*
USA	28	12	adults	60*
USA	29	330	3—60	<20 = 60%, <40 = 20%* <80 = 10%, >80 = 10%
Canada	3	110 nsm	20—65 f	11.8*
		185 sm	20—65 f	23.6
		62 nsm	20—65 m	12.6
		301 sm	20—65 m	27.9
Sweden	37	175	adults	14*
Sweden	4	19	14	20*
Spain	15	245	0—12	14**
Belgium	30	27	adults	21**
Germany	2	355	11—70	55*
Germany	1	30	20—40	42*
Austria	31	43	<65	32**
Austria	32	100	50% >50	73**
Czechoslovakia	33	30	22—58	220*
Hungary	34	22	11—52	245*
Hungary	35	26	adults	265*
Hungary	36	25	adults	54*
Our data		164 m	18—60	64*
		154 f	18—60	34*

nsm = nonsmoker, sm = smoker, f = female, m = male, IgE measured by: $^{+}$ = Phadebas IgE test; $^{++}$ = ELISA; * = IgE PRIST; ** = PACIA; *** = RIA

The IgE values in the IgG preparations show that IgE is usually a trace component. Tovo et al. [17] suggested that the IgE present in the IgG given intravenously can be the cause of some side effects. Considering that the IgG preparations are produced from large pools of plasma, the presence of natural anti- IgE antibodies is likely [18, 19]. These latter can form complexes after injection capable of enhancing the known biological effects. In a recent study Paganelli et al. [19] presented data suggesting that IgE plus anti-IgE content of intravenous IgG preparations might be responsible for some minor side effects, although no evidence of immediate-type reactivity was found.

When whole blood transfusion is given, a high IgE level in the donor blood may imply the infusion of leukocytes carrying high numbers of adsorbed IgE molecules. A possible reaction of such cell-bound IgE with anti-IgE factors may result in symptoms of intolerance. Transfusion of leukocyte-depleted blood can solve this problem.

The method of purification of IgG seems to be very important in view of the IgE content. The fractionation by ethanol seems to cofractionate most of the IgE. On the other hand, the IgE type hypersensitivity to allergens and drugs can be transferred not only by donor bone marrow cells to recipients [20], but, temporarily, by preformed IgE type antibodies, too. This can cause side-effects not predicted by any recorded characteristic of the recipients. As a conclusion, we draw attention to the fact that apparently healthy blood donors may carry high circulating IgE levels, therefore a more through evaluation of allergic symptoms in the donor's anamnesis appears advisable. Procedures eliminating major portions of plasma IgE the final product may promote avoiding possible side effects due to IgE.

Acknowledgements

We would like to thank Mayer Lászlóné for her skillful technical assistance and Enikő Kudari for her secretarial help.

References

1. Lamerz, R., Fateh-Moghadam, A.: Immunglobulin E. *Klin. Wschr. 52*, 1 (1974).
2. Barsoum, A. L., Kuwert, E. K.: Circulating IgE Levels in a Normal Human Population. *Z. Immun.-Forsch. 152*, 388 (1977).
3. Holford-Strevens, V., Warren, P., Wong, C., Manfreda, J.: Serum total immunoglobulin E levels in Canadian adults. *J. All. Clin. Immunol. 73*, 516 (1984).
4. Kjellman, N-I. M., Johansson, S. G. O., Roth, A.: Serum IgE levels in healthy children quantified by a sandwich technique (PRIST). *Clin. Allergy 6*, 51 (1976).
5. Tomcsányi, A., Fekete, B., Schweiger, O., Kertész, L.: Unterschiede für Immunglobulin E im Serum, bestimmt mit kompetitivem bzw. Papier-Radioimmunosorbenttest: Beseitigung durch Vorbehandlung mit Polyethylenglycol. *J. Clin. Chem. Clin. Biochem. 18*, 145 (1980).
6. Solling, H., Dinesen, B.: The development of a rapid ELISA for IgE utilizing commercially available reagents. *Clin. Chim. Acta 130*, 71 (1983).

7. Marsh, D. G., Goodfriend, L., Bias, W. B.: Basal Serum IgE Levels and HLA Antigen Frequencies in Allergic Subjects. I. Studies with Ragweed Allergen Ra3. *Immunogenetics 5*, 217 (1977).
8. Marsh, D. G., Bias, W. B.: Basal Serum IgE Levels and HLA Antigen Frequencies in Allergic Subjects. II. Studies in People Sensitive to Rye Grass Group I and Ragweed Antigen E and of Postulated Immune Response (Ir) Loci in the HLA Region. *Immunogenetics 5*, 235 (1977).
9. Lebowitz, M. D., Barbee, R.: Family Concordance of IgE, Atopy, and Disease. *J. All. Clin. Immunol. 73*, 259 (1984).
10. Johansson, S. G. O., Berglund, A., Kjellman, N.-I. M.: Comparison of IgE values as determined by different solid phase radioimmunoassay methods. *Clin. All. 6*, 91 (1976).
11. Ormonroyd, P., Robertshaw, D.: A comparison of the Phadebas and Phadezym IgE paper immunosorbent test kits. *Clin. Allergy 13*, 51–55 (1983).
12. Gerrard, J. W., Heiner, D. C., Mink, J., Meyers, A., Dosman, J. A.: IgE levels in smokers and nonsmokers. *Ann. Allergy 44*, 261 (1980).
13. Warren, C. P. W., Holford-Stevens, V., Wong, C., Manfreda, J.: The relationship between smoking and total immunoglobulin E levels. *J. All. Clin. Immunol. 67*, 370 (1982).
14. Muranaka, M., Suzuki, S., Koizumi, K., Takafuji, S., Miyamoto, T., Ikemori, R., Tokiwa, H.: Adjuvant activity of diesel-exhaust particulates for the production. *J. All. Clin. Immunol. 77*, 616 (1986).
15. Berciano, F. A., Crespo, M., Bao, C. G., Alvarez, F. V.: Serum levels of total IgE in non-allergic children. *Allergy 42*, 276 (1987).
16. D'Amelio R., Fattorossi A., Berti R., Rossi P., Paganelli R., Castagliuolo P. P.: Serum, IgG, IgA, IgE salivary IgA levels and lung function in a healthy male population from the Italian air force: a preliminary study. *Ann. Allergy 53*, 432 (1984).
17. Tovo, P-A., Gabiano, C.,Roncarolo, M. G., Altare, F.: IgE Content of Commercial Intravenous IgG Preparations. *Lancet 1*, 458 (1984).
18. Inganas, M., Johansson, S. G. O., Bennich, H.: Anti-IgE Antibodies in Human Serum: Occurrence and Specificity. *Int. Arch. All. App. Immunol. 65*, 51 (1981).
19. Paganelli, R., Quinti, I., D'Offizi, G. P., Papetti, C., Cabello, A., Aiuti, F.: A Study of IgE in Immunoglobulin Preparations for Intravenous Administration. *Vox Sang. 51*, 87 (1986).
20. Walker, S. A., Riches, P. G., Wild, G., Ward, A. M., Shaw, P. J., Desai, S., Hobbs, J. R.: Total and allergen-specific IgE in relation to allergic response pattern following bone marrow transplantation. *Clin. Exp. Immunol. 66*, 633 (1986).
21. Zwingenberger, K., Feldmeier, H., Stevens, W. J. ,Steiner, A.: Antibodies of the IgE and IgG isotype, serum IgE and circulating immune complexes in schistosomiasis intercalatum. *Parasitol. Res. 73*, 259 (1987).
22. Polmar, S. H., Wladmann, T. A., Balestra, S. T., Jost, M. C., Terrz, W. D.: Immunoglobulin E in Immunologic Deficiency Diseases. *J. Clin. Invest. 51*, 326 (1972).
23. Hunder, G. G., Gleich, G. J.: Immunoglobulin E (IgE) Levels in Serum and Synovial Fluid in Rheumatoid Arthritis. *Arthr. Rheum. 17*, 955 (1974).
24. Thomas, M. R., Steinberg, P., Votaw, M. L., Bayne, N. K.: IgE levels in Hodgkin's disease. *Ann. Allergy 37*, 416 (1976).
25. Buckley, R. H., Becker, W. G.: Abnormalities in the Regulation of Human IgE Synthesis. *Immunol. Rev. 41*, 288 (1978).
26. Kraemer, et al.: In Vitro Studies of the Hyper-IgE Disorders: Suppression of Spontaneous IgE Synthesis by Allogenic Suppressor T Lymphocytes. *Clin. Imm. Immunopathol. 25*, 157 (1982).
27. Barbee, R. A., Halonen, M., Lebowitz M., Burrows, B.: Distribution of IgE in a community sample: correlation with age, sex and allergen skin test. *J. All. Clin. Immunol. 68*, 106 (1981).
28. Rebhun, J., Quismorio, F., Dubois, E., Heiner, D.: Systemic lupus erythematosus activity and IgE. *Ann. All. 50*, 34 (1983).

29. Grater, W. C., Pavuk, J., Budd, C.: Value of Immunoglobulin E (IgE) in the Private Practice of Allergy. Eight years experience: 1973–1981. *Ann. Allergy 50*, 317 (1983).
30. Sindic, C. J. M., Magnusson, C. G. M., Laterre, E. C., Masson, P. L.: IgE in the Cerebrospinal Fluid. *J. Neuroimmunol. 6*, 319 (1984).
31. Tatra, G., Lasnik, E.: Circulating IgE Levels in Patients with Gynecological Tumors. *Klin. Wochenschr. 63*, 699 (1985).
32. Vinzenz, K., Pavelka, R., Schönthal, E., Zekert, F.: Serum Immunoglobulin Levels in Patients with Head Neck Cancer (IgE, IgA, IgM, IgG). *Oncology 43*, 316 (1986).
33. Hrncir, Z., Tichy, M., Simsa, J., Salavec, M., Vavrine, J.: Levels of Immunoglobulin E (IgE) in Paired Examinations of Serum and Synovial Fluid in Rheumatoid Arthritis and Reactive Synovitis of Local Origin. *Clinica Chimica Acta 81*, 293 (1977).
34. Gyeney L., Böhm U., Merétey K.: A few characteristics of the immune status of patients before tonsillectomy. *Fül-orr-gégegyógy. 23*, 37 (1977) (In Hungarian).
35. Mészáros Cs., Debreczeni M., Mahunka Iné.: The testing of total IgE level in skin diseases. *Bőrgyógy. Venerol. Szle. 61*, 1 (1985) (In Hungarian).
36. Mikecz, K., Sonkoly, I., Mészáros, Cs., Szegedi, Gy.: Serum IgE Level in Systemic Lupus Erythematosus. *Acta Med. Hung. 42(1–2)*, 59 (1985).

Haematologia 22 (3), pp. 161–167 (1989)

Relationship Between Adenosine Deaminase Activity (ADA) and Inosinemonophosphate (IMP) Accumulation in Fresh Erythrocytes Incubated with Adenosine and Dipyridamole

M. KOPFF, I. ZAKRZEWSKA, J. KLEM, M. STRZELCZYK

Department of Biochemistry, Academy of Medicine,
90-647 Lodz, Plac 9-Maja 1, Poland

(Received 16 November, 1987; accepted 20 June, 1988)

Fresh human erythrocytes were incubated in two media: a) adenosine (10 mM), pyruvate (10mM), phosphate (50 mM) (APP medium); b) APP medium enriched with 100 μmol/l dipyridamole (APPD) medium. The amount of IMP in fresh erythrocytes was 0.18 ± 0.09 μmol/g Hb, after incubation in APP medium it was 1.52 ± 0.78 μmol/g Hb, and after incubation in APP medium it was 1.52 ± 0.78 μmol/g Hb, and after incubation in APPD the amount was 5.28 ± 0.94 μmol/g Hb.

ADA activity was measured simultaneously. The mean activity ($\pm$SD) of ADA fresh red cells was 1.29 ± 0.36 U/g Hb, after 2 h incubation in APP medium it was 1.71 ± 0.38 U/g Hb, and after 2 h incubation in APPD medium an activity of 2.68 ± 0.95 U/g Hb was found. A highly significant correlation between the accumulation of IMP and the activity of ADA in fresh erythrocytes ($r = 0.93$; $p = < 0.001$) and in erythrocytes incubated in APPD medium ($r = 0.97$; $p = < 0.001$) was found.

Keywords: adenosine deaminase activity, inosinemonophosphate accumulation, fresh erythrocytes

Introduction

Adenosine deaminase (ADA); adenosine aminohydrolase (EC 3.5.4.4) are widely distributed enzymes which catalyze the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine respectively. This enzyme plays a key role in the normal catabolic pathway of adenine nucleotides and in the production of hypoxanthine in the purine salvage pathway. Clinical interest in this enzyme has been revived by the discovery of a syndrome of severe humoral and cellular immunodeficiency associated with deficiency of ADA in erythrocytes [5].

Dipyridamole; a pyrimido-pyrimidine (2,6-bis [diethanolamino]-4,8-dipiperidino-pyrimido-(5,4-d)-pyrimidine; persantin) vasoactive substance is a well known inhibitor of nucleoside uptake in various animal cells [8, 10, 17]. Persantin first of all inhibits the uptake of adenosine into red blood cells [2]. Inhibition of adenosine deaminase is well recognized, too [3].

Already previously we found a 50% inhibition of 8^{14}C-adenosine penetration into red blood cells in the presence of dipyridamole in APPD medium (containing adenosine pyruvate and inorganic phosphate) [9]. The incorporation of 8^{14}C-ade-

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

nosine into nucleotides of erythrocytes by this drug was 18% [9]. Dipyridamole' however, did not inhibit the synthesis of IMP, but even greatly increased it, which has remained unexplained so far.

The aim of the present work was a) to examine the effect of a two hour incubation of fresh erythrocytes in APP medium enriched with 100 μmol/l dipyridamole on ADA activity and IMP accumulation; b) to study the relationship between the two events.

Materials and Methods

Fresh human blood was collected from healthy donors into heparinized glass bottles. Erythrocytes were washed in an excess of 0.9% saline solution and treated with perchloric acid for removing soluble phosphate compounds [14]. Incubation of erythrocytes was performed at 37 °C in (1) APP medium, i.e. a solution containing 10 mmol/l adenosine, 10 mmol/l pyruvate, 50 mmol/l inorganic phosphate and 75 mmol/l NaCl, or (2) APPD medium, i.e. APP medium containg 100 μmol/l dipyridamole. The pH of the two media was adjusted to 7.35 and they were added to the washed erythrocytes to obtain a haematocrit of about 20%. After a two hour incubation, samples of erythrocytes were removed and rapidly chilled in an ice-bath. The cells were washed three times with an excess of cold 0.9% NaCl. Acid soluble phosphate compounds were extracted in the elsewhere described manner [14]. Separation of phosphate compounds was achieved by means of column ion-exchange chromatography with Dowex-1, in the formate form [13]. Hypoxanthine and adenine nucleotides in eluates were determined spectrophotometrically [20]. Identification of IMP was performed as described previously [20, 21]. The results were expressed in μmol nucleotide/g Hb.

Before and after incubation of erythrocytes in APP and APPD media, activity of adenosine deaminase in lysates was measured by modified Hopkinson's method [7]. The enzyme activity was assayed kinetically by a coupled enzymatic system, in which adenosine is converted into inosine with uric acid as the final product by the action of nucleoside phosphorylase and xanthine oxidase. The reaction mixture had a total volume of 3.06 ml and contained 1.0 mmol/l of adeno-

Table 1

Comparison of ADA activities in control — fresh erythrocytes and after incubation in APP and APPD media

	ADA activity U/g Hb at 37 °C mean +− SD	n	p
Control (fresh RBC)	1.29+−0.36	17	
Incubation in APP medium	1.71+−0.38	17	<0.001
Incubation in APPD medium	2.68+−0.95	17	<0.001

Table 2

Comparison of IMP accumulation in control erythrocytes and after incubation in APP and APPD media

	Content of IMP μmol/g Hb	n	p
Control (fresh RBC)	0.18+−0.09	17	
Incubation in APP medium	1.52+−0.78	17	<0.05
Incubation in APPD medium	5.28+−0.94	17	<0.001

sine, 0.04 U of xanthine oxidase (from cow milk, in 3.2 mol/l ammonium sulfate, Boehringer Mannheim GmbH), 0.1 mol/l of Na-phosphate buffer pH 7.5 and 50 μl lysate of erythrocytes. Formation of uric acid was monitored at 293 nm and 37 °C. A unit of ADA activity was defined as the enzyme activity that catalysed the conversion of 1 μmol of adenosine to uric acid in 1 min at 37 °C ($\varepsilon = 12.5 \times 10^3$ liter/mol × cm).

For statistical evaluation of the results, means and standard deviations were calculated. The coefficient correlations were tested for significance with the Student's *t*-test. Only $p < 0.05$ was regarded to be significant.

Results

In fresh human erythrocytes 0.18±0.09 μmol/g Hb of IMP was found. After a two hour incubation of erythrocytes in APP medium, the contents of IMP increased to 1.52±0.78 μmol/g Hb. The incubation of erythrocytes in APPD medium resulted in further increase of IMP to 5.30±0.94 μmol/g Hb.

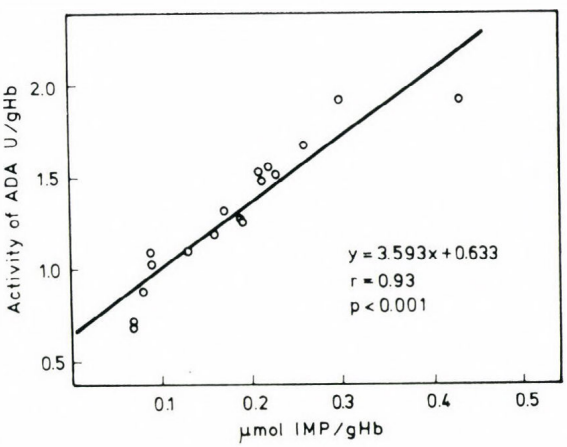


Fig. 1. Relationship between accumulation of IMP (μmol/g Hb) and ADA activity (U/g Hb) in fresh erythrocytes

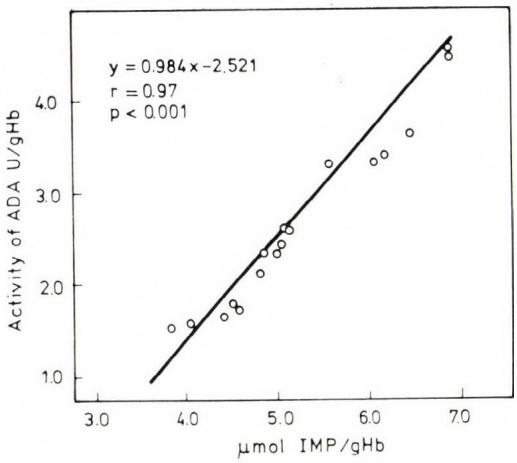


Fig. 2. Relationship between accumulation of IMP (μmol/g Hb) and ADA activity (U/g Hb) in erythrocytes after 2 hours of incubation in APPD medium

Activity of ADA in fresh erythrocytes was 1.29 ± 0.36 U/g Hb at 37 °C, after a 2 hour incubation of erythrocytes in APP medium it increased to 1.71 ± 0.38 U/g Hb, after 2 hours incubation in APPD a further increase to 2.68 ± 0.95 U/g Hb was observed. The results are summarized in Tables 1 and 2.

A highly significant correlation ($r = 0.93$; $p < 0.001$) between the activity of ADA and accumulation of IMP in fresh human erythrocytes (Fig. 1) and correlation ($r = 0.97$; $p < 0.001$) between the activity of ADA and accumulation of IMP in erythrocytes incubated in APPD medium (Fig. 2) were found.

Discussion

Purine bases and their nucleosides easily penetrate the red cell membrane [18]. However, in view of the fact that adenosine passes across the cell membrane quickly, its intracellular concentration is regulated by the enzymes which metabolize it [11, 12]. Inside the red cell adenosine was subjected to two processes:

a) phosphorylation which leads to the formation of AMP and later ADP and ATP, and

b) deamination to inosine [8].

The inosine thus formed may undergo direct phosphorylation to IMP. According to Banaschak [1] this is a side pathway, and only a small amount of inosine undergoes such a transformation. The main pathway of inosine transformation is probably its phosphorolytic cleavage to hypoxanthine and ribosephosphate. Under the influence of hypoxanthine-guanine phosphoribosyltransferase (HGPRT EC 2.4.2.8) and with a contribution 5-phosphoribosyl-1-pyrophosphate (PRPP) hypoxanthine becomes transformed into IMP [21, 22].

According to Parks et al. [16] the key factor deciding whether adenosine will undergo phosphorylation or deamination is the relative value of Km of adenosine for two enzymes: adenosine kinase (EC 2.7.1.20) and adenosine deaminase (EC 3.5.4.4).

We have shown that dipyridamole inhibits about 50% of 8^{-14} C-adenosine penetration into red cells incubated in APPD medium [9]. Bretschneider et al. [2] have shown that in the case of blood platelets the inhibition of penetration is higher and reaches ca 80%. Authors generally agree that dipyridamole inhibits the adenosine penetration to erythrocytes and reduces the incorporation 8^{-14} C-adenosine into nucleotides and synthesis of phosphate compounds. Zachara suggests [25] that the main cause of the slower rate of phosphorylated compound synthesis in erythrocytes incubated in APPD medium is the insufficiency of Pi inside the red cells. This is because dipyridamol considerably reduces the penetration of that ion through red cell membranes [6, 19, 25].

The reduction of nucleotide synthesis did not affect IMP. The quantity of IMP in the APPD medium incubated erythrocytes was 3.5 times higher than in those incubated in APP medium, i.e. 5.30 μmol/g Hb and 1.52 μmol/g Hb, respectively. This increase of IMP synthesis is surprising and difficult to explain. IMP may be generated by AMP deaminase (EC 3.5.4.6) from AMP or from inosine formed of adenosine by adenosine deaminase.

In our present investigation we studied the influence of incubation of fresh human erythrocytes in APP and APP with dipyridamole media on the activity of adenosine deaminase. During incubation of erythrocytes in APP medium a statistically significant (31%) ADA activity increase was found ($p < 0.001$). Incubation of erythrocytes in APPD medium caused still greater ADA activity (increase – about 93%), also statistically significant ($p < 0.001$). So dipyridamole does not inhibit ADA activity under these conditions, but stimulates it, and this increased activity of ADA may be responsible for the increased IMP synthesis. Dipyridamole is a competitive inhibitor of ADA, and at a concentration of 100 μmol/l can be displaced from the active centre by adenosine, which is in excess in APPD medium 10 mmol/l.

Bunag et al. [3] proved that persantin only inhibitits the action of adenosine deaminase in whole human blood, but not in haemolysates. This is also true for incubating media containing an excess of adenosine. Correlation was found between the accumulation of IMP and the activity of ADA in fresh human erythrocytes ($n = 17$) ($r = 0.93$; $p < 0.001$) and in erythrocytes incubated in APPD medium ($n = 17$) ($r = 0.97$; $p < 0.001$). This correlation suggests that adenosine deaminase is involved in the increase of IMP accumulation in erythrocytes incubated in APPD medium.

References

1. Banaschak, H. :Mechanismen der Inosinmonophosphat-Bildung im Menschlichen Erythrozyten. *Acta Biol. Med. Ger. 18*, 581 (1967).
2. Bretschneider, H. J., Frank, A., Kochsiek, V., Scheler, R.: Die Wirkung eines Pyrimido--Pyrimidin Derivates auf die Sauerstoffversorung des Herzmuskels. *Arznneimittel-Forsch. 9*, 49 (1959).
3. Bunag, R. D., Douglas, C. R., Imai, S. and Berne, R. M.: Influence of a pyrimidopyrimidine derivative on deamination of adenosine by blood. *Circ. Res. 15*, 83 (1964).
4. Dresse, A., Chevolet, C., Delapierre, D., Masset, H., Weisenberger, H., Bozler, G., Heinzel, G.: Pharmacokinetics of oral dipyridamole (persantin) and its effect on platelet adenosine uptake in man. *Eur. J. Clin. Pharmacol. 23*, 229 (1982).
5. Giblett, E. R., Anderson, J. E., Cohen, F., Pollara, B., Meuwissen, H. J.: Adenosine deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet 2*, 1067 (1972).
6. Gerlach, E., Deuticke, B., Duhm, J.: Phosphat-permeabilitat und Phosphat-Stoffwechsel Menschlicher Erythrozyten und Möglichkeiten ihrer Experimentallen Beeinflüssung. *Pflugers. Arch. 280*, 243 (1964).
7. Hopkinson, D. A., Cook, P. J. L. and Harris, H.: Further data on the adenosine deaminase (ADA) polymorphism and a report of a new phenotype. *Ann. Hum. Genet. 32*, 361 (1969).
8. Kolassa, N. and Pfleger, K.: Adenosine uptake by erythrocyte of man, rat and guinea-pig and its inhibition by hexobendine and dipyridamole. *Biochem. Pharmac. 24*, 154 (1975).
9. Kopff, M., Zakrzewska, I., Klem, J., Zachara, B.: Effect of dipyridamole on adenosine incorporation into hypoxanthine nucleotides of fresh human red cells. *Haematologia 19*, 89 (1986).
10. Liu, M. S. and Feinberg, H.: The effect of persantin on nucleoside metabolism of the perfused rabbit heart. *Biochem, Pharmac. 22*, 1118 (1973).
11. Lowy, B. A., Williams, M. K., London, J. M.: Enzymatic deficiens of purine nucleoside synthesis in human erythrocytes. *J. Biol. Chem. 237*, 1622 (1962).
12. Lum, C. L., Marr, R., Plagemann, P. G. W., Wohlhueter, R. M.: Adenosine transport and metabolism in mouse leukemia cells and in canine thymocytes and peripherial blood leukocytes. *J. Cell Physiol. 101*, 173 (1979).
13. Mills, G. O., Burger, D. O., Schneider, M., Levin, C.: Ion exchange analysis of organic phosphate compounds of erythrocytes from individuals with neoplastic diseases. *J. Lab. Clin. Med. 58*, 725 (1961).
14. Morell, S. A., Ayers, V. E., Greenwalt, T. J.: Linear gradient elution of nucleotides from Dower-1 formate: application to the erythrocytes. *Anal. Biochem. 3*, 285 (1962).
15. Pinilla, M., Luque, J., Santos-Ruiz, A.: Inhibition de l'incorporation de l'adenosine par le dipyridamole: influence sur le metabolisme du 2,3-diphosphoglycerate dans l'erythrocyte de l'homme. *Ann. Pharmac. Franc. 31*, 503 (1973).
16. Parks, Jr. R. E., Brown, R.: Incorporation of nucleosides into the nucleotide pools of human erythrocytes. Adenosine and its analogs. *Biochemistry 12*, 3294 (1973).
17. Roos, H. and Pfleger, K.: Kinetics of adenosine uptake by erythrocytes and the influence of dipyridamole. *Molec. Pharmac. 8*, 417 (1972).
18. Whittam, J.: The high permeability of human red cells to adenine and hypoxanthine and their ribosides. *J. Physiol. 154*, 614 (1960).
19. Zachara, B.: The distribution of inorganic phosphate in blood conserved at 4 °C in acid--citrate-dextrose with adenosine and persantin. *Experientia 27*, 1287 (1973).
20. Zachara, B., Lewandowski, J.: Isolation and identification of inosine triphosphate from human erythrocytes. *Biochim. Biophys. Acta 353*, 253 (1974).
21. Zachara, B.: In vitro synthesis of inosine triphosphate in human erythrocytes. *J. Biochem. 76*, 891 (1974).

22. Zachara, B.: The effect of inosine, pyruvate and inorganic phosphate on 2,3-diphosphoglycerate, adenine and hypoxanthine nucleotides synthesis in outdated human erythrocytes. *J. Lab. Clin. Med. 85*, 436 (1975).
23. Zachara, B.: Adenosine as a source for hypoxanthine nucleotides synthesis in human erythrocytes. *Vox Sang. 17*, 453 (1975).
24. Zachara, B.: Synthesis of ITP and ATP and regeneration of 2,3-DPG in human erythrocytes incubated with adenosine, pyruvate and inorganic phosphate. *Transfusion 17*, 628 (1977).
25. Zachara, B.: Uptake of adenosine by outdated human erythrocytes incubated with dipyridamole. XI[th] International Congress of Biochemistry, July 8–13, (1979), Toronto, Canada, Abstracts 06-8-R58.

Haematologia 22 (3), pp. 169—174 (1989)

Clinical Features of Hepatic Sequestration in Sickle Cell Anaemia

P. HERNÁNDEZ, E. DORTICÓS, E. ESPINOSA, X. GONZÁLEZ, E. SVARCH

Instituto de Hematología e Inmunología, Apartado 8070,
Habana 8, Ciudad Habana, Cuba

(Received 31 May, 1988; accepted 22 July, 1988)

Episodes of hepatic sequestration were seen in four patients with sickle cell anaemia (SCA). One case presented a severe and fatal sequestration crisis whereas the others showed mild episodes associated with less striking haematological and clinical changes. These clinical pictures are similar to those seen in the splenic sequestration crises of children with SCA. Different causes of liver enlargement in SCA suggest that the clinical spectrum representing intrahepatic trapping of blood could range from the acute sequestration crisis to chronic sequestration events which very probably should play any role in the pathogenesis of the hepatomegaly frequently found in these patients.

Keywords: sickle cell anaemia, hepatic sequestration, painful crisis, hepatic crisis

Introduction

Infants and young children with sickle cell disease may suddenly have intrasplenic trapping of vast amounts of blood, which result in the so-called acute splenic sequestration crisis [1, 2]. Sudden trapping of blood in the liver may also occur [3]. This complication has been recently emphasized and it has been considered that hepatic sequestration in sickle cell anaemia (SCA) may be more common than currently realized and may result in considerable morbidity [4, 5].

In this paper the clinico-haematological features of four homozygous sickle cell disease patients with different degrees of hepatic sequestration are presented, and several causes of liver enlargement with intra hepatic trapping of blood in SCA are discussed.

Case reports

Case 1

A 25 year old male who had repeatedly suffered painful crises since childhood experienced mild leg pain in the morning one day before hospitalization. He was treated with non-narcotic analgesics at home. This symptomatic therapy provided complete pain relief and in the evening he was asymptomatic. The following morning he felt very weak, faint and complained of abdominal discomfort. The parents

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

noted that he was extremely pale. On admission he was moribund, in profound circulatory collapse with a distended abdomen and a tender liver extending to the pelvis brim. The haemoglobin concentration was 2.1 g/dl with 18% reticulocyte count. He died shortly after a blood transfusion was started. Permission for autopsy was not obtained.

Case 2

A 17 year old male was admitted to hospital with fever, weakness and mild jaundice. A week before admission his haemoglobin had been 7.2 g/dl with 10% reticulocyte count, the liver was palpable 2 cm below the right costal margin.

On admission respiratory and cardiovascular examinations gave normal results, and his liver was palpable 4 cm below the right costal margin. Initial investigations showed: haemoglobin concentration 7.8 g/dl with 16% reticulocyte count; white blood cell (WBC) count 9.4×10^9/l with 31% neutrophils, 4% monocytes and 65% lymphocytes, platelet count 322×10^9/l. The peripheral blood smear showed 9 nucleated red blood cells/100 WBC. The serum urea, creatinine, glucose and urinalysis findings were within normal limits. Hepatitis B virus surface antigen (HBs Ag) was negative. Repeated blood cultures and a chest X-ray were negative. He was treated with bed rest and intravenous fluids. Four days later he was afebrile, but his liver was enlarged 6 cm below the right costal margin, and the haemoglobin concentration had fallen to 6.8 g/dl with a reticulocyte count of 20%. Liver function tests showed bilirubin concentration 3.5 mg/dl (normal = 0.2–1.2 mg/dl) with 1.9 mg/dl indirect fraction; serum glutamic pyruvic transaminase (SGPT) activity 28 IU/L (normal = 0–16 IU/l), and serum alkaline phosphatase (SAP) activity 75 IU/L (normal = 25–92 IU/l).

Two units of packed red cells were transfused. His haemoglobin concentration rose to 9.2 g/dl, the liver rapidly regressed to 4 cm and his liver function impro-

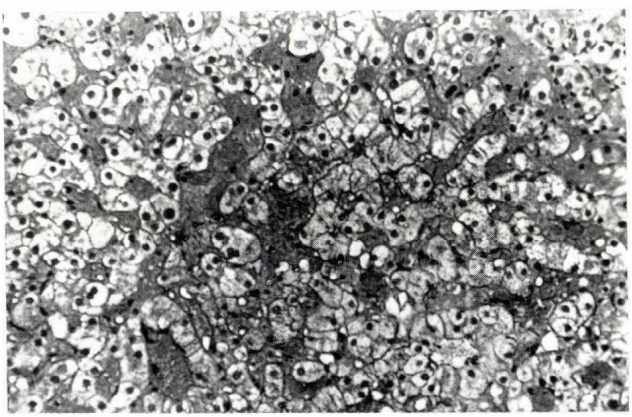


Fig. 1. Liver biopsy showing sequestration of large number of sickled erythrocytes in the hepatic circulation (Fuchsin & Ponceau 2R, × 200)

ved, but, because the liver remained enlarged, a laparoscopy and liver biopsy were performed. At laparoscopy the liver was congested. The most prominent microscopic hepatic feature was distention of the sinusoids by red blood cells. Mild round cell infiltration was seen in some portal areas (Fig. 1). Over several weeks his liver spontaneously returned to 2 cm.

Case 3

A 27 year old male was admitted with severe weakness and mild jaundice. Four days earlier he had generalised bone pain, dark urine and was slightly jaundiced. He was successfully treated with intravenous fluids and analgesics, after which the painful crisis disappeared and the urine became clear. His haemoglobin concentration at presentation was 8.9 g/dl with a reticulocyte count of 11%. The liver extended 3 cm below the right costal margin. On admission, examination showed tender hepatomegaly which was palpable 6 cm below the right costal margin. Results of initial investigations were: haemoglobin concentration 7.6 g/dl with 19% reticulocyte count; bilirubin concentration 8.0 mg/dl with 6.3 mg/dl indirect fraction; SGPT activity 17 IU/l and SAP activity 73 IU/l; HBs Ag was negative; glucose – 6 – phosphate dehydrogenase (G6PD) activity was 14.9 IU/g Hb, and the electrophoretic pattern was G6PD B. He was only treated with bed rest and intravenous fluids. Over the subsequent few days there was clinical improvement, although his liver remained 5 cm below the right costal margin. His haemoglobin concentration rose spontaneously to 8.9 g/dl with 10% reticulocyte count, and his bilirubin concentration fell to 4.8 mg/dl with 3.6 mg/dl indirect fraction, SGPT activity was 20 IU/l and SAP activity 56 IU/l.

Because hepatomegaly remained, the patient underwent laparoscopy and liver biopsy. At laparoscopy the enlarged red smooth surface liver with mild fibrotic areas was observed. Liver biopsy disclosed markedly dilated sinusoids packed with

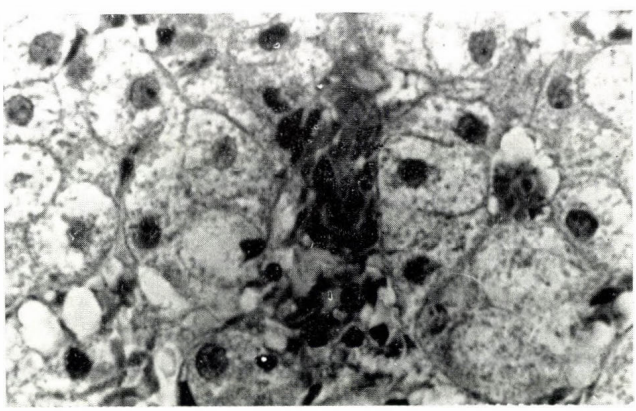


Fig. 2. Liver biopsy specimen showing sickling of red cells in dilated hepatic sinusoids (Fuchsin & Ponceau 2R, × 450)

red blood cells, most of which were sickled. Additional findings included scarce focal areas of mild parenchymal degeneration and necrosis which tended to be centrolobular and mild round cell infiltration in few portal areas (Fig. 2).

A month after hospitalization his liver was palpable 4 cm below the right costal margin, and the haemoglobin concentration was 8.4 g/dl. Over several weeks the liver returned to 3 cm.

Case 4

A 28 year old female was hospitalized with generalised bone pain. Respiratory and cardiovascular examinations gave normal results. Her liver was palpable 2 cm below the right costal margin.

Initial investigations showed: haemoglobin concentration 6.9 g/dl with 9% reticulocyte count; WBC 13.3×10^9/l with 87% neutrophils 1% monocytes and 12% lymphocytes; platelet count 200×10^9/l. The peripheral blood smear showed 9 nucleated red blood cells/100 WBC. She was treated with bed rest, analgesics and intravenous fluids. Two days later her bone pain had disappeared, but she was weak and complained of abdominal discomfort. Her liver was enlarged 10 cm below the right costal margin and the haemoglobin concentration had fallen to 3.4 g/dl with a reticulocyte count of 10%. The bilirubin concentration was 6.1 mg/dl with 4.1 mg/dl direct fraction. Four units of packed red cells were transfused.

The following day she was pyrexial, and a chest radiograph showed slight cardiac enlargement and mild inflammatory infiltrates in both lungs. Repeated blood cultures and sputum cultures were negative. She was treated with intravenous fluids and broad spectrum antibiotics. In a few days there was a complete resolution of pulmonary infiltrates and she became afebrile. Two weeks later her liver had returned to 2 cm, bilirubin concentration showed normal values, and haemoglobin concentration was 9.7 g/dl with a reticulocyte count of 8%.

Discussion

In a study of 100 patients with SCA (50 adults and 50 children) performed in our institution, all cases were noted to have enlarged liver with a mean value of 3.8 cm below the right costal margin in adults and of 3 cm in children [6, 7]. As it has been previously observed [8], hepatomegaly was soft, smooth, not tender, and there was no evidence that the liver had been displaced downwards, neither was there any evidence of a raised central venous pressure to account for the enlargement. In some patients liver biopsy was performed. Most adult patients studied in the steady state and those studied immediately after recovery of generalised bone pain, showed distended hepatic sinusoids containing sickle red blood cells. In children, during the steady state, nearly half the patients had distended and congestive hepatic sinusoids most of them containing sickle red blood cells [6, 7]. These histological data give evidence that trapping of red blood cells in hepatic sinusoid in SCA patients is a very frequent event even during the steady state.

Liver enlargement in SCA patients has been emphasized by various authors [3, 8–11] including the observation that the liver may suddenly increase in size during episodes of acute vaso-occlusive crises being usually smooth and tender, and frequently returning to normal in a few days [9].

Recently, episodes of acute hepatic enlargement associated with a rapid fall in haemoglobin concentration have been reported in SCA patients [4, 5] showing a clinical picture similar to that seen in the splenic sequestration crisis of early life. Concerning acute splenic sequestration, in addition to the classical life-threatening attacks, minor episodes associated with less striking haematological and clinical changes have often been recognised [2]. These minor episodes usually resolve spontaneously.

Evaluation of clinico-haematological features of our cases and of those recently reported [4, 5] suggests that the clinical picture of acute hepatic sequestration, like acute splenic sequestration, includes attacks of severe and sometimes fatal sequestration, and the more frequent mild episodes associated with less striking haematological and clinical changes and, furthermore, acute hepatic sequestration should be excluded whenever a fall in haemoglobin concentration with an increase in the reticulocyte or in normoblast count occurs in SCA patients.

We agree with the opinion that acute hepatic sequestration in SCA is probably more common than is recognized, since this complication – mainly the mild episodes – may be overlooked.

Factors precipitating episodes of acute splenic sequentration have not been identified. The role of bacterial infection in the pathogenesis of this event is difficult to define, but present evidences suggest that bacterial infection is not an important precipitating factor and it is possible that viral infection may be more significant [2].

Of the three recently reported cases with acute hepatic sequestration, in one with several episodes of hepatic sequestration this complication was always preceded by a painful crisis; in another it was preceded by an infection of the chest, and, in the third, by an upper respiratory tract infection and a painful crisis. Of our 4 cases 3 had painful crisis before the hepatic complication and in the third fever of probable viral origin preceded the sequestration event. It seems possible that at least in these cases infection and vaso-occlusive crises may have played certain role as precipitating factors.

Patients with SCA may have also attacks characterized by hepatomegaly and jaundice often called "hepatic crisis" [12, 13]. The most prominent and constant microscopic feature observed in the liver of these cases has been severe distention of the sinusoids by sickled red blood cells. Occasional bile stasis and mild centrolobular necrosis have been seen, but no evidence of obstruction in the biliary system has been described [6, 12–14].

The sinusoidal stagnation of sickle red blood cell in the liver may decrease circulation through hepatic sinusoids with possible implication in the pathogenesis of hepatic crisis [13–16].

All these facts taken into account suggest that the clinical spectrum represent-

ing intrahepatic trapping of blood in SCA could range from the acute sequestration crisis to chronic sequestration events, which very probably should play some role in the pathogenesis of the hepatomegaly frequently found in these patients. According to previously observed histological hepatic features, the so-called "hepatic crisis" could be located between these two extreme ends of the spectrum as a clinical expression of the disease where subacute sequestration episodes may exist. Further studies on this matter surely will contribute to a better understanding of the hepatic complications of SCA.

References

1. Seeler, R. A., Shwiaki, M. Z.: Acute splenic sequestration crises in young children with sickle cell anemia. *Clin. Pediatr. 1*, 701–704 (1972).
2. Topley, M. J., Rogers, D. W., Stevens, M. C., G. Serjeant, G. R.: Acute splenic sequestration and hypersplenism in the first five years in homozygous sickle cell disease. *Arch. Dis. Childr. 56*, 765–769 (1981).
3. Konotey-Ahulu, F. I. D.: The sickle cell diseases. Clinical manifestations including the "sickle crisis". *Arch. Int. Med. 133*, 611–619 (1984).
4. Hatton, C. S. R., Buch, C., Weatherall, D. J.: Hepatic sequestration in sickle cell anaemia. *Brit. Med. J. 290*, 744–745 (1985).
5. Gutteridge, C., Newland, A. C., Sequeira, J.: Hepatic sequestration in sickle cell anaemia. *Brit. Med. J. 290*, 1214–1215 (1985).
6. Espinosa, E., Barreto, G., Cabrera, J., Hernández, P.: El hígado en la anemia drepanocítica: Un estudio clínico patológico en cincuenta pacientes. *Sangre 30*, 945 (1985).
7. Quintero, I., Svarch, E., Villaescusa, R., Cubero, D.: Hepatopatía en niños con anemia drepanocítica. Reporte preliminar. *Sangre 26*, 526 (1981).
8. Serjeant, G. R., Richards, R., Barbor, P. R. H., Milner, P. F.: Relatively benign sickle-cell anaemia in 67 patients aged over 30 in the West Indies. *Brit. Med. J. 3*, 86–91 (1968).
9. Karayalcin, G., Rosner, F., Kim, K. Y., Chandra, P., Aballi, A. J.: Sickle cell anemia.–Clinical manifestation in 100 patients and review of the literature. *Amer. J. Med. Sci. 269*, 51–68 (1975).
10. Green, T. W., Conley, C. L., Berthrong, M.: The liver in sickle cell anemia. *Bull. Johns Hopkins Hosp. 92*, 99–127 (1953).
11. Konotey-Ahulu, F. I. D.: The liver in sickle-cell disease: Clinical aspects. *Ghana Med. J. 8*, 104–118 (1969).
12. Diggs, L. W.: Sickle cells crises. *Amer. J. Clin. Path. 44*, 1–19 (1965).
13. Sheehy, T. W.: Sickle cell Hepatopathy. *South. Med. J. 70*, 533–538 (1977).
14. Song, Y. S.: Hepatic lesions in sickle cell anemia. *Am. J. Pathol. 33*, 331–351 (1957).
15. Hernández, P., Carnot, J. Cruz, C., Dorticós, E., Espinosa, E., González, A., Santos, M. N., Villaescusa, R.: Circulating immune complexes in sickle cell hepatic crises. *Acta Haematol. 65*, 15–20 (1981).
16. Villaescusa, R., Santos, M. N., Espinosa, E., Hernández, P.: Circulating immune complexes in sickle cell anaemia. *Haematologia 17*, 185–191 (1986).

Haematologia 22 (3), pp. 175—180 (1989)

α-Thalassemia Changes the Cell Density Profile in Sickle Cell Anaemia

M. TEJUCA[1], G. MARTINEZ[1], R. FERREIRA[1], A. HERNÁNDEZ[1], L. FERRER[1], A. GOICOCHEA[1], A. C. HERNÁNDEZ[1], R. COYULA[2], M. WADE[3], B. COLOMBO[4]

[1] Institute of Hematology and Immunology, Apdo. 8070
Habana 8, Cuba
[2] University of Havana, Faculty of Biology
[3] Ministry of Public Health, Medical Care Division, Habana, Cuba
[4] Institute of Cell Biology, Via Romagnosi 18/a, Rome, Italy

(Received 16 November, 1987; accepted 23 September, 1988)

Several factors are implied in the haematological and clinical picture of sickle cell anaemia. Attention has been focused on the concomitant presence of $-\alpha$-thalassemia and high levels of HbF, but contradictory results have been reported in different populations.

We compared the blood cell density profile, obtained by the phtalate esther method, of normal subjects with those of patients with sickle cell anaemia – with or without heterozygous α-thalassemia.

We found that the density profile of both groups of patients differs from normal subjects, and that a difference can also be demonstrated between normal α genotype patients with sickle cell anaemia and patients with heterozygous α-thalassemia.

These results are in agreement with the findings obtained in other countries in which a gene from Caucasian to African populations have been demonstrated, and are different from the results obtained in populations of more pure African ancestry. It can be suggested, therefore, that these data, in addition with findings of other authors in different geographical areas, support the hypothesis that the genetic make up plays an important role in the haematologic and clinical picture of sickle cell anaemia.

Keywords: α-thalassemia, cell density profile, HbF, sickle cell disease

Introduction

Sickle cell anaemia is a genetic disease characterized by an extremely variable clinical expression, due to genetical and environmental factors. α-thalassemia associated with sickle cell anaemia has been the subject of many studies. These studies have reported α-thalassemia to lower mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean haemoglobin concentration (MCHC) [1, 2]. These changes are assumed to be beneficial for patients with sickle cell anaemia, since the corpuscular haemoglobin concentration (CHC) is an important factor for the kinetics [3] and extent [4] of intracellular polimerization. Thus, the proportion of dense erythrocytes, which are a fraction of cells containing irreversible sickled cells (ISC), should also be reduced. In fact, recent studies by

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Noguchi et al. [5] and Baudin et al. [6] showed when sickle cell anaemia is associated with homozygous α-thalassemia (α-/α-) the high proportion of dense cells and the broad distribution in erythrocyte density are reduced. The density profile, however, in cases of sickle cell anaemia heterozygous for α-thalassemia (α-/αα) did not differ from that observed in subject of normal α genotype with sickle-cell disease. On the contrary, Fabry et al. [7] found a significant reduction in the number of dense cells in cases of (α-/αα) genotype compared to sickle cell anaemia subjects of normal (αα/αα) genotype.

For further information we compared the density profile of sickle-cell patients with normal number of α-genes (αα/αα) to subjects heterozygous for α-thalassemia (α-/αα) with phtalate ester method of Danon and Marikovsky [8].

Materials and Methods

We studied 34 patients with sickle cell anaemia heterozygous for α-thalassemia (α-/αα), 31 patients with sickle-cell anaemia and a normal α genotype (αα/αα) attending the Institute of Hematology and Immunology in Havana, and 31 subjects with a normal haemoglobin phenotype and normal α genotype. These patients had had no crisis episodes and had not received blood transfusions during the preceeding three months.

The analysis of the α genes was carried out by restriction endonuclease analysis of the DNA obtained from peripheral blood leukocytes. DNA was digested with Bam HI, electrophoresed on agarose gel, transferred to a nitrocellulose filter and hybridized with a ^{32}dATP nick-translated α-globin specific plasmid (JW 101) as previously described [9].

Phatalate esters were prepared by the method of Danon and Marikovsky [8] by mixing various concentrations of dimethyl phtalate (Sp. gr. 1.189) and N-butyl phtalate (Sp. gr. 1.042), purchased from Fischer Scientific Co. In this way 20 stock solutions of specific gravities ranging from 1.060 to 1.136, with increments of 0.004 between successive solutions were prepared. For each density point a microcapillary tube was loaded with approximately 5 mm of phtalate ester, filled with blood and clay-sealed at the phtalate end. After centrifugation at 12.000 RPM for 7 min at room temperature the height of the packed cells above and below the phtalate layer were measured and the "fraction under" calculated as follows:

"fraction under" = height (mm) under/total height (mm).

The phtalate ester profile was obtained by representing the "fraction under" (i.e. the proportion of cells is more dense than the marker) as a function of the density. The histogram for the density distribution corresponds to the first derivative of the curve thus obtained. The profiles were further characterized using the parameters selected by Noguchi et al. [5]:

1) the D_{50} represents the median cell density;
2) the R_{60} indicates the spread in the density, i.e. the density range obtained after subtracting the lightest 20% and most dense 20% of cell fractions, and

3) the percent of packed cells (density > 1.12 which corresponds to a CHC > 37 g/dl) that is an indication of the proportion of very dense cells.

Fetal haemoglobin (HbF) was determined by the alkali denaturation method of Betke et al. [10].

Results

The average density profiles of normal individuals, sickle-cell patients with a normal α gene number (αα/αα) and patients with heterozygous α-thalassemia (α-/αα) are shown in Fig. 1 in which the respective density distribution is also plotted.

Table 1 summarizes the analysis of the D_{50}, the R_{60} and the percent of packed cells showing a density > 1.12 in the above three groups. These results show a

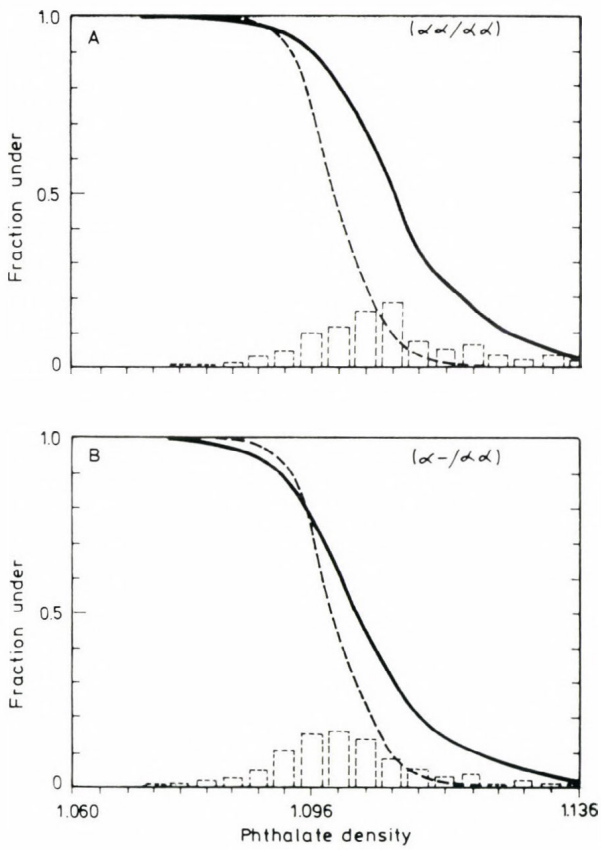


Fig. 1. Mean density profiles (A) for normal individuals and individuals with sickle cell anemia without α-thalassemia (αα/αα) and (B) with heterozygous α-thalassemia (α— /αα). The profile for normal individuals is indicated by dashed lines. The histograms for the density distributions are from patients

Table 1

The median density (D50), the R60 and the percent of packed cells 1.12 for normal individuals and HbSS patients without α-thalassemia (αα/αα) and with heterozygous α-thalassemia (α-/αα)

	AA (n = 31)	SS (αα/αα) (n = 34)		A (α−/αα) (n = 31)
D 50	1.100± 0.004	1.110± 0.004	(p < 0.001)	1.105± 0.006
R 60	0.007± 0.001	0.019± 0.003	N.S.	0.018± 0.006
Percent of packed cells >1.12	0.2± 0.4	17.7± 7.3	(p < 0.01)	11.7± 7.5

D 50 and R 60 are expressed in density units
The three paremeters differ significantly ($p < 0.001$) between the group of normal individuals and both subgroups of HbSS patients.
N.S.: Not significant

significant difference ($p < 0.001$) between both subgroups of sickle-cell patients and the group with a normal haemoglobin phenotype.

When the two subgroups of patients were compared we found that the density profile for the group of (α-/αα) genotype is shifted toward lower values. The D_{50} was reduced and fewer dense cells were observable (Fig. 1 and Table 1), but the spread in density distribution was not statistically different between the two subgroups of patients.

We calculated the Pearson Correlation Coefficient between the HbF levels and the percent of dense cells in both groups of sickle cell patients, and found that in the group of patients with normal (αα/αα) genotype there was an inverse correlation ($r = -0.408$ at $p < 0.05$). However, the correlation was not significant in (α-/αα) genotype patients. Logarithmic transformation of the HbF values was not used since we corrected its normal distribution using the Kolmogorov-Smirnov test.

It is of interest that while a difference in the correlation dense cells vs. HbF could be demonstrated, there was no statistical difference between the percentages of HbF in the two groups (αα/αα = 4.1 ± 2.5% and α-/αα = 4.0 ± 3.5%).

Discussion

The results of this study confirm the data of others [5, 11] which demonstrate an increase in the percent of dense cells and a concomitant broader range of erythrocyte density in patients with sickle-cell disease compared to normal subjects (Fig. 1 and Table 1).

On the other hand, conflicting results have been published on the erythrocyte density distribution observed in sickle-cell patients with a normal (αα/αα) genotype

and in patients in whom the disease is associated with heterozygous (α-/αα) thalassemia. Noguchi et al. [5] and Baudin et al. [6] found no significant different between the two groups, whereas Fabry et al. [7] found that the percentage of dense cells determined by the Percoll-Stractan method is higher in (αα/αα) genotype patients than in those of (α-αα) genotype. Embury et al. [12] analysing the effect of the α--globin gene number on erythrocyte density used a twelve-step analytic Stractan gradient and found that the percentage of dense cells in the presence of four α-gens is higher than in cases of sickle cell anaemia associated with 3 or 2 α-globin genes.

Our results are in agreement with these studies and show that in cases of sickle cell anaemia of (α-/αα) genotype individuals the density profile is shifted to the left compared to those with a normal number of α-genes. This indicates that the cells have a lower D_{50} and that a smaller percent of dense erythrocytes (CHC > 37 g/dl) is present, although the density distribution is still broader than in normal subjects (Fig. 1).

These changes in density distribution toward lower values are in agreement with the results of Higgs et al. [1] and Embury et al. [2] who found a significant reduction of the MCHC in sickle cell patients with heterozygous α-thalassemia. Thus the difference in MCHC is reflected in the density cell profile.

Our results are in keeping with the hypothesis that the beneficial effects of α-thalassemia in sickle cell disease are due to a reduction of the MCHC. Other unknown genetic and/or environmental factors may also play an important role in the clinical variability of sickle cell disease [13].

It has been suggested that HbF level is one of the factors influencing the clinical picture, since HbF has been shown to inhibit sickling [14]. Thus high levels of HbF are expected to decrease the production of dense cells. Our results show that there is an inverse correlation between the percent of dense cells and the percent of HbF in non-thalassemia patients only. No correlation was found in (α-/αα) genotype sickle cell patients. These findings agree with the results of Fabry et al. [7]. They suggested that either the decrease of MCHC values and the inhibitory effect of HbF are not additive, or the preservation of splenic function for a longer period of time in (α-/αα) patients makes the removal of dense cells in sickle cell patients with low levels of HbF more efficient.

The results presented in this paper intend to add further information on the heterogeneity of the haematological picture and on the effects of the concomitant presence of α-thalassemia in sickle cell disease.

The contradictory results obtained in different populations [1, 2, 5–7] are not easily explained, but the fact that patients with high interbreeding with Caucasian populations [15, 16] show similar results when compared to populations of a more pure African ancestry [5, 6], suggests that the genetic make-up plays an important role in the haematologic and clinical picture of sickle cell anaemia.

References

1. Higgs, D. R., Aldridge, B. E., Lamb, J., Clegg, J. B., Weatherall, D. J., Hayes, R. J., Grandison, Y., Lowrie, Y., Mason, K. P., Serjeant, B. E., Sarjeant, G. R.: The interaction of alpha — thalassemia and homozygous sickle — cell disease. *N. Engl. J. Med. 306*, 1441 (1982).
2. Embury, S. H., Dozy, A. M., Miller, J., Davis, J. R., Jr., Kleman, K. M., Preisler, H., Vichinsky, E., Lande, W. N., Lubin, B. H., Kan, Y. W., Mentzer, W. C.: Concurrent sickle-cell anemia and α-thalassemia: Effect on Severity of Anemia. *N. Engl. J. Med. 306*, 270 (1982).
3. Hofrichter, J., Ross, P. D., Eaton, W. A.: Kinetics and mechanism of deoxyhemoglobin S gelation: a new approach to understanding sickle cell disease. Proc. *Natl. Acad. Sci. USA. 71*, 4864 (1974).
4. Noguchi, C. T., Torchia, D. A., Schechter, A. N.: Determination of deoxyhemoglobin S. polymer in sickle erythrocytes upon deoxygenation. *Proc. Natl. Acad. Sci. USA 77*, 5487 (1980).
5. Noguchi, C. T., Dover, G. J., Rodgers, G. P., Serjeant, G. R., Antonarakis, S. E., Anagnou, N. P., Higgs, D. R., Wheatherall, D. J., Schechter, A. N.: Alpha thalassemia changes erythrocyte heterogeneity in sickle cell disease. *J. Clin. Invest. 75*, 1632 (1985).
6. Baudin, V., Pagnier, J., Labie, D., Girot, R., Wajcman, H.: Heterogeneity of sickle cell disease as shown by density profiles: Effects of fetal hemoglobin and alpha thalassemia. *Haematologia 17*, 177 (1986).
7. Fabry, M. E., Mears, J. G., Patel, P., Schaefer-Rego, K., Carmichael, L. D., Martínez, G., Nagel, R. L.: Dense cells in sickle cell anemia. The effects of gene interaction. *Blood 64*, 1042 (1984).
8. Danon, D., Marikovsky, Y.: Determination of density distribution of red cell population. *J. Lab. Clin. Med. 64*, 668 (1964).
9. Martínez, G., Ferreira, R., Hernández, A., Di Rienzo, A., Felicetti, L., Colombo, B.: Molecular characterization of HbH disease in the Cuban population. *Hum. Genet. 72*, 318 (1986).
10. Betke, K., Marti, H. R., Schlicht, I.: Estimation of small percentages of foetal haemoglobin. *Nature 184*, 1877 (1959).
11. Oda, S., Oda, E., Tanaka, K. R.: Relationship of density distribution and pyruvata kinase electrophoretic pattern of erythrocytes in sickle cell diseases and other disorders. *Acta Haematol. 60*, 201 (1978).
12. Embury, S. H., Clark, M. R., Monroy, G., Mohandas, N.: Concurrent sickle cell anemia and alpha-thalassemia: Effect on pathological properties of sickle erythrocytes. *J. Clin. Invest. 73*, 116 (1984).
13. Steinberg, M. H., Hebbel, R. P.: The clinical diversity of sickle cell anemia: Genetic and cellular modulation of disease severity. *Am. J. Hematol. 14*, 405 (1983).
14. Bertles, J. F., Rabinowitz, R., Dobler, J.: Hemoglobin interaction: Modification of solid phase composition in the sickling phenomenon. *Science 169*, 375 (1970).
15. Reed, T. E.: Caucasian genesin American Nagroes. *Science 165*, 762 (1969).
16. González, R., Ballester, J. M., Estrada, M., Lima, F., Martínez, G., Wade, M., Colombo, B., Vento, R.: A study of the Genetical Structure of the Cuban Populalion: Red Cell and Serum Biochemical Markers. Am. J. Hum. Genet. 28, 585 (1976).

Haematologia 22 (3), pp. 181–188 (1989)

Preparation of Leukocyte-poor Platelet Concentrates for Transfusion by Filtration Through a New Cellulose Acetate Filter

K. KOERNER

DRK-Blutspendezentrale Ulm, Oberer Eselsberg 10, D-7900 Ulm, FRG

(Received 9 March, 1988; accepted 10 August, 1988)

The preparation of leukocyte-poor platelet concentrates using a new cellulose acetate filter was evaluated. The platelet loss was 9% when freshly prepared platelet concentrates were filtered. The platelet loss was more variable and about 16% ($p < 0.05$) when 5 day old platelets were filtered. The leukocyte removal was not influenced by storage time. The mean absolute number of leukocytes was about 1×10^7 and 3×10^7 for freshly prepared or 5 day old platelet concentrates, respectively ($p < 0.05$).

The leukocyte-poor platelet concentrate prepared by the described filtration procedure may be used as a blood component in transfusion therapy to prevent or delay platelet alloimmunization.

Keywords: filtration, HLA-alloimmunization, leukocyte-poor platelet concentrates, platelet function

Introduction

Poor survival of the transfused platelets because of HLA-alloimmunization is often seen in thrombocytopenic patients after repeated platelet transfusions [1, 2]. 35–90% of the multitransfused patients have problems due to anti-HLA antibodies [3], whereas refractoriness caused by platelet specific antibodies is less a problem [4, 5]. The incidence of HLA-alloimmunization may be reduced by the transfusion of leukocyte-poor platelet concentrates (PC) [6–8]. It has been shown that filtration of PC currently is the most efficient method with regard to removal of leukocytes and platelet yield [9, 17]. We have evaluated a new filter Miropore PLT made from cellulose acetate for preparing leukocyte-poor PC.

Materials and Methods

Preparation of PC

PC from random donors were prepared from 500 ml whole blood in CPD-Al as described previously [10]. Briefly, whole blood was centrifuged at 2,400*g* for 4 min. Platelet-rich plasma (PRP) was then expressed into a satellite bag until reaching the buffy coat about 1 cm above the red cell layer. Then the PRP was centrifuged

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

at 3,100*g* for 20 min. The platelet button was left undisturbed for 1 h at 20–24 °C and 70 ml of plasma was left on the platelet button. PC were stored with end-over-end agitation (Helmer rotator) at 6 rpm for 5 days in the 5-day platelet bag at 20–24 °C (Biotest Pharma, Frankfurt, FRG) [11].

Just before removing the leukocytes by filtration, 6 or 7 PC were pooled.

Filtration

Miropore PLT was used (manufacturer: Miramed S.p.A., Mirandola, Italy, code 620 000 000; batch 87 10 03) for removing leukocates from PC. The filter was made from cellulose acetate. This material has a well defined adsorbent activity for leukocytes, lymphocytes and polymorphonucleated cells. The filter functions as a depth filter, its volume is about 150 ml. The filter was applied according to the instruction of the manufacturer. The filter was primed with 150 ml saline to wet it and to expell air. Thereafter, the pooled PC, volume 420 ml, was passed through the filter without pressure. The filter was then rinsed with 120–150 ml of saline. Total filtration processing time, including priming and washing of the filter, was about 15–20 minutes.

A total number of 192 random PC, each obtained from 500 ml blood collected in CPD-Al were studied.

Platelets were counted on a TOA counter (TOA-Medical-Electronics, Japan). Leukocytes were counted manually in duplicate by microscope. In vitro platelet function was determined by aggregation induced by ADP or collagen and by platelet response to hypotonic shock as described previously [12]. The morphologic score was evaluated by the method of Kunicki et al. [13] using the following sequence of changes: discs, spheres, dendrites and ballons. The precentage of each morphologic type, assessed by phase microscopy, was multiplied by a series of arbitrary factors as follows: discs × 4, spheres × 2, dendrites × 1, ballons × 0. The morphologic score was defined as the total of the four numbers thus derived. At least 200 platelets were determined. The most superior morphology, or 100% discs, was scored as 400.

Experiments

1. Filtration of fresh PC, after preparation, up to 1 day old
2. Filtration of old PC, stored for 5 days
3. Filtration of PC with increasing white cell contamination (capacity of filter)
4. The possibility of removing leukocytes from the filter by additional rinsing with saline and knocking at the filter
5. In vitro platelet function pre- and post filtration.

Statistical analysis

The p values were calculated with Student's *t* test. Results with p less than or equal to 0.05 were considered to be significant.

Results

Filtration of fresh PC and PC stored for 5 days

The yield of platelets and the removal of leukocytes is shown in Tables 1 and 2.

When the PC was filtered on the day of preparation, the platelet loss was about 9%. However, when the PC filtered after 5 days of storage, the platelet loss was about 16% ($p < 0.05$) showing a variable platelet loss of 5–28%. No difference was observed in the leukocyte removal of fresh or stored PC ($p > 0.05$). The absolute number of leukocytes is about $1-3 \times 10^7$, in 25% of filtration there were no detectable leukocytes in the counting chamber. To evaluate the absolute number of the leukocytes after filtration, the filtered PC was centrifuged once more at 3,000*g* for 30 min to pellet the residual leukocytes. The absolute number of leukocytes was below 6×10^6 per filtered platelet pool.

Table 1

Leukocyte-poor platelet concetrate by filtration of *fresh platelets (filtration after preparation)*
total number of platelet concentrates: 72

Pool (n)	Platelets $\times 10^{11}$		Yield (%)
	pre	post	
12	4.22±0.61	3.85±0.57	91±2 (range: 88–94%)
	leukocytes $\times 10^8$		loss (%)
12	1.07±0.4	0.12±0.05*	>85

* in 4/12 no detectable leukocyte in the counting chamber

Table 2

Leukocyte-poor platelet concentrate by filtration, storage time: 5 days, total number of platelet concentrates: 84

Pool (n)	Platelets $\times 10^{11}$		Yield (%)
	pre	post	
14	4.0±0.68	3.38±0.61	84±7 (range: 72–95%)
	leukocytes $\times 10^8$		loss (%)
14	2.0±1.21	0.30±0.27*	>80

* in 4/14 no detectable leukocyte in the counting chamber
Leukocytes after centrifugation of the filtered platelet concentrate (3.000 *g*/30 min): 6×10^6 leukocytes ($0.8-12 \times 10^6$)

Table 3

Platelet yield after filtration in relation to initial platelet number; n = 20 pools (platelet storage time: 5 days)

Platelet before filtration $\times 10^{11}$	Platelet yield after filtration %
3.14	75
3.37	95
3.42	74
3.50	72
3.52	91
3.55	88
3.63	82
3.67	85
3.72	89
3.73	88
3.77	80
3.80	89
3.81	83
4.08	95
4.39	84
4.49	88
4.59	92
4.71	86
5.08	93
5.78	76
mean:	84±7%;
±s.d.:	range: 72–95%
median: 84	

The variable results of the filtration of stored platelets were analyzed for initial platelet number and platelet yield after filtration (Table 3). There is no correlation (r = 0.1178) of platelet yield and initial platelet number.

Capacity of the filter for leukocyte removal

The capacity of removal of leukocytes of the filtered PC is shown in Table 4. Increasing volumes of buffy coat cells (leukocytes separated one day after blood donation) were added to the pooled PC. There is a good removal of 98% of leukocytes up to 2.5×10^9 white cells. Normally, these numbers of leukocytes are never reached in single platelet concentrates when preparation is under good quality control, but in cytapheresis preparations this number is not uncommon.

Table 4

Capacity of leukocyte removal of Miropore PLT. Increasing numbers of leukocytes were added to platelet concentrates; total number of platelet concentrates: 42, storage time: 5 days (pool n = 4)

Platelets $\times 10^{11}$		Yield %	Leukocytes $\times 10^{8}$		Loss %
pre	post		pre	post	
3.72	3.30	89	13.4	0.11	99
3.55	3.13	88	17.5	0.45	97
3.42	2.53	74	21.3	0.34	98
3.52	3.22	91	24.7	0.46	98
storage time: fresh platelets, after preparation (pool n = 2)					
3.93	3.45	88	11.1	0.19	98
2.69	2.51	93	12.3	<0.1	99

Stability of the adsorbed leukocytes

The washing out rate of leukocytes and platelets by additional rinsing of the filter with 100 ml saline and by knocking the filter was studied. No leukocytes were found in the filtrate by manual counting in the chamber, and $2.2 \pm 0.4\%$ of the filtered platelets were additionally recovered (n = 7).

Platelet function after filtration

The in vitro platelet function pre- and post filtration was studied. There was no significant change in the morphological score after filtration (Table 5.1). The hypotonic shock response is slightly, but statistically not significantly, reduced after filtration. The same result was seen when platelet aggregation was induced by 50 μM and 5 μM ADP. The aggregation response induced by collagen, however, was reduced to 15% and 25% using 5 μg and 2.5 μg collagen, respectively.

Discussion

The benefit of leukocyte depletion of blood components in reducing HLA alloimmunization was shown in some studies [6–8].

Recent studies of Murphy et al. [2] confirmed these results, where 16% of patients with acute leukaemia undergoing initial chemotherapy and receiving leukocyte-poor blood components developed lymphocytotoxic antibodies, compared with 48% of patients in a control group receiving non-leukocyte-depleted blood components. Combining the use of HLA-matched donors for platelet transfusion with leukocyte-poor blood components, none of the patients developed lymphocytotoxic antibodies in this study [2]. Recently Andreau et al. [14] found that in

Table 5

In vitro platelet function pre-and postfiltration of fresh platelets (filtration after preparation)

5.1. *Morphologic score* (Kunicki et al.): n = 7		
pre filtration:	287±17	
post filtration:	282±14	$p > 0.05$
5.2. *Hypotonic shock response (HSR)* n = 8		
	Vmax (A620 nm) x 10^{2}	%
filtration pre	13.2±2.2	100
post	12.7±2.5	96
5.3. *Aggregation induced by ADP*		
	Vmax (cm/min)	%
50 μM ADP		
pre	8.8±0.9	100
post	8.4±1.1	95±9
5 μM ADP		
pre	4.9±1.1	
post	4.8±1.4	96±16
5.4. *Aggregation induced by collagen*		
	Vmax (cm/min)	%
5 μg		
pre	7.6±1.2	
post	6.4±0.8	85±9
2.5μg		
pre	5.4±0.8	
post	4.1±1.0	75±15

oncohaematologic patients filtration of PC using cotton-wool filters reduced HLA-immunization in polytransfused patients significantly ($p < 0.05$).

Leukocyte-poor PC can be prepared by differential centrifugation [15, 16] by a special platelet pooling system [9] or by filtration through cotton-wool filters [17, 18]. Using cellulose acetate filters (Sepacell^{R}), the platelet recovery was only 40% [19]. A new polyester depth filter, Sepacell R-500A made by Asahi Medical Co., Ltd. (Tokyo, Japan) removed approximately 98% of the platelets from stored PC.

The new Miropore^{R} PLT filter evaluated in this study resulted in good removal of leukocytes of PC compared to cotton-wool filters [9]. The higher leukocyte count after filtration of stored PC, which was not significantly different compared

to the leukocyte count after filtration of fresh PC, may be caused by leukocyte fragments. The only change of the in vitro function tested was seen in the aggregation response induced by collagen. Whether this phenomenon influences in vivo recovery and function, is to be shown in transfusion studies. The platelet yield but not the leukocyte removal depends on the storage time of PC. Therefore, if stored PC is filtered, the platelet yield has to be considered for adequate platelet increments in the patients.

Acknowledgements

The technical assistance of P. Sahlmen and B. Zimmermann is gratefully acknowledged. We thank H. Barro for typing the manuscript.

References

1. Howard, J. E., Perkins, H. A.: The natural history of alloimmunization to platelets. *Transfusion 18*, 496–503 (1978).
2. Murphy, M. F., Metcalfe, P., Thomas, H., Eve, J., Ord, J.; Lister, T. A., Waters, A. H.: Use of leukocyte-poor blood components and HLA-matched platelet donors to prevent HLA-alloimmunization. *Br. J. Haematol. 62*, 529–534 (1986).
3. Murphy, M. F., Waters, A. H.: Immunological aspects of platelet transfusions. *Br. J. Haematol. 60*, 409–414 (1985).
4. Pegels, J. G., Bruynes, E. C. E., Engelfriet, C. P., Borne, A. E. G. Kr. von dem: Serological studies in patients on platelet and granulocyte substitution therapy. *Br. J. Haematol. 52*, 59–68 (1982).
5. Waters, A. H., Minchinton, R. M., Bell, R., Ford, J. M., Lister, T. A.: A cross-matching procedure for the selection of donors for alloiummunized patients. *Br. J. Haematol. 48*, 59–68 (1981).
6. Eernisse, J. G., Brand, A.: Prevention of platelet refractoriness due to HLA-antibodies by administration of leukocyte-poor blood components. *Exp. Haematol. 7*, 77–83 (1981).
7. Schiffer, C. A., Dutcher, J. P., Aisner, J., Hogge, D.; Wiernik, P. H., Reilly, J. P.: A randomized trial of leukocyte-depleted platelet transfusion to modify alloimmunization in patients with leukemia. *Blood 52*, 815–820 (1983).
8. Robinson, E. A. E.: Single donor granulocytes and platelets. *Clin. Haematol. 13*, 185–216 (1984).
9. Koerner, K., Kubanek, B.: Comparison of three different methods used in the preparation of leukocyte-poor platelet concentrates. *Vox Sang. 53*, 26–30 (1987).
10. Koerner, K., Sahlmen, P., Stampe, D.: In vitro effect on stored red blood cells and platelets after a 15-hour delayed refrigeration of whole blood prior to component preparation in CPD-AD. *Vox Sang. 50*, 141–145 (1986).
11. Koerner, K.: Platelet function during storage in PVC bags with increased gas permeability. In: Smit Sibinga, Das, Greenwalt (eds): *Future developments in blood banking*, Nijhoff, Boston 1986, pp. 75–82.
12. Koerner, K.: Platelet function of room temperature platelet concentrates stored in a new plastic material with high gas permeability. *Vox Sang. 47*, 406–411 (1984).
13. Kunicki, T. J., Tuccelli, M., Becker, G. A., Aster, R. H.: A study of variables affecting the quality of platelets stored at room temperature. *Transfusion 15*, 414–421 (1975).
14. Andreu, G., Dewailly, Leberre, C., Carre, M. C., Paitre, M. L., Fauchet, R., Tardivel, R., Devers, I., Soreau, E., Lam, Y., Boccaccio, C.,Piard, N., Genetet, B.: Prevention of

HLA immunization with leukocyte-poor packed red cells and platelet concentrates obtained by filtration. *Transfusion 27*, 512 (1987).
15. Cott, M. E., Oh, J. H., Vroon, D. H.: Isolation of leukocyte-free platelets from standard platelet concentrates by centrifugation. *Transfusion 26*, 272–273 (1986).
16. Herzig, R. H., Herzig, G. P., Bull, M. J., Decter, J. A., Lohrmann, H. P., Stout, F. G., Yankee, R. A., Graw, R. J.: Correction of poor platelet transfusion responses with leukocyte-poor HLA-matched platelet concentrates. *Blood 46*, 743–750 (1975).
17. Sirchia, G., Parravicini, A., Rebulla, P., Bertolini, F., Morelati, F., Marconi, M.: Preparation of leukocyte-free platelets for transfusion by filtration through cotton-wool. *Vox Sang. 44*, 115–120 (1983).
18. Fisher, M., Chapman, J. R., Ting, A., Morris, P. J.: Alloimmunisation to HLA antigens following transfusion with leukocyte-poor and purified platelet suspensions. *Vox Sang. 49*, 331–335 (1985).
19. Elias, M. K., Wolf de, J. Th. M., Pol, H., Das, P. C., Smit-Sibinga, C. Th.: Comparison of Leukocyte-depleted platelet concentrates (PC) by filtration and its clinical use. *Transfusion 27*, 531 (1987).
20. Snyder, E. L., De Palma, L., Napychank, P.: Use of polyester filters for the preparation of leukocyte-poor platelet concentrates. *Vox Sang. 54*, 21–23 (1988).

Haematologia 22 (3), pp. 189–193 (1989)

Functional Determination of C 1 Esterase Inhibitor in Fractions Derived from Plasma

A. G. BENNY, C. M. BRIMER

Auckland Regional Blood Service, Auckland Hospital, Private bag, Auckland, New Zealand

(Received 21 March, 1988; accepted 10 August, 1988)

A traditional esterolytic assay, a commercial chromogenic assay and an in-house chromogenic assay were compared for suitability in estimating C 1 esterase inhibitor (C 1 INH) activity in plasma and fractions obtained during preparation of a clinical concentrate of C 1 INH. The esterolytic and both of the chromogenic assays showed similar C 1 INH activity in unfractionated plasma samples. However, the esterolytic assay was significantly influenced by the buffer pH and concentration in fractionated plasma samples, and this often resulted in a significant overestimation of C 1 INH in some fractions. Similarly, the commercial C 1 INH assay kit did not always reliably estimate C1 INH activity in fractions derived from plasma. A modified in-house functional assay was found to be more reliable in measuring C 1 INH in fractionated plasma samples. There was a good correlation between this activity assay and immunological assay estimates of C 1 INH in fractions derived from plasma.

Keywords: C 1 esterase inhibitor, clinical concentrates, functional assay

Introduction

There are a number of published functional assays for estimation of C 1 INH in normal plasma and patients with hereditary angioneurotic edema [1, 2, 3, 4]. The traditional assay is based on the hydrolysis of an amino acid ester titrating released H+ as a means of following the activity of C 1 esterase (C 1s) [1]. More recently specific chromogenic peptide substrates for C 1s have been identified, and the use of these substrates in a functional chromogenic C 1 INH assay has been reported [2]. Most of these methods have been developed for estimation of C 1 INH in plasma. In our experience both the esterolytic and chromogenic methods are well suited for the determination of C 1 INH in both normal and abnormal plasma samples. However, we have often noted some inconsistencies when different methods were used to estimate C 1 INH in fractions derived from plasma. These fractions may contain significantly different concentrations of buffers and salts and differing levels of potentially interfering contaminating proteins.

In this report we used the traditional esterolytic assay, a commercial chromogenic kit, an in-house chromogenic assay and the standard immunological assay for C 1 INH. These assays are compared for reliability in estimating C 1 INH levels in fractions obtained during preparation of a clinical C 1 INH concentrate.

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Materials and Methods

Plasma samples: Individual blood samples were collected by clean venepuncture into 10 ml Vacutainer brand tubes (Becton-Dickinson, USA) containing 0.1 x volume of 0.13 M Trisodium citrate. The samples were centrifuged and the obtained plasma stored at −30 °C until assayed. A pool of 20 plasma donations prepared as a freeze-dried in-house coagulation factor standard was used for calibration of all assays on a day to day basis.

Reagents: CNBr activated Sepharose CL6B (Pharmacia, Uppsala, Sweden); human IgG prepared from outdated plasma by ion exchange chromatography on DEAE-Sephadex as described by Hoppe et al. [5]; chromogenic peptide substrate H-D-Valine-Serine-Arginine-pNa (KabiVitrum, Sweden); acetyl-tyrosine-ethyl ester (ATEE) and aprotonin (Sigma Chemical Co., St. Louis, MD, USA); polybrene (Aldrich Chemical Co. Inc., Milwaukee, WI, USA) were used. All other chemicals and reagents were analytical laboratory reagent grade from either Serva, Heidelburg, FRG or Sigma Chemical Co., St. Louis, MD, USA.

C 1 esterase preparation: Highly purified C 1 esterase was prepared by affinity chromatography on Human IgG Sepharose as described by Assimeh et al. [6]. The purified, active C 1s was diluted with 0.15 M NaCl to give an activity in the standard assay of 4.5 μM pNa released/min/ml (extinction coefficient for pNa = 9920).

C 1 INH concentrate preparation: C 1 INH was prepared from 15–30 l pools of normal human plasma by a modification [7] of the methods of Vogelaar et al. [8] and Wickerhauser et al. [9]. Samples were taken at each stage of the process and stored at −30 °C until assayed.

In-house C 1 INH assay: A modification of Wiman and Nilsson [2] was used. 500 μl Tris buffer (0.1 M Tris-HCl pH 7.3, containing 50 mg/l polybrene and 100 mg/l aprotonin) was preincubated with 100 μl C 1s and 50 μl of sample for 5 min at 37 °C. After preincubation, 50 μl of pre-warmed 5 mM chromogenic substrate was added and the reaction was monitored at 37 °C in a 10 nm path length cuvette at 405 nm in a Shimadzu UV 160 spectrophotometer. The linear change in absorbance over the first 90 seconds of the assay was calculated by the kinetic rate reaction program of the spectrophotometer. This activity was calibrated against a standard curve constructed by using several dilutions of a normal plasma pool.

ATEE assay: was performed by a modification of the method of Levy and Lepow [1]. The change in H+ concentration in the buffer mixture was continuously monitored by an Orion semi-micro pH probe coupled to an Orion 811 pH meter (Orion Research, Cambridge, MA, USA) and a Cole Parmer single channel chart recorder (Cole Parmer Instrument Co., Chicago, IL, USA). The linear change in H+ concentration was converted to units C 1 INH/ml by calibration against a normal plasma pool.

Commercial kit: The Berichrom C 1 Inactivator chromogenic peptide substrate assay kit was obtained from Behring Institute (Hoechst, FRG) and used exactly as recommended by the manufacturer. Two different batches of kit (Lot No. T8478 Expiry 12/88 and Lot No. 18987 Expiry 12/89) were used.

C 1 INH antigen assay: Electroimmunoassay of C 1 INH was performed as described by Laurell [10] using rabbit anti-human C 1 esterase inhibitor (Dakopatt, Denmark).

Results

The mean value and standard deviation of 25 random normal plasma samples assayed by each of the functional methods is shown in Table 1. All assays showed similar normal plasma levels of C 1 INH. The functional and immunological assay methods were used to assess C 1 INH levels in fractions obtained from 6 production batches of a C 1 INH concentrate (Table 2). For clarity, the data were corrected for a starting plasma C 1 INH level for each assay of 100%. Both the commercial chromogenic kit and the ATEE assays overestimated the C 1 INH in several fractions. In addition, both assays were unreliable and gave poor reproducibility, particularly with fractions containing salt concentrations greater than 0.18 M (final

Table 1

Mean assay values and standard deviations for C 1 INH activity in random normal plasma samples

n = 25	Behring assay (U/ml)*	ATEE assay (U/ml)*	In-house assay (U/ml)*
	0.87+/−0.1	0.98+/−0.13	1.09+/−0.14

* (C 1 INH units per ml of normal plasma calibrated against a 20 donor pool of normal plasma)

Table 2

Comparative analyses of C 1 INH recovery in fractions from production batches of concentrate

n = 6	Behring assay (%)	ATEE assay (%)	In-house assay (%)	Antigen assay (%)
Starting plasma	100	100	100	100
Supernatant plasma	165	30	34	29
DEAE eluant	39	50	27	40
PEG supernatant	NR*	30	32	41
PEG precipitate	NR*	23	4	5
Final concentrate	95	71	25	30

(Values are expressed as a percentage of the total amount of C 1 esterase inhibitor present in the starting plasma)

*NR — No result due to extreme non-linearity of the assay.

Table 3

Correlation coefficients of assays for the estimation of C 1 INH in fractions derived from plasma

	In-house	ATEE	Antigen
ATEE	0.81	—	—
Antigen	0.98	0.85	—
Behring	0.21	0.30	0.131

concentrate fraction). The correlation between the results obtained with the different assay methods for fractions from the C 1 INH concentrate preparation are presented in Table 3. There was a poor correlation between the Behring commercial assay and all the other assays. The in-house chromogenic method gave a good correlation with the immunological assay at all stages of the process. The ATEE assay correlated well with the in-house and antigen assays except in the fractions containing increased NaCl concentration.

Discussion

The in-house assay gave a good, reproducible linear standard curve and highly repeatable estimations of C 1 INH activity in both plasma samples and plasma derived fractions. The assay was not significantly influenced by different buffers in the different fractions or by moderate variation in the pH or the salt concentration of the fractions. Therefore a correction for the background non-C1s catalysed chromogenic activity observed in some fractions was possible. In contrast, the commercial chromogenic assay was supplied with the enzyme as part of the buffer reagent. Thus, the estimation of the background chromogenic activity was not possible. Moreover, it was impossible to construct a reliable standard curve using dilutions of normal plasma in saline, as the reproducibility of both the dilutions and the saline blank was very poor.

The ATEE assay, the in-house chromogenic functional assay and the immunological method all gave good linear standard curves over the range of 0.1 – 1.2 units C 1 INH per ml.

The antigen assay gave repeatable estimation of the total C 1 INH content in both plasma and plasma fractions, and a high coefficient of correlation of 0.98 with the in-house activity assay. For this reason the immunological assay is a useful and reliable routine method for analysis of fractions obtained during concentrate preparation.

We found the chromogenic substrate method of Wiman and Nilsson [2] to be the most reliable for the functional determination of C 1 INH activity in normal or abnormal plasma samples and in fractions derived from plasma.

Acknowledgements

The authors wish to thank the Blood Foundation of New Zealand and the Telethon Foundation (Year for the Disabled) for financial support. We also thank Dr. D. G. Woodfield, Medical Director, ARBC for his support and guidance.

References

1. Levy, L. R., Lepow, I. H.: Assay and properties of serum inhibitor of C 1 esterase. *Proc. Soc. Exp. Biol. Med. 10*, 608 (1959).
2. Wiman, B., Nilsson, T.: A new simple method for determination of C 1 esterase inhibitor activity in plasma. *Clin. Chim. Acta. 128*, 359 (1983).
3. Cullman, W., Kovary, P. M., Dick, W., Czarnetzki, B., Echternach-Happle, K.: A rapid method for functional determination of C 1 esterase inhibitor in plasma. *Clin. Chim. Acta. 119*, 237 (1982).
4. Schapira, M., Silver, L. D., Scott, C. F., Colman, R. W.: New and rapid functional assay for C 1 inhibitor in Human plasma. *Blood 59*, 719 (1982).
5. Hoppe, H. H., Mester, T., Hennig, W., Krebs, H. J.: Prevention of Rh-immunization. Modified production of IgG anti-Rh for intravenous application by ion exchange chromatography (IEC). *Vox Sang 25*, 308 (1973).
6. Assimeh, S. N., Bing, D. M., Painter, R. H.: A simple method for the isolation of the sub--components of the First component of complement by affinity chromatography. *J. Immunol. 113*, 225 (1974).
7. Benny, A. G., Brimer, C. M.: A chromatographic procedure for preparation of a high purity C 1 Esterase Inhibitor concentrate. *J. Chromatog. (Biomedical Applications) 433*, 363 (1988).
8. Vogelaar, E. F., Brummelhuis, H. G. J., Krijnen, H. W.: Contributions to the optimal use of Human blood III. Large scale preparation of human C 1 esterase inhibitor concentrate for clinical use. *Vox Sang. 26*, 118 (1974).
9. Wickerhauser, M., Williams, C., Kolen, B. L., Busby, T. F.: Heat stability of lyophilised C 1 inactivator concentrates. *Vox Sang. 53*, 1 (1987).
10. Laurell, C-B., Quantitative estimation of proteins by electrophoresis in agarose gels containing antibodies. *Anal. Biochem. 15*, 45 (1966).

Haematologia 22 (3), pp. 195–199 (1989)

Central Nervous System Acute Promyelocytic Leukaemia: a Report of Three Cases

R. MARRA[1], S. STORI[1], L. PAGANO[1]*, G. FIORITONI[1], C. RABITTI[2], S. SICA[1], G. LEONE[1], G. TORLONTANO[1], B. BIZZI[2]

[1] Istituto di Semeiotica Medica, Universita Cattolica S. Cuore Roma, Cattedra di Ematologia, Chieti-Pescara

[2] Istituto di Anatomia ed Istologia Patologica, Universita Cattolica S. Suore, Largo Agostino Gemelli 8, I-00168 Roma, Italy

(Received 27 May, 1988; accepted 22 September, 1988)

Authors report 3 cases of acute promyelocytic leukaemia, in which the central nervous system was involved in the first period of relapse. The clinical features of these patients are discussed. Authors conclude that central nervous system involvement in acute promyelocytic leukaemia must be considered a very bad prognostic sign, because in these cases a rapidly progressive course may be expected.

Keywords: acute promyelocytic leukaemia, central nervous system

Introduction

The incidence of central nervous system (CNS) involvement during the course of acute non-lymphoblastic leukaemia (ANLL) has become more common [1, 2, 3]. It is more frequent in M5 F.A.B. subtype patients [4, 5], whereas it is a rare event in the course of acute promyelocytic leukaemia (APL); in fact, to date only few cases of APL with CNS involvement have been described [6, 7, 8, 9, 10, 11]. In our study we report 3 cases of APL who developed CNS infiltration during the first relapse of leukaemia.

Case report

Between January 1983 and June 1986, 21 cases of APL were seen for the first time in the Divisions of Haematology of the Catholic University, Rome and Pescara Hospital. Among the 12 patients who achieved complete remission (CR) 1 died in CR, 3 are still in CR and 8 had a bone marrow relapse; in 3 of the latter CNS involvement developed.

* To whom correspondence should be addressed.

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Case No. 1

A 51 year old man was first seen on August 3, 1983, with a one-month history of fever and asthenia. Physical examination did not reveal any relevant pathological sign. A haemogram showed a haemoglobin level of 13.3 g/dl, a leukocyte count of 1.700/mm^3 without peripheral blasts and a platelet count of 176.000/mm^3. Bone marrow examination demonstrated 55% malignant promyelocytes and a diagnosis of acute promyelocytic leukaemia was made.

Cytogenetic study was not available because of poor examination material. The coagulation study was within the normal range. As the patient was resistant to the treatment with 2 courses of Daunoblastin (DNR) 60 mg/mq 1→3, a second line therapy with pOMp combination was started [12] (3 courses). CR was achieved in November 1983, and a consolidation therapy with TRAP combination [13] was begun. All therapy was stopped in June 1984. The patient remained in CR until January 1985 when asthenia, restlessness, vomiting and fever (40.7 °C) arose. The patient died in February 1985, unresponsive to chemotherapy (high doses of Aracytin). Autopsy revealed a diffuse leptomeningeal promyelocyte leukaemic infiltration.

Case No. 2

A 57 year old woman was first seen in August 8, 1985 for fever and mucocutaneous haemorrhages. Physical examination showed hepatomegaly and submandibular lympadenopathy. Haemoglobin level was 6.7 g/dl; leukocyte count 7.700/mm^3 with 92% blasts; platelet count was 17.000/mm^3. Malignant Auer-positive promyelocytes consisted of about 80% bone marrow cells. Cytogenetic study was no

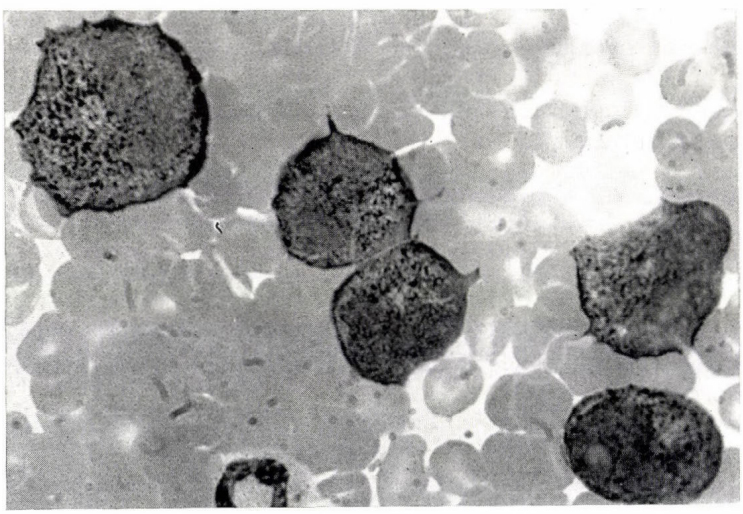


Fig. 1. Case N°2 — malignant promyelocytes in CSF (May—Grundwald—Giemsa 400x)

performed. Coagulation study demonstrated a mild disseminated intravascular coagulipathy (DIC). Cerebrospinal fluid (CSF) examination did not reveal any abnormality. The patient was given DNR 2 mg/kg on days 1 to 6 and achieved CR in September 1985. Consolidation therapy was performed with 4 courses of DAT combination [14]. The therapy was stopped on December 26, 1985. In August 1986 the patient presented headache followed by diplopia, paresis of VI and VII pairs of cranial nerves, papillhoedema and polineuritis. CT scan did not demonstrate any pathological findings. Haemogram and coagulation parameters were normal. The patient refused admittance to the hospital until 20 days later, when cutaneous haemorrhages appeared. CSF examination showed malignant promyelocytes (Fig. 1); the bone marrow was also infiltrated by the same cells. The patient received Methotrexate intrathecal therapy and Aracytin systemic therapy, but died due to sepsis after a few days.

Case No. 3

A 53 year old man was first seen on February 17, 1986 with a 15 day story of fever, asthenia and mucocutaneous haemorrhages. Physical examination revealed a mild hepatomegaly, but neither splenomegaly nor adenopathy. A haemogram showed a haemoglobin level of 7.8 g/dl; a leukocyte count of 42.000/mm^3 with 70% blasts and a platelet count of 50.000/mm^3. Bone marrow examination revealed the presence of 90% Auer-positive malignant promyelocytes. The bone marrow cytogenetic study demonstrated a 46 xy+(15; 17) (q22; q11) and 47 xy+8 (15; 17) pattern. Coagulation study showed a DIC. The patient received DNR 2/m/kg

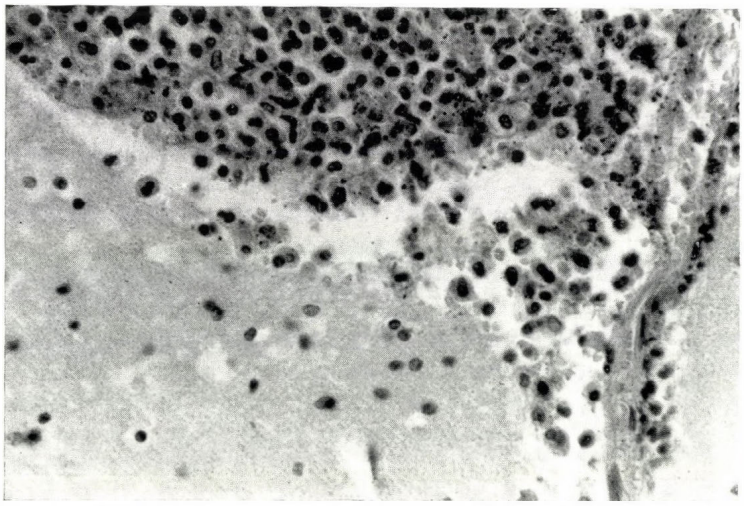


Fig. 2. Case N°3 — infiltration of cerebral and cerebellar nervous parenchyma by malignant promyelocytes (May—Grundwald—Giemsa 250x)

on days 1 to 6 and achieved CR in March 1986; then he was given a consolidation therapy with DAT combination. In May 1986, at the 3rd course the chemotherapy was stopped because of viral hepatitis. On August 6, 1986, a bone marrow examination was performed while the patient did not take any medicine. It showed 2% of blasts; the cytogenetic study did not reveal any abnormality. On August 22, 1986, the patient was admitted to a surgical division of our hospital because of a 10 day history of low back pain followed by an abdominal tenderness suspected to be an intestinal occlusion. After 2 days he developed a spastic paraplegia. A bone marrow examination showed a promyelocytic infiltration and the first coagulation study did not reveal any abnormality. A CSF examination showed the presence of a large number of blastic cells (glicorrachia 47 mg/dl; proteinorrachia 1328 mg/dl; malignant promyelocytes 408/mm^3). On August 25, 1986, the patient had a seizure and hyperthermia (41.7 °C). The patient died on August 26, 1986, before antiblastic therapy could be started. Autopsy showed a diffuse cerebral and cerebellar involvement; the infiltration was massive and subarachnoidal space was filled with "a band" of promyelocytic cells, without direct invasion of cerebral or cerebellar nervous parenchyma and without forming "a mass" (Fig. 2).

Discussion

In our patients at the onset of ANLL no clinical or laboratory features were present which might have been prognostic for CNS involvement. Bone marrow cytogenetic study was performed only in the patient with 42.000/mm^3 leukocytes; this demonstrated the existence of a chromosomic abnormality. Trisomia 8 has been reported to be the second most frequent cytogenetic abnormality in cases of acute myelomonocytic leukaemia with CNS involvement [9]. For this reason it is worthwhile to perform diagnostic lumbar puncture periodically in APL patients with this cytogenetic abnormality in order to evaluate the relationship between CNS infiltration and trisomia 8.

To date only few cases of APL with CNS involvement have been described [6, 7, 8, 9, 10, 11], and some studies with a large number of APL patients do not report cases of neuromeningosis [15, 16]. Therefore the relatively high incidence of CNS infiltration in our patient may be casual. However, it must be considered that leukaemic patients with CNS involvement sometimes have only CSF blastic cells without clinical signs of neuromeningosis [5]. As lumbal puncture is not routinely performed, the frequency of this complication in APL could be underestimated. This fact, together with the rapid progression of the leukaemia observed in our patients with CNS involvement and in the cases reported in the literature seem to make a diagnostic lumbar puncture advisable when CR is achieved and therapy is terminated.

References

1. Ruggero, D., Baccarani, M., Zaccaria, A., Gobbi, M., Tomasini, I., Tura, S.: Nervous system involvement in adult acute myeloid leukemia: a report of five cases. *Haematologica 62*, 3, 317 (1977).
2. Shoenfeld, Y., Rothenberg, M., Berliner, S., Gallant, L. A., Garfinkel, D., Shaklal, M.: Absence of predictive parameters for CNS involvement in adult non-lymphocytic leukemia at the time of diagnosis. *Scand. J. Haematol. 28*, 335 (1982).
3. Tobelem, G., Jacquillant, C., Chastang, C., Aucler, M. F., Lechevallier, T., Weil, M., Daniel, M. T., Flandrin, G., Haroussean, J. L., Schaison, G., Bairon, M., Bernard, J.: Acute monoblastic leukemia: a clinical and biological study of 74 cases. *Blood 55*, 71 (1980).
4. Meyer, R. J., Ferreira, P. P. C., Cuttner, J., Greenberg, M. L., Goldberg, J., Holland, J. F.: Central nervous system involvement at the presentation in acute granulocytic leukemia. *Am. J. Med. 68*, 691 (1980).
5. Peterson, B. A., Brunning, R. D., Bloomfield, C. D., Hurd, D. D., Gan, J. A., Peng, G. T., Goldman, A. I.: Central nervous system in acute nonlymphocytic leukemia. *Am. J. Med. 83*. 464 (1987).
6. Audenbert, A. A., Dousset, I., Krulik, M., Zylberait, D., DeBray, J.: Méningite leucémique au cours d'une leucémie aigué promyélocytaire. *Sem. Hop. Paris 56*, 325 (1980).
7. Bernard, J., Lasneret, J., Chome, J., Levy, J. P., Boiron, M.: A cytological and hystological study of acute promyelocytic leukemia. *J. Clin. Path. 16*, 319 (1963).
8. Pogliani, M. E., Fowst, C., Marozzi, A., Salvatore, M., Polli, E. E.: Cerebral parenchimal involvement in acute promyelocytic leukemia. A case report. *Haemotologica 73*, 71 (1988).
9. Holmes, R., Keating, M. J., Cork, A., Broach, Y., Trujillo, J., Dalton, W. T., McCredie, K. B., Freireich, E. J.: A unique pattern of central nervous system leukemia in acute myelomonocytic leukemia associated with inv (16) (pl3q32). *Blood 65*, 1071 (1985).
10. Pui, C. H., Dahl, G. R., Kalwinsky, D. K., Look, A. T., Mirro, J., Dodge, R. K., Simone, J. V.: Central nervous system leukemia in children with acute non lymphoblastic leukemia. *Blood 66*, 1062 (1985).
11. Solano-Vercet, C., Escudero, A., Fernandez-Ranada, J. M.: Meningeal relapse in acute promyelocytic leukemia. *Acta Haematol. 70*, 137 (1983).
12. Leone, G., Pagano, L., Marra, R., Landolfi, R., Cudillo, L., Storti, S., Mango, G., Alfano, G., D'Onofrio, G.: Combination of 6-mercaptopurine, vincristine, methotrexate and prednisone in resistant or relapsed acute non lymphoid leukemia. *Cancer Treat Rep. 71*, 751 (1987).
13. Gluckberg, H., Cheever, M. A., Firewell, V. T., Fefer, A., Sole, G. E., Thomas, E. D.: High dose combination chemotherapy for ANLL in adults. *Cancer 48*, 1073 (1981).
14. Gale, R. P., Cline, M. J.: High remission induction rate in acute myeloid leukemia. *Lancet 1* (8010), 497 (1977).
15. Kantarjian, H. M., Keating, M. J., Walters, R. S., Estey, E. H., McCredie, K. B., Smith, T. L., Dalton, W. T., Cork, A., Trujillo, J. M., Freireich, E. J.: Acute promyelocytic leukemia. *Am. J. Med. 80*, 789 (1986).
16. Jones, M. E., Saleem, A.: Acute promyelocytic leukemia. A review of literature. *Am. J. Med. 65*, 673 (1978).

Abstracts

Macrophage recognition of periodate-treated erythrocytes: Involvement of disulfide formation of the erythrocyte membrane proteins. M. Beppu, H. Ochiai and K. Kikugawa (Tokyo College of Pharmacy, Tokyo, Japan). *Biochim. Biophys. Acta 979*, 35 (1989).

Upon exposure to 2 mM periodate at 0°C for 15 min, mouse erythrocytes underwent membrane lipid oxidation, oxidation of cell surface sialyl residues into aldehyde-bearing derivatives, and oxidation of SH groups of the membrane proteins into disulfides. The periodate-treated erythrocytes exhibited a remarkable increase in rosette attachment to resident mouse peritoneal macrophages in the absence of serum. The relationship between the oxidation of the membrane constituents and the macrophage recognition of these cells was investigated. Periodate treatment of erythrocytes in the presence of butylated hydroxytoluene, an inhibitor of lipid oxidation, did not affect the subsequent attachment of the erythrocytes to the macrophages. Reduction of the periodate-treated erythrocytes with borohydride or cyanoborohydride did not affect the erythrocyte attachment. Neuraminidase treatment of erythrocytes before periodate did not affect the attachment either. On reduction of the disulfides of the membrane proteins with dithiothreitol, the periodate-treated erythrocytes lost their ability to attach to the macrophages. Erythrocytes treated with an SH--oxidizing agent, diamide, were then examined for the macrophage recognition. The diamide-treated cells also showed rosette attachment to the macrophages in the absence of serum, but did not when reduced with dithiothreitol. These results indicate that oxidation of the SH groups of the membrane proteins to disulfides causes reversible membrane changes that macrophages recognize, and it is this mechanism that is responsible for the macrophage recognition of the periodate-treated erythrocytes.

G. Gárdos

Covalent modification of serum transferrin with phospholipid and incorporation into liposomal membranes. P. Afzelius, E. J. F. Demant, G. H. Hansen and P. B. Jensen (Department of Biochemistry C, Panum Institute, University of Copenhagen, Copenhagen, Denmark). *Biochim. Biophys. Acta 979*, 231 (1989).

A method is described for incorporation of water-soluble proteins into liposomal membranes using covalent protein-phospholipid conjugates in detergent solution. A disulfide derivative of phosphatidylethanolamine containing a reactive N-hydroxysuccicinimide ester group is synthesized, and the derivative is reacted with serum transferrin in deoxycholate-containing buffer. Disulfide-linked transferrin-phosphatidylethanolamine conjugates containing up to 6 mol phospholipid/mol protein are prepared. The amphiphilic conjugates have solubility properties very similar to integral membrane proteins. The conjugates self-associate to form protein micelles of narrow size distribution (Stokes radii 6—7 nm), and in the presence of excess phospholipid (egg phosphatidylcholine), they readily incorporate into liposomal membranes upon removal of deter-

gent. Stable incorporation into liposomes requires the introduction of two molecules of phosphatidylethanolamine into the transferrin. Using the disulfide linker to release transferrin from the liposomes, evidence is presented for a function of the phosphatidylethanolamine as an anchor-molecule into the liposomal lipid. Optimal conditions for preparation of homogeneous liposomes with diameters in the range 30–125 nm and with a varying content of transferrin are defined. The liposomes appear well suited for studies on liposome-cell membrane interactions.

G. Gárdos

Room temperature electron spin resonance of superoxide dismutase-loaded liposomes and erythrocytes. A direct approach to the interaction of O_2^- with cells. L. Marcocci, I. Mavelli, A. Di Guilio, J. Z. Pedersen, A. Desideri and G. Rotilio (Department of Biology, Tor Vergata University of Rome, Rome, Italy). *Biochim. Biophys. Acta 979*, 99 (1989).

Human erythrocytes were enriched with bovine superoxide dismutase by fusion with liposomes containing the entrapped enzyme. Liquid solution ESR of intact cells at room temperature was used to measure directly the increase in the superoxide dismutase content. From the spectral characteristics (g-value and hyperfine splitting tensor), the structural integrity of the Cu site of the enzyme was found to be unaffected by the liposome preparation procedure or the incubation with cells. Changes in the ESR signal size were used to test directly the interaction of superoxide with the enzyme entrapped in liposomes or delivered to erythrocytes. It was found that the liposome-entrapped enzyme does not react with externally generated O_2^-, but once delivered to red blood cells this reaction can take place. This is the first demonstration of O_2^--scavenging activity by superoxide dismutase delivered into an intact cell structure and is therefore to be considered as strong evidence for activity of this enzyme under in vivo conditions.

G. Gárdos

Inhibition of inorganic anion transport across the human red blood cell membrane by chloride-dependent association of dipyridamole with a stilbene disulfonate binding site on the band 3 protein. B. Legrum and H. Passow (Max Planck Institute für Biophysik, Frankfurt am Main, FRG). *Biochym. Biophys. Acta 979*, 193 (1989).

The inhibition of inorganic anion transport by dipyridamole (2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido [5,4-d] pyrimidine) takes place only in the presence of Cl^-, other halides, nitrate or bicarbonate. At any given dipyridamole concentration, the anion flux relative to the flux in the absence of dipyridamole follows the equation: $J_{rel} = (1 + \alpha_2 [Cl]^- / (1 + \alpha_4 [Cl^-])$ where α_2 and α_4 are independent of $[Cl^-]$ but dependent on dipyridamole concentration. At high $[Cl^-]$ the flux approaches α_2/α_4, which decreases with increasing dipyridamole concentration. Even when both $[Cl^-]$ and dipyridamole concentrations assume large values, a small residual flux remains. The equation can be deduced on the assumption that Cl^- binding allosterically increases the affinity for dipyridamole binding to band 3 and that the bound dipyridamole produces a non-competitive inhibition of sulfate transport. The mass-law constants for the binding of Cl^- and dipyridamole to their respective-binding sites are about 24 mM and 1,5 μM, respectively (pH 6.9, 26 °C). Dipyridamole binding leads to a displacement of 4,4'-dibenzoylstilbene-2,2'-disulfonate (DBDS) from the stilbenedisulfonate binding site of band 3. The effect can be predicted quantitatively on the assumption that the Cl^--promoted dipyridamole binding leads to a competitive replacement of the stilbenedisulfonates. For the calculations, the same mass-law constants for binding of Cl^- and dipyridamole can be used that were derived from the kinetic studies on Cl^--promoted anion transport inhibition. The newly described Cl^- binding site is highly selective with respect to Cl^- and other monovalent anion species. There is little competition with SO_4^{2-}, indicating that Cl^- binding involves other than purely electrostative forces. The affinity of the binding site to Cl^- does not change over the pH range 6.0 – 7.5. Dipyridamole binds only in its deprotonated

state. Binding of the deprotonated dipyridamole is pH-independent over the same range as Cl^- binding.

B. Sarkadi

Membrane proteins in senescent erythrocytes. T. Suzuki and G. L. Dale (Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, CA, USA). *Biochem. J. 257*, 37 (1989).

The examination of erythrocyte senescence has been facilitated by recent advances in techniques for the isolation of aged red cells. One of these methods, which uses biotinylated rabbit erythrocytes, has been used to examine the state of membrane proteins in effete cells. These aged red cells were found to have normal rations of α-spectrin and β-spectrin as well as normal levels of ankyrin. The observation concerning ankyrin is particularly important due to the sensivitity of this protein to proteolysis and the postulated action of proteinases in the aging process. The senescent erythrocytes were also found to have an altered ratio of bands 4.1a and 4.1b without any apparent change in the total level of 4.1. In addition, the analysis of the aged cell membranes did not show any large-molecular-mass aggregated protein at the origin of the SDS/polyacrylamide gels, indicating a lack of transglutaminase activity in the senescence process for rabbit erythrocytes. These results indicate that aging of the rabbit erythrocyte is not accompanied by gross proteolytic degradation or transglutaminase-catalysed cross-linking of membrane components.

Ilma Szász

Ca^{2+}-mediated activation of human erythrocyte membrane Ca^{2+}-ATPase. K. S. Au, M. F. Lee and Y. L. Siu (Department of Biochemistry, University of Hong Kong, Hong Kong). *Biochim. Biophys. Acta 978*, 197 (1989).

Ca^{2+}-ATPase of human erythrocyte membranes, after being washed to remove Ca^{2+} after incubation with the ion, was found to be activated. Stimulation of the ATPase was related neither to fluidity change nor to cytoskeletal degradation of the membranes mediated by Ca^{2+}. Activation of the transport enzyme was also unaffected by detergent treatment of the membrane, but was suppressed when leupeptin was included during incubation of the membranes with Ca^{2+}. Stimulation of the ATPase by a membrane-associated Ca^{2+}-dependent proteinase was thus suggested. Much less 138kDa Ca^{2+}-ATPase protein could be harvested from a Triton extract of membranes incubated with Ca^{2+} than without Ca^{2+}. Activity of the activated enzyme could not be further elevated by exogenous calpain, even after treatment of the membranes with glycodeoxycholate. There was also an overlap in the effect of calmodulin and the Ca^{2+}-mediated stimulation of membrane Ca^{2+}-ATPase. While K_m(ATP) of the stimulated ATPase remained unchanged, a significant drop in the free-Ca^{2+} concentration for half-maximal activation of the enzyme was observed.

B. Sarkadi

Inhibition of the glycolytic pathway by methylglyoxal in human platelets. G. Leoncini, M. Maresca and E. Buzzi (Istituto Policattedra di Chimica Biologica, Universita di Genova, Genova, Italy). *Cell Biochem. Funct. 7*, 65 (1989).

The incubation of human platelets with methylglyoxal and glucose produces a rapid transformation of the ketoaldehyde to d-lactate by the glyoxalase system and a partial reduction in GSH. Glucose utilization is affected at the level of the glycolytic pathway. No effect of the ketoaldehyde on glycogenolysis and glucose oxidation through the hexose monophosphate shunt was demonstrated. Phosphofructokinase, fructose 1,6-diphosphate (Fl, 6DP) aldolase, glyceraldehyde 3-phosphate dehydrogenase and 3--phosphoglycerate mutase were mostly inhibited by methylglyoxal. A decrease in lactate and pyruvate formation and an accumulation of some glycolytic intermediates (fructose 1,6 diphosphate, dihydroxyacetone phosphate, 3-phosphoglycerate) was observed. Moreover methylglyoxal induced a fall in the metabolic ATP concentration.

Since methylglyoxal is an intermediate of the glycolytic bypass system from dihydroxyacetone phosphate to d-lactate, it may be assumed that ketoaldehyde exerts a regulating effect on triose metabolism.

Ágnes Enyedi

Release of Ca^{2+} by inositol 1,4,5-triphosphate in platelet membrane vesicles is not dependent on cyclic AMP-dependent protein kinase. F. O'Rourke, G. B. Zavoico and M. B. Feinstein (Department of Pharmacology, University of Connecticut Health Center, Farmington, CT, USA). *Biochem. J. 257*, 715 (1989).

In contrast with previous reports, it was found that membrane-protein phosphorylation by the catalytic subunit (CS) of cyclic AMP-dependent protein kinase had no effect on Ca^{2+} uptake into platelet membrane vesicles or on subsequent Ca^{2+} release by inositol 1,4,5-trisphosphate (IP_3). Furthermore, IP-20, a highly potent synthetic peptide inhibitor of CS, which totally abolished membrane protein phosphorylation by endogenous CS, also had no effect on either Ca^{2+} uptake or release by IP_3. Commercial preparations of protein kinase inhibitor protein (PKI) usually had no effect, but one preparation partially inhibited Ca^{2+} uptake, which is attributable to the gross impurity of the commercial PKI preparation. IP_3-induced release of Ca^{2+} was also unaffected by the absence of ATP from the medium, supporting the conclusion that Ca^{2+} release by IP_3 does not require the phosphorylation of membrane protein.

Ágnes Enyedi

Stimulation of phosphatidylinositol 4,5-bisphosphate phospholipase C activity by phosphatidic acid. S. Jackowski and C. O. Rock (Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee, USA). *Arch. Biochem. Biophys. 268*, 516 (1989).

Phosphatidic acid was a potent activator of the phosphatidylinositol 4,5-bisphosphate (PtdIns-P_2) phospholipase C activity associated with human platelet membranes. Lysophosphatidic acid was half as active as phosphatidic acid, and shortening the fatty acid chain reduced the effectiveness of the corresponding phosphatidic acid. Compounds lacking either the phosphate group (diacylglycerol or phorbol ester) or the fatty acid (glycerol phosphate) were not activators. When the negative charge was contributed by a carboxyl group (fatty acid or phosphatidylserine), stimulation of phospholipase C was weak but detectable. Structural analogs of phosphotidic acid (lipopolysaccharide, lipid A, and 2,3-diacylglucosamine 1-phosphate) were less effective but also enhanced PtdIns-P_2 hydrolysis. Phosphatidic acid potentiated the activation of phospholipase C by α-thrombin, chelators, and guanine nucleotides. Phosphatidylinositol 4-phosphate and PtdIns-P_2 were also effective activators of PtdIns-P_2 degradation. Other phospholipids were without effect. The production of inositol 1,4,5-trisphosphate and diacylglycerol via the activation of phospholipase C provides a rationale for the cellular responses evoked by phosphatidic acid and the ability of this phospholipid to potentiale and initiate hormonal responses.

Ágnes Enyedi

Book Review

BLOOD SUBSTITUTES. Edited by Thomas M. S. Chang and Robert P. Geyer. 1989. 736 pages, bound, illustrated. $ 99.75 (U.S. and Canada); $ 119.50 (All other countries). ISBN: 0–8247–8027–2. (Prices subject to change without notice.)

The AIDS epidemic has accentuated such haematological problems as a shortage of donor blood and possible complications with transfusion blood. As a result, clinicians and researchers have become increasingly interested in developing blood substitutes for transfusions as well as for therapeutic uses and research.

BLOOD SUBSTITUTES—*written by 350 leading international experts*—covers a wide variety of important clinical topics, including transfusion, blood replacement, hemorrhagic shock, exchange transfusion, cancer therapy, heart and brain ischemia, surgery, inhalation anesthesia, and organ perfusion.

The result of the *Third International Symposium on Blood Substitutes* organized by the Artificial Cells and Organs Research Centre of McGill University, BLOOD SUBSTITUTES gives a balanced, comprehensive perspective of the subject unavailable elsewhere . . . indicates future trends in such areas as perfluorochemical, stroma-free, microencapsulated, conjugated, and bovine hemoglobins, synthetic hemes, red blood cell engineering, and cofactor incorporation . . . evaluates commercial and experimental emulsion in biological research . . . and much more!

READERSHIP: Hematologists; biochemists; cardiovascular surgeons; physiologists; blood bank personnel; biotechnologists; oncologists; pharmacologists; cytologists; biomedical engineers; protein and polymer chemists; critical care and emergency room physicians; transplantation and artificial organs biomedical researchers; ancillary reading for graduate/medical courses in hematology, physiology, or surgery; and professional seminars and continuing education courses on hemorrhagic shock, AIDS, transfusion, and trauma, emergency, or cardiovascular surgery.

Printed in Hungary

Akadémiai Kiadó és Nyomda Vállalat, Budapest

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 brackets. 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.

Authors are entitled to 50 reprints free of charge.

HAEMATOLOGIA
VOLUME 22 · NUMBER 3 **198**

Contents

Petri, I. B., Gyódi, É., Kaiser, G., Kassai, M., Petrányi, G. Gy.: Dynamism of the development of "Fc receptor-blocking antibodies" after immunization with buffy coat 135

Invernizzi, R., Perugini, O., De Fazio, P., Bonetti, F., Nespoli, L.: Cytochemistry of dipeptidylaminopeptidase IV in T lymphoblastic leukaemia — lymphoma 143

Merétey, K., Jakab, Á., Szilassy, K., Medgyesi, G. A.: IgE levels in normal human sera and IgG preparations 151

Kopff, M., Zakrzewska, I., Klem, J., Strzelczyk, M.: Relationship between adenosine deaminase activity (ADA) and inosinemonophosphate (IMP) accumulation in fresh erythrocytes incubated with adenosine and dipyramidole 161

Hernández, P., Dorticós, E., Espinosa, E., Gonzáles, X., Svarch, E.: Clinical features of hepatic sequestration in sickle cell anaemia 169

Tejuca, M., Martinez, G., Ferreira, R., Hernández, A., Ferrer, L., Goicochea, A., Hernández, A. C., Coyeula, R., Wade, M., Colombo, B.: α-thalassemia changes the cell density profile in sickle cell anaemia 175

Koerner, K.: Preparation of leukocyte-poor platelet concentrates for transfusion by filtration through a new cellulose acetate filter 181

Benny, A. G., Brimer, C. M.: Functional determination of C 1 esterase inhibitor in fractions derived from plasma 189

Marra, R., Stori, S. Pagano, L., Fioritoni, G., Rabitti, C., Sica, S. G. L., Torlontano, G., Bizzi, B.: Central nervous system acute promyelocytic leukemia: a report of three cases 195

Abstracts

Book Review

Index: 26.861

VOLUME 22 • NUMBER 4 • 1989

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

///VSP///

UTRECHT, THE NETHERLANDS,
TOKYO, JAPAN

AKADÉMIAI KIADÓ, BUDAPEST

HAEMATOLOGIA

Haematologia is an international journal publishing original papers on all clinical and theoretical aspects of haematology. In addition to regular papers, book reviews and abstracts of selected papers from other periodicals also are a special feature of the journal.

Haematologia, published at quarterly intervals, provides the reader with complex and up-to-date information.

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

Copy Editor:
D. Keszthelyi

Distributor for

Albania, Bulgaria, China, Cuba, Czechoslovakia, German Democratic Republic, Korean People's Republic, Mongolia, Poland, Romania, U.S.S.R., Viet-Nam, and Yugoslavia:

K U L T U R A
Hungarian Foreign Trading Company
P.O.B. 149, H-1389 Budapest 62, Hungary

For all other countries subscriptions should be sent to

VSP
P.O.B. 346
3700 AH ZEIST, The Netherlands

Subscription price for volume 22:

DM 330,– (including postage)

Haematologia is covered in Current Contents/Clinical Practice, ISI/Biomed and the SCI.

VOLUME 22 · NUMBER 4

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

VSP UTRECHT, THE NETHERLANDS, TOKYO, JAPAN

AKADÉMIAI KIADÓ, BUDAPEST

1989

Haematologia 22 (4), pp. 201–214 (1989)

Epidemiology of Leukaemia*

JEAN BERNARD

Institut de Recherches sur les Maladies du Sang,
Hôpital Saint-Louis, 2 Place du Docteur-Fournier,
75475 Paris Cedex 10, France

(Received 5 September, 1988; accepted 28 September, 1988)

The history of the epidemiology of leukaemia provides us with models which are very useful even today and their implications for the future are still more important. Thus, in the present study, we shall review the history and present state of the epidemiology of leukaemia, as well as its future prospects.

In the fifth century B. C. Hippocrates already advocated studying the climatic, geographical and physical environment as well as the behaviour of individuals and of their eating and drinking habits in order to understand the origin of a disease.

At present, the epidemiology of leukaemia is still based on these ancient precepts.

Keywords: leukaemia

History of the epidemiology of leukaemia

There are three periods in the history of the epidemiology of leukaemia. Each will be described here with reference to recent developments.

First period

The first period was marked by three main features:

- the description of occupational and therapeutic leukaemia,
- virus-induced leukaemia in animals, and
- the concept of geographical haematology.

Occupational leukaemia

A. *Radiation.* The history of the various types of leukaemia induced by radiation, which must be considered first, includes two opposite evolutionary trends and one uncertain trend.

* Lecture presented at the XXII. Congress of the International Society of Haematology, Milano, 1988

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Table 1

Chinese study on cancer among medical diagnostic X-ray workers

	Diagnostic X-ray workers 27.011	Other medical specialists 25.782
Leukaemias	30	8
Lung cancer	22	25

1. First off all, a net *reduction* in the frequency of radioleukaemia among physicists (for instance, among the members of the Curie family, with their glorious but tragic record of five Nobel Prizes and two cases of leukaemia), radiologists and patients (including those with spondylosis, children irradiated during pregnancy and above all the number of patients with cancer of the corpus of the uterus).

Where protection is not correct and radiation exposure remained high as in China in 1950, the risk of leukaemia persisted, as shown below by the large Chinese study concerning cancer among medical diagnostic X-ray workers (Table 1)

2. Secondly, the recent increase in the frequency of *leukaemia caused by war injuries*, with the three findings made at Hiroshima:

- the rise in the number of cases of leukaemia,
- the periods elapsing before their manifestation, and
- the unequal degrees of sensitivity of the persons exposed to war injuries.

3. Thirdly, the element of uncertainty, and the fact that it is at present impossible to evaluate correctly the possible effects of the peaceful uses of nuclear energy and of accidents in nuclear industries.

Radiogenic leukaemia revisited:

Table 2

Frequency of leukaemia after radiotherapy for cancer of the corpus uteri

First cancer	Leukaemia
All sites	1.6
Trunk sites	2.4
Breast cancer	2.2
Cancer of the corpus uteri	10.3

Table 3

Rate of clonal chromosomal aberrations in patients with radiotherapy related leukaemias

Leukaemias	Parients	Clonal chromosomal aberrations
A. N. L. L.	8	3
A. L. L.	3	2
C. G. L.	3	3

The clinical forms and evolution of radiogenic leukaemia vary, depending on its etiology. In a recent study entitled Radiogenic Leukaemia Revisited a distinction was drawn between the two classes of leukaemia observed in Japan after the atomic bomb explosions: 1) the class similar to the cases observed after irradiation for cancer of the uterus, which are the same as the de novo adult types, 2) the class similar to the cases observed after irradiation for spondylitis, often preceded by a period of myelodysplasia, and expressed in erythromyeloblastic and megakaryoblastic forms.

The frequency of leukaemia after radiotherapy for cancer of the corpus uteri has also been evaluated and found to be as shown in Table 2.

B. *Chemicals and Industry*. The second main type of occupational leukaemia is that induced by chemicals, particularly *benzene*. The stages in the history of benzene-induced leukaemia include.

– the first descriptions,

– the complete study which, together with Leon Braier, we presented at the Second International Congress of Haematology in Cambridge in 1950.

The importance of the cytogenetic studies has frequently been noted.

In a recent work e.g. Philip and Pedersen-Bjorgaard (1988) found the following rate of clonal chromosomal aberrations in patients with radiotherapy related leukaemias (Table 3):

– subsequent debates, and

– the present state.

A recent remarkable Chinese study by Yen, Zhang and Wang (1987) seems to make it possible to end the controversy regarding the effects of chemical substances.

Table 4 summarizes the results of the comparison they made between cohorts comprising equal numbers of workers exposed or not exposed to benzene respectively.

Recently Flandrin et al. (1984, 1987) have discovered that patients with hairy cell leukaemia were frequently exposed to radiations and chemicals. It may be compared with another B-cell neoplasm, myeloma, the frequency of which was augmented in Hiroshima whereas B-CLL does not seem to be caused by such events.

Table 4

Comparison of workers exposed to and not exposed to benzene

Plants using benzene	Plants not using benzene
233	83
Workers exposed to benzene	Workers not exposed to benzene
28.460	28.257
Years of exposure	Years of non exposure
178.556	199.200
Number of leukaemia cases	Number of leukaemia cases
30	*4*
(14/100.000)	(2/100.000)

In connection with the history of chemically-induced leukaemia I should mention the experimental leukaemia induced in the rat by intramedullar injection of tar by Jean Bernard between 1933 and 1936, or of hydrocarbons by Storti in 1936 (Bernard 1934, 1950).

Therapeutic leukaemia. The number of leukaemia cases induced by certain types of chemotherapy is much larger than the number of cases of occupational leukaemia.

Over the past twenty years, research in this field has revealed:

1. the existence of therapeutic forms and their relative frequency, which varies from 2 to 10%;

2. the diversity of the diseases treated, expecially Hodgkin's disease and various forms of myeloma, as well as non-malignant diseases such as polyarthritis and psoriasis. Curiously, there have been few or no cases of therapeutic leukaemia after treatment for chronic lymphocytic leukaemia;

3. the nature of the medical drugs responsible, including first and foremost long alkilating chemotherapy (chlorambucil, melphalan) extending over considerable periods but not necessarily very aggressive.

4. the two kinds of diversity characterizing the preleukaemic period: diversity of duration (from one to ten years) and diversity of expression, in most cases completely silent, sometimes with successive phases of medullary insufficiency and oligoblastosis before leukaemia, and sometimes with non-specific chromosomal abnormalities

5. the very severe nature of therapeutic leukaemia, which is usually myeloblastic and poorly responsive to treatment, and

6. the different etiologies of post-therapeutic leukaemia and lymphoma, of leukaemia as a complication of chemotherapy, lymphoma as a complication of bone marrow transplantation with immune deficiency.

Although cigarette smoke is not generally thought to be associate with increased leukaemia risk, some studies have reported increased risk for leukaemia among smokers.

In a large cohort study in the United States, leukaemia risk among smokers compared with non smokers was increased by 40% at ages 45–64 years and by 70% at ages 65–79 years. In contrast in a 10 years follow up study of 40.000 patients, British physicians found leukaemia mortality for smokers to be lower than for non smokers.

Viruses and leukaemia in animals

The role of viruses in the etiology of animal leukaemia was first acknowledged in 1908 by Ellerman and Bang.

Subsequent research was orientated in three directions:

1. In *chicken*, for the diverse forms of avian leukaemia, analysis of epidemiological data and (at least for one variety of chicken) Marek's disease, and lastly, for effective vaccination.

2. In rodents, particularly laboratory *mice*, with the discovery of the role of viruses thanks to Ludwig Gross (1951) and subsequently, remarkable studies of the relationship between viruses and leukaemic cells.

3. In wild and domestic animals not kept in the laboratory, including cattle, pigs, dogs and especially cats. The remarkable studies by Jarret (1984) in this field revealed:

a) the role of retroviruses, especially FeLV in the cat.

b) the responsibility of retroviruses like FeLV for both malignant and non-malignant diseases including, in the latter case, anaemia, aplasia and sterility.

c) knowledge of the various modes of transmission, which in chickens vary according to the species (congenital via the egg, or later, by contact, inhalation or ingestion) and in cats according to whether they live in a group or isolated.

d) knowledge of certain genetic co-factors for cattle and pigs.

e) the absence – in the present state of the art – of any relationship between the epidemiologies of these forms of animal leukaemia and the epidemiology of human leukaemia. However, it is to be mentioned that bovine leukaemia virus can possibly be transmitted to sheep as indicated by Stirizinger.

Geographical haematology and leukaemia

The concept of geographical haematology proposed in Paris in 1963 can be expressed in the following simple terms: "The characteristics of a man's blood depend on the place where he lives and the place where his ancestors lived." Later, the same principle inspired Doll's important work on the geography of cancer.

The uneven geographical distribution of leukaemia was acknowledged in the initial studies inspired by this concept. The criticism they aroused (imprecise diagnosis, uncertainty, inadequate classification) gave rise to more rigorous epidemiological research, and several types of geographical inequality were subsequently defined:

1. The unequal frequency of leukaemia in the different age groups was confirmed, with peaks in childhood and old age.

The following findings were made in this connection:

a – The frequency of acute leukaemia in children seems to be a recent phenomenon.

b – In all countries, age is an important prognostic factor. The evolution of acute lymphoblastic leukaemia in children, which is often favourable, contrasts with the persistent gravity of the same type of leukaemia in adults.

c – Hodgkin's disease is frequent in adults and rare in children in Europe and North America, but the contrary is true for Africa and South America (where it is rare in adults and frequent in children).

Two findings were recently reported on:

a) the relationship between economic status and the age at which peak frequency is observed for leukaemia:

2 to 5 years in wealthy countries, and

10 to 15 years in poor countries (Africa, India, Madras).

b) the relationship between economic status and the frequency of leukaemia among the general population:

In Ibadan, e.g. a relationship was observed between socioeconomic status and the rise in the frequency of leukaemia among children.

2. The uneven geographical distribution of leukaemia was confirmed in countries with haematologist experts of the same rank.

– The frequency of the disease in Sweden is double of that of in Italy.

– In the United States, large differences were found from one state to the other, and leukaemia was rare among American Indians.

– In Italy, there were also great differences between the various provinces.

– In Britain, leukaemia was especially frequent in the Midlands, and lymphoma in the rural districts of Yorkshire.

3. This uneven geographical distribution of leukaemia depends, perhaps, on the patient's occupation.

– In this respect some peculiar facts have been observed but not interpreted, e.g.:

– The high frequency of Waldenström's disease among Heads of State: 4 cases – 1/25 – i.e. 10.000 times more frequent than among the general population.

– The reports (as yet unconfirmed) that leukaemia is frequent among hairdressers and wood workers.

– The frequency of leukaemia in various rural populations, including farmers in Nebraska (no connection with epizootic disease among cattle), and New Zealand farmers.

– Despite certain claims to the contrary, the frequency of leukaemia among doctors who are not radiologists and among male nurses is no higher than among the general population.

– Contradictory data have been reported about the frequency of leukaemia in male nurses working in hospital haematology departments:

increased risk	Finland
no risk	Sweden

– Higher frequency of leukaemia was reported among children whose parents work in certain type of plants

occupational exposure of the father to:
chlorinated solvents,
spray paint,
dyes and pigments;
father working in industries manufacturing:
transportation equipment;
parents using pesticides in the home.

4. Much more reliable, although as yet unexplained, are the data concerning the unequal distribution of the different types of leukaemia and related diseases, and more especially

a – the rareness of chronic lymphocytic leukaemia in China, Japan and South-East Asia and the relative frequency of this form of leukaemia among young adults in Africa.

b – the relative frequency of acute myeloblastic leukaemia and African children's chloroma, in both Uganda and Nigeria, and the appearance in Africa of chronic myeloid leukaemia at a younger age than elsewhere.

c – and above all, the different geographical distributions of leukaemia and lymphoma as if the former were linked to wealth and the latter to poverty (leukaemia in Israel, lymphoma in Gaza).

Second period

The second period in the history of leukaemia may be termed the Burkitt period. It is hardly necessary to recall the importance of the research done by Denis Burkitt in Uganda, under very difficult conditions, in a country of lions, giraffes, bougainville and tribal feuds; his first studies, the first description of a geographical tumour, the confirmations and consecrations, the new orientation he gave to the epidemiology of lymphoma, and beyond lymphoma, to leukaemia and cancer as a whole.

Burkitt's discoveries had two main consequences:

1. in fundamental biology, they revealed the role of the Epstein–Barr virus, the oncogenes, the immunoglobulin genes and the links connecting them.

2. in epidemiology proper, they gave rise to the notion of multifactorial causes, including observations in which he discovered the importance of the type of cradle – covered or uncovered –, thereby permitting or blocking the access of vector insects and their bites; the importance of the way in which "infants"

pap is prepared, because prior chewing by the mother transmits the virus; the fortunate consequences of the rise in the general population's standard of living in Tanzania, including the reduction in the frequency of Burkitt's lymphoma.

More generally, this type of lymphoma has been a veritable Rosetta stone for the study of leukaemogenesis and of multifactorial carcinogenesis in general.

Third period

The third period in the history of leukaemia was initiated by Japanese and American discoveries later extended in several senses.

The *discovery* phase

– began with the communication at the 16th International Congress of Haematology in Tokyo regarding the frequency of T-cell lymphoid leukaemia in South Japan, particularly on the Islands of Kyushu (Takatguki, 1976).

– next discovery was made by Gallo in 1980 on the HTLV retrovirus, isolated from a cell line derived from fungoid mycosis and then from a case of Sézary's syndrome.

– the subsequent discovery stated that the same retrovirus was the origin of leukaemia in Japan.

The discovery of the first retrovirus was extremely important as it completed the circle of retroviruses present in all animal species, from fish to primates and man.

The *extension* phase can be discussed in four senses:

1. the extension of *methods* of detection thanks to the progress of serological techniques for the detection of antibodies against viral proteins or against certain virus-specific polypeptides. This detection enabled healthy carriers to be identified and consequently, there again, to acknowledge that etiology may be multifactorial, i.e.

– to note the existence of familial clusters, and

– to rule out any connection with the HLA system.

2. *Geographical extension*

– first of all, in Japan, extension from the coastal area of Kyushu, the most south-westerly of the four main Japanese islands, to the neighbouring islands, Okinawa and the Pacific coastal areas. These areas have in common a rural environment, hot climate and abundant rainfall.

Concomitantly with the observations regarding this extension, Japanese authors gave a precise description of the disease:

– it appears in the age-group of the sixties, and the two sexes are equally affected;

– its manifestations are lymphadenopathies, hepatomegaly, skin lesions and hypercalcaemia;

– sometimes its form is leukaemic, and sometimes that of a non-leukaemic lymphoma;

– it is histologically characterized by plenohomorphous lymphoma lesions with giant cells with a cerebriform nucleus;

– its evolution is rapid, and death is often due to an opportunistic infection.

Outside Japan, the extension of leukaemia has been observed:

– In China, along the Yang Tse river, where climatic conditions are the same as those in Japan, the proportions of subjects with positive antibodies against HTLV are in

men:	2 to 5%
monkeys:	8 to 15%

– In Korea this proportion in man is very small, whereas in the Caribbean islands high.

– In Central and West Africa:

Gabon:	6%
elsewhere:	2%

Catarrhinian African monkeys had positive antibodies against HTLV.

These studies of the areas of extension, expecially those conducted in Japan, show the importance of two types of survey:

– familian surveys. Comparing, for instance, the frequency of seropositiveness among healthy members of the families of leukaemic subjects. This frequency is

high for T-cell leukaemia,
nil for myeloblastic leukaemia.

– surveys dealing with migrants, in which the results for T-cell leukaemia suggest contamination in childhood.

The results of recent Japanese studies may be recalled here concerning:

1. Epidemiological feature of HTLV I carriers and incidence of ATL in a ATL endemic island, (A report of a community based cooperative study in TSUSHIMA, JAPAN) (Tajima et al., 1987).

2. Mother to child transmission of HTLV I establishing that seropositive children are all born from seropositive mothers (Kasunara et al., 1987.)

3. *Anatomicoclinical* extension, when in the same geographical area the same HTLV I virus is recognized as the origin of spastic paralysis and other serious diseases of the nervous system.

4. *Virological* extension, when viruses are isolated which are at once close to and different from the HTLV I virus.

In general, one can no longer accept the claim made in 1970 that retroviruses constitute the final common pathway of all human and animal carcinogenesis and leukaemogenesis. Modern epidemiologists consider that viruses are leukaemogenic agents in the environment, in the same way as radiation and chemical agents.

Present data

Such is the history of the epidemiology of leukaemia.

As to the present, there are strong points and weaknesses: the strong points reside in the data acquired and the weaknesses in the gaps in present knowledge.

Data acquired

Two kinds of diversity have been established by the epidemiological studies made so far:

Firstly, *the diversity of the etiological factors*, which can be classified into two categories:

1. general (radiation and chemical substances), and
2. specific to an animal species, such as viruses, which are of two kinds:

– Herpes viruses (such as the one found in Marek's disease, or the Epstein–Barr virus),

– retroviruses (e.g. HTLV I or FeLV) with different epidemiologies for each type of virus.

The importance of virus-induced types of leukaemia should be stressed in three aspects:

– the hope for the eventual finding of antiviral vaccines,

– the fact that they permit early detection of the types of leukaemia concerned, as well as that

– the treatment should suppress the action of certain cofactors at various stages in leukaemogenesis.

Secondly, *the diversity of the combinations of etiological agents*. Sometimes several different etiological agents are involved, the model of this type of etiology being that of Burkitt's disease (Epstein–Barr virus + haematozoon + chromosomal abnormality + poverty).

Other cases comprise a combination of two factors:

– this was demonstrated, for instance, in Henry Kaplan's famous experiments with radioleukaemia involving radiation + a virus.

– such a combination probably applied to certain cases of leukaemia at Hiroshima (fortunately, it was not always present).

– its existence is uncertain in other cases like T-cell leukaemia in Japan and in the Caribbean, which might be due either to HTLV I alone or to its combination with other factors.

Weaknesses of the present situation: inadequate elements and uncertainties

The field in which our knowledge is insufficient includes epidemiology.

Epidemiology. For a long time, this was confined to the study of infectious diesases, but great progress has been made in this field in the past few years and epidemiology has become a veritable science. However, it still suffers from the diversity and divergence of the methods used, which means, for instance, that the

rigorous precision of molecular biology must be combined with the uncertain nature of surveys concerning cultural factors.

The *inadequate classification* of the various forms of leukaemia accounts for many of the difficulties encountered in epidemiological survey. (Not so long ago, a symposium of the Blood Cell Club could be entitled "Classification of Unclassified Types of Leukaemia"!)

The description of the forms of T-cell leukaemia in Japan is one of the first examples of the importance of these new immunological and cytogenetic criteria. For instance, a work on the geography of chronic myeloid leukaemia, published after this description, was constructively based on the absence or presence of the Philadelphia Chromosome.

Future Prospects — New Lines of Research

The strong and weak points in the present state of the art as regards the epidemiology of leukaemia are the elements that will determine the main lines of future research in this field as the end of the century approaches. This research will be oriented in three directions – induction, prediction and correlation.

Induction

The question arises whether the data established for a rare type of leukaemia peculiar to a limited geographical region have more general implications. For instance, will studies of T-cell leukaemia remain confined to the uncommon form found in south Japan and the Caribbean, or will they prove to be of more general interest? This could be the case if one or more of the following conditions were to occur:

– if new surveys reveal a great extension of the geographical area affected by T-cell leukaemia;

– if, above all, the general body of data acquired because of the existence of this form enables it to be considered as a model;

– if the epidemiological methods to which it has given rise make it possible to establish new facts concerning the various stages in the genesis of the different types of leukaemia and their physiopathology, and lastly,

– if relationships are established between epidemiological research on leukaemia and other fields of epidemiological and haematological research.

Prediction – limitations and potentialities.

1. Until now, prediction has been limited to a few cases, namely,

– the frequency of Down Syndrome leukaemia, which we had already noted in 1955, even before trisomy 21 was discovered, and which was later extensively confirmed. At the same time, leukaemia was also frequently reported among children with other chromosomal abnormalities;

– frequent concordant leukaemia among monozygous twins (incidence 1/5 as compared with 1/20.000 in the general populations);

– the frequency of leukaemia – or rather lymphoma – during congenital agammaglobulinaemia and Chediak' syndrome, and the frequency of chronic lymphoid leukaemia in patients with rheumatoid arthritis.

2. A prediction that will have to be extended during the next few years. The relationship established in Eastern China between the fact of belonging to an HLA subgroup and developing cancer of the rhinopharynx is exemplary. The results of their first surveys, conducted in 1965 with J. Dausset and F. Kourilsky, were not significant. However, further information has been acquired since then. Thus Bortin, d'Amara, Bach, Rim and Van Rood who studies 1834 cases of acute leukaemias have shown:

a) a significant rise in the frequency of the presence of the CW3 and CW4 antigens in acute leukaemia and,

b) a drop in the frequency of the presence of the AW19 antigen.

The progress made in acquiring knowledge of the HLA system and in passing from immunology to molecular biology should provide the stimulus for new investigations of the HLA system or other as yet undiscovered predictive system.

Correlation – the most important line of research.

This involves correlating epidemiological findings with those acquired in other fields implicated in exploring the causes of leukaemia: such correlations will necessarily require changes in methods and concepts.

In other fields of pathology, the correlations established between epidemiology and other branches of science were very simple for a long time. These branches included:

– bacteriology in the case of plague,
– toxicology in that of lead poisoning, and
– chemistry for diseases involving haemoglobin.

For certain other pathological states, constant effects were established in order to assess the respective responsibilities of environmental factors and innate characteristics.

As regards the epidemiology of leukaemia, the whole situation changed with the progress made in virology and the improved knowledge of the biology and mode of action of viruses, with the progress achieved in cytogenetics, rapidly followed by the description of the chromosomal abnormalities peculiar to each form of leukaemia, and with the strides made in the field of immunochemistry, and above all with the discovery of oncogenes.

It will not be possible in the future – in fact it is already no longer possible – to study the epidemiology of chronic and acute leukaemia *en bloc*. Correlations will have to be established between epidemiological data and the data supplied by the study of the environment on the one hand, and of chromosomal abnormalities, viral intervention and oncogenes on the other.

On this kind of solid basis, the epidemiology of leukaemia will be able to fulfil its objectives of prediction and prevention. In addition, it will perhaps

become possible to elaborate effective vaccines – at least against certain forms of leukaemias and certainly to limit its frequency by improved knowledge of risk factors.

References

1. Austin, H., Delezell, E., Cole, P.: Benzene and leukemia (a review of the literature and a risk assessment *Am. J. of Epidemiology 127*, 419 (1988).
2. Barnes, N. et al.: Distribution of leukemia, lymphoma and allied disease in parts of Great Britain. *Leukemia 1*, 78 (1987).
3. Bernard, J.: L'érythroleucémie expérimentale provoquée par le goudron. *Le Sang. 8*, 28 (1934).
4. Bernard, J. et Braier, L.: Les leucémies benzéniques. *Proc. of the 3th Int. Cong. of Haemat.* Aout, 251 (1950).
5. Bernard, J., Mathe, G., Decorme, J. C., et Barnaud, O.: Les leucoses des trés jeunes enfants. *Arch. Franc. Pédiatrie 12*, 470 (1955).
6. Bernard, J.: Esquisse d'une hématologie géographique. *Nouv. Rev. Fr. Hémat. 3*, 51 (1963).
7. Blair, A., White, D. W.: Leukemia cell types and agricultural practices in Nebraska. *Arch. Envir. Health 40*, 211 (1985).
3. Boivin, J. F., Hutchinson, G. B., Evans, F. B., Abou-Daoud, K. T., Junod, B.: Leukemia after radiotherapy for first primary cancers of various anatomic sites. *Am. J. of Epidem. 123*, 993 (1986).
9. Bortin, M. M., d'Amaro, J., Bach, F. H., Rimm, A. A., Van Rood, J. J.: HLA association with leukaemia. *Blood 70*, 227 (1987).
10. Brandt: Environmental factors and leukemia. *Med. Oncol. and Tumor Pharmacother. 2*, 7 (1985).
11. Chan, Li, C., Chen, P. M., Powles, R., Saragas, E., Wiezemann, L. W., Groffe, N., Greaves, M. F.: Molecular lesion in chronic granulocytic leukemia is highly conserved despite ethnic and geographical variations. *Leukemia 1*, 486 (1987).
12. DeMoor, P., Boogaerts, M., and Couwagia, A.: More familial leukemia in ALL patients with both unexplained high transcorten levels and an HLA antigen CW3. *Brit. J. Haematol 69*, 225 (1988).
13. Duncan, N. M., Wiggins, C. L., Samat, J. M., Key, C. R.: Chlidhood cancer epidemiology in new Mexico's American Indians, Hispanic whites and non- hispanic whites. *J. M. C. I. 76 (6)*, 1013 (1986).
14. El-Bolkainy, M. N. et al.: Epidemiology of lymhoma and leukemia in Egypt. In: *Pathogenesis of leukemia and lymphoma.* Raven Press New York, 1984.
15. Flandrin, G., Castaigre, S., Sigaux, S., et Degas L.: Respects of treating 53 hairy cell leukemia problems with alpha interferon. *Leukemia 2*, 326 (1987).
16. Flandrin, G., Sigaux, S., Sebahaun, G. et Bouflabbo, R.: Hairy cell leukemia. Clinical presentation and follow up of 211 patients. *Seminar in Oncology 11*, 458 (1984).
17. Fleming, A. F.: A bibliography of the leukemia in Africa 1904–1985. *Leuk. Res. 10 (11)*, 1355 (1986).
18. Gallo, R. C., Blattner, W. A.: Human T cell leukemia/lymphoma viruses ATL and AIDS. In: *Important advances in oncology.* (D. Vita, ed.) Lippincott Company, Philadelphia, 1985, p. 104.
19. Gessain, A., de The, G. et al.: Leucémia/lymphome T de l'adulte associé au virus HTLV1 en Martinique, á propos de deux cas. *Nouv. Rev. Fr. d'Hématol. 28*, 107 (1986).
20. Gilbert, R. D., Kaarabus, C. D., Mills, A. C.: Acute promyelocytic leukemia. A childhood cluster. *Cancer 1* 59 (5) 933 (1987).
21. Goguel, A., Cavigrean, A. et Bernard, J.: Les leucémies benziniqués de la region parisienne entre 1950 et 1965. Etude de 50 observations. *Nouv. Rev. Fr. d'Hématol. 7*, 475 (1967).

22. Hakulimen, T., Isomaki, H., Knekt, P.: Multiple tumor incidence in patients with rheumatoid arthritis or allied disorders. *J. Chronic. Disease 38*, 775 (1985).
23. Iwanaga, M., Fujiwara, S. et al.: High prevalence of adult T cell virus infection in family members with adult T cell leukemia in non-endemic Hokkaido district. *Hokkaido Igaku Zasshi 6*, 876 (1985).
24. Jarrett, O., and Onioms, D. E.: Retro virus in leukemia of animals and man. In: Hematology—Leukemia. Butterworth International Medical Review 1, 1984.
25. Kikuchi, M. et al.: Virus associated adult T cell leukemia (ATL) in Japan. Clinical, histological and immunological study. *Hematology Oncology 4*, 67 (1984).
26. Kourilsky, F., Dausset, J., Feingold, H., Dupuy, J. M. et Bernard, J.: Etude de la répartition des antigénes leucocytaires chez des malades atteints de leucémia aigue en rémission. In: *Advances in Transplantation.* Munsgaard, Copenhague, 1968, p. 515.
27. Laval, G., Suillet, G., Phizippen et Tuyns, A. Facteurs d'environnement et leucémie aigue chez l'enfant. A propos d'une étude cas-témoins réalisée dans la région Rhone-Alpes. *Pédiatrie 43*, 59 (1988).
28. Linet, M. S., The leukemias epidemiologic aspects. Monogr. Epidem. *Bio-statistic 6*, 293 (1985).
29. Muir, C. S., and Staszewsli, J.: Geographical epidemiology and migrant studies. In: *Biochemical and Molecular Epidemiology of Cancer* 1986, p. 135.
30. O'Conor, G. T.: Geographical variations in the occurrence of leukemias and lymphomas. Summary and comments. In: *Pathogenesis of leukemias and lymphomas.* Raven Press, New York 1984.
31. Owor, R. Geographical distribution of malignant lymphomas and leukemias in Uganda. In: Pathogenesis of leukemias and lymphomas, Raven Press, New York 1984.
32. Philip, P. and Pedersen-Bjogaard, J.: Cytogenetic, clinical and cytologic characteristics in radiotherapy related leukemias. *Cancer Genetic, Cytogenetic. 31*, 227 (1988).
32. Ramot, B., Benbassat, I. et al.: The epidemiology of childhood acute lymphoblastic leukemias and non Hodgkin lymphomas in Israel between 1976—1981. *Leuk. Res. 8*, 691 (1984).
33. Sandler, D. F. et Collman, G. W.: Cytogenetic and environmental factors in the etiology of the acute leukemia in adults. *Am. J. of Epidemiology 128*, 1017 (1987).
34. Sasco, A. J., de The, G.: Epidémiologie des leucémies et lymphomes. In: Encyclopédie des cancers, hémopathies malignes. Flammarion, Médecine, Sciences, Paris 1986, p. 3.
35. Savitz, A., Calle, E. C.: Leukemia and occupational exposure to electromagnetic fluids. *J. Occup. Med. 29* (I) 47 (1987).
36. Stirizinger, T.: Experimental bovine infection in sheep. Host response and control by vaccination. *Diss. Abstr. Int. (B) 46*, 2971 (1986).
37. Tajima, K., Tominaga, S., Suchi, T.: Malignant lymphoma in Japan. Epidemiological analysis in adult T cell leukemia/lymphoma. *Hematol. Oncol. 4 (1)*, 31 (1986).
38. Tajima, K., Tominaga, S., Suchi, T. et al.: HTLV1 carriers among migrants from an ATL endemic area to ATL non endemic metropolitan area in adults. *Int. J. Cancer 37 (3)*, 383 (1986).
39. Tajima, K., Kamura, S. J., Ito, S., Nagatomo, M., Kinoshita, K. I., Ikeda, S.: Epidemiological features of HTLV1 carriers and incidence of ATL in an ATL endemic island; a report of the community based cooperative study in Tsushima, Japan. *Int. J. Cancer 40*, 741 (1987).
40. de The, G.: Virus et cancers humains. Aspects fondamentaux et espoirs de prévention. *C. R. Soc. Biol. 180*, 249 (1986).
41. Vernant, J. C., Gessain, A., de The, G.: Paraparesies spastiques tropicales en Martinique. Haute prévalence d'anticorps HTLV1. *Presse Médicale 15*, 419 (1986).
42. Wang, J. X., Boice, J. D., Li, B. X., Zhang, J. Y., Fraoment, J. F.: Cancer among medical diagnostic X-ray workers in China. *Journal of the Natl. Cancer Institutes 80*, 341 (1988).
43. Yen, P. Z., Shamg, J. Z., Fu, Z. I., Ji, N. C. et al.: Leukemia in benzene workers. A retrospective cohort study. *Brit. J. Int. Med. 44 (2)*, 124 (1987).

Haematologia 22 (4), pp. 215–231 (1989)

Molecular Basis of Red Cell Enzymopathies Associated with Hereditary Nonspherocytic Hemolytic Anemia*

S. MIWA

Okinaka Memorial Institute for Medical Research, 2–2–2
Toranomon, Minato-ku, Tokyo 105, Japan

(Received 10 September, 1988; accepted 30 September, 1988)

In the past few years, very rapid advances have been made in the field of red cell enzymopathies associated with hereditary nonspherocytic hemolytic anemia, particularly in molecular basis. Nucleotide sequence and amino acid sequence of normal human red cell enzymes have been clarified in phosphofructokinase, aldolase, triosephosphate isomerase, phosphoglycerate kinase, pyruvate kinase, diphosphoglycerate mutase, glucose 6-phosphate dehydrogenase, adenylate kinase and adenosine deaminase. Furthermore, in aldolase-, triosephosphate isomerase-, diphosphoglycerate mutase-, glucose 6-phosphate dehydrogenase-, and adenylate kinase deficiency, single nucleotide changes which cause single amino acid substitutions and finally hemolysis, have been found.

Keywords: aldolase deficiency, erythroenzymopathies, glucose 6-phosphate dehydrogenase deficiency, hereditary nonspherocytic hemolytic anemia, molecular basis, phosphoglycerate kinase deficiency, pyruvate kinase deficiency, red cell enzymopathies

Introduction

Since the discovery of glucose 6-phosphate dehydrogenase deficiency in 1956 [1], and of pyruvate kinase deficiency in 1961 [2], red cell enzymopathies associated with hereditary nonspherocytic hemolytic anemia (HNSHA) have been extensively investigated [3, 4]. Up to the present, fourteen red cell enzyme deficiencies in the Embden–Meyerhof glycolytic pathway, the Rapoport–Luebering cycle, the hexose monophosphate pathway, glutathione metabolism and nucleotide metabolism have been found to cause HNSHA. In addition, a marked increase of apparently normal red cell enzyme, adenosine deaminase is also known to cause HNSHA. With the exception of adenosine deaminase overproduction, kinetic and electrophoretic studies have shown that most, if not all, red cell enzymopathies associated with HNSHA are considered to result from the production of structurally abnormal and functionally defective enzymes. Understanding the pathogenesis of these disorders requires knowledge of the amino acid sequences of both normal and abnormal enzymes, but it had not been easy to purify and to characterize these enzymes because of their low levels in red cells.

* Plenary lecture presented at the XXII. Congress of the International Society of Haematology, Milano, 1988

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

So far, the primary structures of red cell phosphoglycerate kinase [5, 6], adenylate kinase [7], triosephosphate isomerase [8] and diphosphoglycerate mutase [9] have been determined using protein samples. Purified protein samples from one electrophoretic variant as well as three patients deficient in phosphoglycerate kinase activity have clearly shown that the deficiency is caused by substitution of a single amino acid [10–13]. Gene cloning of these enzymes have been difficult because messenger RNA (mRNA) is present in trace amounts, between 0.01 and 0.1% of the total mRNA in reticulocytes. However, in these few years, recent advances in recombinant DNA technology have made possible the isolation of the genes for human aldolase A [14], phosphoglycerate kinase [15], glucose 6-phosphate dehydrogenase [16, 17], adenosine deaminase [18–20], phosphofructokinase [21, 22], triosephosphate isomerase [23], diphosphoglycerate mutase [24] and pyruvate kinase [25]. In some of them, genomic DNA analysis has been made. Quite recently, single base changes which resulted in single amino acid substitutions have been clarified in patients with triosephosphate isomerase deficiency [26], aldolase deficiency [27], diphosphoglycerate mutase deficiency [28], glucose 6-phosphate dehydrogenase deficiency [29–31] and adenylate kinase deficiency [32].

Table 1

Summary of the molecular basis of red cell enzymes, deficiency or overproduction which can cause hereditary non-spherocytic hemolytic anemia

Names of red cell enzymes	Nucleotide and amino acid sequences	Single base change and/or single amino acid substitution in patients with HNSHA	Chromosome locus of red cell enzyme (Chromosome number)
Hexokinase	−	−	1
Glucosephosphate isomerase	−	−	19
Phosphofructokinase	+	−	M 1, L 21
Aldolase	+	+	16 [99]
Triosephosphate isomerase	+	+	12
Phosphoglycerate kinase	+	+	X
Pyruvate kinase	+	−	L (≒ R) 1
2,3-Diphosphoglycerate mutase	+	+	7 [100]
Glucose 6-phosphate dehydrogenase	+	+	X
Gamma-glutamylcysteine synthetase	−	−	?
Glutathione synthetase	−	−	?
Glutathione peroxidase	+ [101]	−	3 [102]
Adenylate kinase	+	+	9
Pyrimidine 5'-nucleotidase	−	−	?
Adenosine deaminase (overproduction)	+	+	20

In this review article, recent advances of molecular biology in the field of red cell enzymopathies associated with HNSHA is reviewed, and the relationship between the enzyme defect and metabolic alteration which results in hemolysis, in part, is summarized based on our own results.

Hexokinase Deficiency

Of all the red cell glycolytic enzymes, hexokinase (Hx) has the lowest activity and is the most age-dependent [33]. There are four isozymes (Hx I, II, III and IV), and the Hx in the red cell is mainly type I, whih has three major forms, Ia, Ib and Ic. In young red cells, Hx Ib is predominant. The mature red cells contain Hx III, which is not found in the fetal cell. The structural gene locus for Hx I is on chromosome 10 [34]. The electorphoretic pattern of the enzyme from two of the patients showed a decrease of Hx I and a relative increase of Hx III [35, 36]. The cloning of hexokinase I has not been made. Hence, the molecular basis of Hx deficiency is unknown.

Glucosephosphate Isomerase Deficiency

A single genetic form of glucosephosphate isomerase (GPI) is synthesized in all cells of the body [33]. The structural gene locus for GPI is on chromosome 19 [37]. The characteristic of GPI deficiency is thermal instability of the enzyme and normal affinity for the substrate, with two exceptions, GPI Cowen [38] and GPI Kaiserslautern [39]. Single amino acid substitutions of genetic GPI variants, namely, Sigh variant [37] and PHI (GPI) 5–1 [40] have been identified, although the primary structure of GPI is unknown.

Phosphofructokinase Deficiency

Phosphofructokinase (PFK) is a tetrameric enzyme, and three types of subunits, muscle-type (M), liver-type (L) and fibroblast or platelet type (F or P) subunits, exist in human tissues [33, 41]. Red cell PFK consists of a heterozygous mixture of five tetramers (M_4, M_3L, M_2L_2, ML_3 and L_4) [42]. Biochemical and immunological studies show that red cell PKF deficiency may involve a defect of either the M or L subunits, and in most cases, the defect probably results from the synthesis of a mutant subunit [43, 44].

Recently, cloning of both human L- and M-type PFKs have been made [21, 22]. M-type PFK has 779 amino acid residues and homologies between human and rabbit M-type PFK were 96% of the amino acids and 89% of the nucleotides in the coding region. Cloned human M- and L-type PFK cDNAs will help to identify the molecular defect in patients with PFK deficiency associated with HNSHA and patients with glycogenosis type VII. Gene loci encoding L- and M-type PFKs reside on chromosome 21 [45], and 1 [46], respectively.

Aldolase Deficiency

Three aldolase isozymes are found in human tissues; A in the muscle and red cells, B in the liver and C in the brain together with A [33]. Recently, genes for aldolases A and B have been cloned and characterized [14, 47–49]. While aldolase B deficiency causes fructose intolerance, aldolase A deficiency causes HNSHA. The latter is very rare, and only two kindreds have been reported [50, 51]. The patient described by Beutler et al. [50] showed many dysmorphic features, with mental and growth retardation. The patient's red cell enzyme showed no structural abnormalities by electrophoretic and kinetic studies. The proband reported by us [51] had only HNSHA. The patient's red cell aldolase was unstable to heat and had an increased Michaelis constant for fructose 1,6-diphosphate. Our case suggested a structural gene mutation. A cooperative study with Kishi et al. [27] succeeded in cloning and characterizing the patient's aldolase A cDNA. Compared with normal human aldolase A cDNA, A → G change was found to occur in the codon for the 128th amino acid, resulting in an amino acid substitution from aspartic acid (GAU) to glycine (GGU). Southern blot analysis of the genomic DNA showed the patient carried a homozygous mutation inherited from his parents. The patient's enzyme from red cell and from cultured lymphoblastoid cells was found to be highly thermolabile. The thermal stability of aldolase A of the patient and of a normal control, expressed in *Escherichia coli* using expression plasmids, confirmed the thermolabile nature of the mutant enzyme. The same ^{128}Gly variant form made by oligonucleotide directed mutagenesis technique also showed thermolability.

^{128}Asp has been shown to be well conserved in all aldolase isozymes examined so far, including human, rat, and rabbit A-type, human, rat and chicken B-type, rat and mouse C-type aldolases and even in *Drosophila* aldolase.

The tertiary structure of rabbit skeletal muscle aldolase A, was clarified by Sygusch et al. in 1987 [52]. Based on the assumption that human aldolase A has an almost similar structure to that of the rabbit A-type due to the high homology, ^{128}Asp is located very close to the β-barrel structure which is important to the enzyme activity expression. Substitution of asparagine which has negative electric charge into chargeless glycine will probably make the enzyme molecule unstable. This will result in the structural change of the enzyme molecule, and hence, in decreased specific activity.

Triosephosphate Isomerase Deficiency

Triosephosphate isomerase (TPI) is a dimeric enzyme; the subunit's size is 26.5 kDa [33]. Human TPI exists in multiple electrophoretic forms probably as the consequence of minor postsynthetic alterations of form A, the three major ones being A, B and C [53]. The TPI A and B isozymes are products of the same structural locus on chromosome 12 [54]. TPI deficiency is a rare disease character-

ized by HNSHA, progressive neurological impairment and in some instances, recurring bacterial infections. In 1985, Maquat et al. [23] determined human TPI cDNA sequence. In 1986, Daar et al. [26] succeeded in determining the molecular basis of this disorder. A TPI allele from two unrelated patients homozygous for TPI deficiency was compared with an allele from a normal control. Each disease-associated sequence harbors a GC → CG transversion in the codon for the 104th amino acid, resulting in an amino acid substitution from glutamate to aspartate. The importance of ^{104}Glu to the enzyme structure and function is implicated by its conservation in the TPI of all species that have been characterized to date, including *Bacillus stearothermophilus*, *Escherichia coli*, *Saccharomyces cervisiae*, coelecanth, chicken, rabbit and man. The Glu → Asp substitution results in a thermolabile enzyme as demonstrated by assays of TPI activity in cultured fibroblasts of each patient and cultured Chinese hamster ovary (CHO) cells that were stably transformed with the mutant alleles. The X-ray crystal structure analysis of chicken TPI indicates that the loss of a side-chain methylene group is sufficient to disrupt the counterbalancing of charges that normally exists within a hydrophobic pocket of the native enzyme.

Phosphoglycerate Kinase Deficiency

Human phosphoglycerate kinase (PGK) in red cells and other tissues, except for sperm, is controlled by a single structural gene located on the long arm of the X chromosome [33]. The complete amino acid sequence of human PGK was determined in 1980 in Yoshida's laboratory [5, 6], and the nucleotide sequence of human PGK cDNA was made in 1983 by Michelson et al. [55]. Enzyme protein analysis of human PGK showed that it consisted of 417 amino acid residues; compared with that predicted from the full-length cDNA, it had an extra lysine residue. Human PGK determined from nucleotide sequence [55] and horse PGK [56] both have 416 amino acids. There are only 11 differences (nonhomology of 3%) between the human and horse sequences. The tertiary structure of horse PGK has been identified [56].

PGK deficiency is associated with HNSHA and also often with mental retardation [33]. PGK II [10], PGK München [11], PGK Uppsala [12] and PGK Tokyo [13] had single amino acid substitution; the correlation between the functional and structural abnormalities of these variants has been clarified based on the tertiary structure of horse PGK. The structural abnormality of PGK II, which is fairly common in Southern Pacific populations and is an asymptomatic variant, is a single amino acid substitution from threonine to asparagine at 352. This substitution does not change the enzyme activity, but causes strong binding of the variant enzyme with citrate. The structural abnormality of PGK München is a single amino acid substitution at 268, from aspartic acid to asparagine. PGK München is associated with red cell enzyme deficiency and heat instability, but shows no signs of hemolysis. The negative charge of an aspartyl residue at the

site of substitution must maintain the stability of the molecule. The structural abnormality of PGK Uppsala is a single amino acid substitution from arginine to proline at 206, and is associated with severe red cell enzyme deficiency, hemolytic anemia and mental disorders. The variant enzyme has lower specific activity, thermal instability, and higher-than-normal Michaelis constant for the substrates. The structural abnormality of PGK Tokyo is a single amino acid substitution, from valine to methionine at 266. PGK Tokyo is associated with enzyme deficiency HNSHA and neurological disturbances. This variant enzyme was purified from cultured lymphoblastoid cells. PGK Tokyo has a lower specific activity, higher--than-normal Michaelis constant for the substrates, acidic shift in pH optimum and thermal instability. The substitutions of PGK Uppsala and PGK Tokyo are proximal to the site of substrate binding. Substitutions at such a position are likely to induce a dislocation of the ATP and ADP binding sites, resulting in markedly disadvantageous enzyme properties and severe clinical symptoms.

Pyruvate Kinase Deficiency

Pyruvate kinase (PK) has four isozymes (L, R, M_1 and M_2) that are encoded by two different genes. The L (liver)- and R (red cell)-type PK differ from M_1 (muscle)- and M_2-type PK by their enzyme kinetic, electrophoretic and immunological properties, and they are under the control of different genes at least in rat [58], and very likely to be so in human. In rat, differential splicing was considered to be involved in the production of L- and R-type PK, and M_1- and M_2-type PK, respectively, from L and M genes [59, 60]. PK cDNA and genomic cDNA from non-human species, namely, yeast PK, chicken M_{12}-PK, rat M_1- M_2-, L- and R--type PK have been isolated by several investigators [59–66]. The structure of cat muscle PK has been analyzed extensively [67]. In man, structural abnormalities of the L-(=R)-type PK are considered to be responsible for HNSHA due to PK deficiency. For the purpose of clarifying the PK deficiency at the gene level, we isolated the partial L-type PK cDNA in 1987 [68], and assigned the human L-type PK gene on chromosome 1 at band q21 [68, 69]. Recently, we succeeded in isolating and determining the full-length sequence of human L-type PK cDNA [25]. The cDNA contains 1629 base pairs of coding for 543 amino acids. The homology between human and rat L-type PK was 86.9% at the nucleotide sequence level, and 92.4% at the amino acid sequence level. It also had 68 base pairs of 5'-noncoding sequence and 734 base pairs of 3'-noncoding sequence. The full-length L-type PK cDNA was placed under the promotor of simian virus 40 and introduced into monkey COS cells. Human L-type PK activity was detected in the extract of COS cells by the classical PK electrophoresis, confirming that this cDNA is the actual structural L-type PK gene. The availability of human full-length cDNA for L-type PK will be useful to clarify the molecular basis of PK deficiency associated with HNSHA.

Leukocyte PK and platelet PK are known to be M_2-type, while red cell PK is R-type. As there are multipotent hematopoietic stem cells in bone marrow, and red cells, leukocytes and platelets differentiate from these stem cells, there must be an isozyme switch from M_2-type to R(= L)-type PK during the differentiation step of erythroid cells. The relationship between erythroid cell maturation and the change of PK isozymes was studied using anti-rat-L-type-PK fluorescent antibody techniques in normal subjects [70], in PK deficiency subjects [71], as well as with K562 cells [72]. The results indicated that the conversion of PK isozymes from M_2-type to L-type in erythroid precursor cells occurred in the early stage of maturation, and that compensatory M_2-type PK production occurred in the erythroblasts of patients with PK deficiency. The experiment using K562 cells showed that after hemin induction, change from M_2-type to L-type PK appeared along with the appearance of hemoglobin F synthesis.

In 1979, the working group in the Expert Panel of Red Cell Enzymes of the International Committee for Standardization in Haematology (ICSH) issued the recommended methods for characterization of PK variants [73]. The procedures enabled us to compare the results obtained in different laboratories. Our results of the enzyme characteristics of 8 PK variants obtained from 8 true homozygous PK deficient patients showed that all of them had high Km for phosphoenolpyruvate, 6 out of 8 had thermal instability, and half of them showed low Ki for the product, ATP, and half of them showed decreased fructose 1,6-diphosphate activation. Although, Zanella et al. [74] recently reported that no correlation was found between the severity of anemia and either the enzyme activity or specific biochemical enzyme abnormalities in 11 compound heterozygous patients, our data showed that in many variants, high Km for phosphoenolpyruvate and thermal instability seemed to play roles in causing HNSHA. It is worth noting that 3 out of 8 had higher than normal enzyme activity and an additional case showed normal activity when PK was assayed using conventional assay procedure which contained high phosphoenolpyruvate concentration. Hence, in order not to overlook such patients, it is necessary to use a much lower substrate concentration for activity assay [73], or to see accumulation of intermediates upstream of the PK step, such as phosphoenolpyruvate, 3-phosphoglycerate and 2,3-diphosphoglycerate.

PK deficiency is the most common enzyme deficiency in the Embden–-Meyerhof glycolytic pathway associated with HNSHA, and more than 300 cases have been reported. We found 63 cases with PK deficiency in Japan.

Recently, Tani et al. [75] succeeded in clarifying the complete nucleotide sequence of M_2-type PK cDNA and assigned the gene locus on chromosome 22 at q22.

2,3-Diphosphoglycerate Mutase Deficiency

2,3-Diphosphoglycerate mutase (DPGM) deficiency has been known to cause either HNSHA [76] or polycythemia [77] due to marked decrease of 2,3--diphosphoglycerate in the red cell. DPGM Créteil was initially found by Rosa

et al. [77], and the patient showed polycythemia. Quite recently, Rosa et al. succeeded in defining the molecular basis of DPGM Crétail.[87]Arginine was replaced by cysteine [28]. [87]Arg is next to the [86]Gln which is presumed to be in the active site of the enzyme. Hence, it is conceivable that a change from arginine to cysteine at the 87th position causes profound ill-effect to the enzyme function. They assume that the patient is a compound heterozygote of this abnormal gene and of a non-expressed gene, although it has not been proven as yet.

Glucose 6-Phosphate Dehydrogenase Deficiency

Human glucose 6-phosphate dehydrogenase (G6PD) was first purified to homogeneity from red cells by Yoshida in 1966 [78]. Single amino acid substitution was described by Yoshida in two variants, G6PD A+ [79] and G6PD Hektoen [80] in 1969 and 1970, respectively, although at that time the primary structure of human normal G6PD was unknown.

Takizawa et al. [29] clarified that a single nucleotide base transition is the molecular basis of the common G6PD A+ variant. AT to GC transition occurred in the variant A+ gene, thus producing the amino acid substitution from asperagine to aspartic acid at 142. The nucleotide change created an additional Fok I cleavage site in the variant A+ gene. Thus, G6PD A+ can be differentiated from that of normal G6DP B subjects by Southern hybridization using a restriction endonuclease Fok I. Hirono and Beutler [30] clarified the molecular basis of G6PD A−, showing that A− mutation has two base changes, of which one is the same as seen in A+ subjects. An additional base transition from G to A at 202 occurred, resulting in a single amino acid substitution from valine to methionine. That G6PD A+ and G6PD A− contain the same mutation strongly suggests that the latter occurred as a result of an additional base change in a carrier of the former.

Quite recently, Vulliamy et al. [31] reported the molecular basis of several other G6PD variants, including a common variant, G6PD Mediterranean.

Adenylate Kinase Deficiency

Adenylate kinase (AK) has two isozymes, AK-1 (muscle type) and AK-II [33]. Red cell AK is AK-I. Human AK-I has been characterized and primary and tertiary structure were analyzed by von Zabern et al. in 1976 [7]. AK-I has been assigned to a gene locus on chromosome 9 and consists of 194 amino acids. Nucleotide sequence of human AK-I cDNA and genomic DNA has been determined by Nakazawa et al. (personal communication). AK deficiency is a very rare disorder [33]. As I had a case with AK deficiency associated with HNSHA [81], we have done a cooperative study with them to clone and characterize the patient's AK-I gene using cultured lymphoblastoid cells of the patient. Pre-

liminary data revealed that a single base change from C to T which resulted in amino acid substitution from arginine to tryptophane at 128 in one allele while the other allele was normal. This finding confirmed the fact that the patient had about 50% of normal red cell AK activity and was thought to be heterozygote. Since chicken AK-I is highly homologous to human AK-I with respect to the amino acid sequence, we introduced the Arg to Trp substitution to chicken AK-I gene at the same position by oligonucleotide-directed mutagenesis. The mutant chicken AK-I expressed in *Escherichia coli* showed a reduced catalytic activity as well as a decreased solubility and a charge in affinity to phosphocellulose. Thus, it seems that the observed C to T transition is a cause of the decrease in AK-I activity of the patient's red cell. Analysis on phosphocellulose chromatography of red cell AK-I of the patient and the parents revealed that the mutant allele of the patient was derived from her mother. As ^{128}Arg is in an α-helix which locates just outside of the active center and substrate binding site, it appears to play an important role in the regulation of enzyme activity. In this case, the question why the heterozygous patient showed HNSHA, but a similar heterozygous sister and the mother did not show HNSHA remains to be solved. Another unknown factor may be responsible for the fact that the proband shows hemolysis in addition to AK deficiency.

In this connection, it is worth mentioning that a case with AK deficiency reported by Beutler et al. [82] in 1983, in spite of marked decrease in the red cell enzyme activity, lacked evidence of hemolysis. Thus, the mechanism of hemolysis in AK deficiency is puzzling.

Adenosine Deaminase Overproduction

Two forms of adenosine deaminase (ADA) have been characterized. The small form (38 kDa) of the enzyme predominates in the spleen, stomach and red cells, while the large form (298 kDa) predominates in the kidney, liver and skin fibroblasts. The small form can be converted to the large form by complexing with a 200 kDa protein known as the ADA binding protein [33]. The structural gene locus for ADA (small form) is on chromosome 20 [83]. Low levels or the absence of ADA is associated with one form of severe combined immunodeficiency disease [84].

Markedly increased ADA activity in red cells develops into HNSHA. The mode of inheritance is autosomal dominant. Only four such families have been reported, including our two [85–88]. We purified red cell ADA from normal subjects and from a patient with increased ADA activity [89]. There were no differences in the enzymatic and chemical properties. The accumulation of ADA in our patient's red cells seemed to be due to increased synthesis of structurally normal emzyme [90].

Clones encoding human ADA were isolated and characterized by three groups [18–20], which enabled the investigation of the molecular mechanisms

of this interesting disease. Chottiner et al. [91] presented the data in a case reported by Valentine et al. [85]. Western blots demonstrated a corresponding increase in red cell ADA. Analysis of genomic DNA revealed no evidence for amplification or major structural changes in the ADA gene. ADA-specific mRNA from a patient's reticulocytes was comparable in size and amount to mRNA from reticulocytes of a normal control subject. Translation of a patient's poly A+ reticulocyte mRNA in a rabbit reticulocyte lysate system and immunoprecipitation of ^{35}S-labelled protein products with anti-ADA antibody yielded a band of 42 kDa that was undetectable in translation products from control reticulocyte mRNAs. These data suggest that the increased ADA activity in red cells in this disorder results from the increased translation of an aberrant ADA mRNA.

In our laboratory, Kanno et al. [88] performed molecular analysis in a recently discovered case. Southern blot analysis, Western blotting of partially purified ADA, as well as dot blot analysis to determine ADA mRNA showed essentially the same results as those of Chottiner et al. However, in a further experiment, the 2.2 kb ADA promotor DNA fragment of these clones was fused to the chloramphenicol acetyl transferase (CAT) gene, and transfected to human erythroid cell line, K562. One out of three of these clones expressed 2.6 times higher CAT activity than the normal ADA promotor fused to CAT gene in K562, but such enhancement was not seen in human non-erythroid cell lines, HL 60 and Raji. From these results, it is likely, though not conclusive, that the 5' promotor fragment of the ADA gene of the patient is responsible for the cell-specific enhancement of protein synthesis. From our own studies, we speculated the two molecular mechanisms. One is increased translational efficiency of aberrant ADA mRNA, and the other is the transient increase of the transcription of ADA gene in the red cell precursor cells of the patient. The latter possibility may occur for the following reasons. First, it takes over 100 hours for the erythroid precursor cells to mature, whereas most of the mRNA species have much shorter half-lives, which range from 30 minutes to 17 hours. Second, the rate of mRNA synthesis rapidly declines in late stages of erythroid maturation. Thus, an accumulation of ADA can occur without an accumulation of reticulocyte ADA mRNA.

Quite recently, Chottiner et al. [92] described in an abstract that Northern blot analysis performed under stringent conditions confirmed a markedly increased amount of reticulocyte ADA mRNA in the patient, and they concluded that this disorder probably occurs at the level of transcription in erythroid precursors. If it is true, then our first hypothesis is wrong while the second hypothesis is similar to their conclusions. However, even so, he molecular basis to cause this increased transcription efficiency needs further study.

Pyrimidine 5'-Nucleotidase Deficiency

Pyrimidine 5'-nucleotidase (P5N) deficiency seems to be one of the more common causes of HNSHA. Swallow et al. [93] and Paglia et al. [94] found, in-

dependently, that P5N-deficient patients had a marked deficiency in P5N activity when uridine monophosphate (UMP), cytidine monophosphate (CMP), or deoxycytidine monophosphate (dCMP) were the substrates, but near-normal levels of activity when deoxyuridine monophosphate (dUMP) or deoxythymidine monophosphate (dTMP) were the substrates. In our laboratory Hirono et al. [95] separated human red cell P5N into two subclasses, P5N-I and -II, by DEAE gel chromatography. The P5N-II from the five patients with this disorder had normal activity. However, the P5N-I from these patients had abnormal properties, and were heterogeneous. These results strongly suggest that the main cause of P5N deficiency is an abnormality of P5N, probably arising from a structural gene mutation. It should be added that when dUMP was used as a substrate, all these P5N deficient patients showed normal P5N activity.

Conclusion

In these few years, very rapid advances have been made with respect to the molecular basis of red cell enzymopathies associated with HNSHA as summarized in Table 1. It is expected that the comming few years will be more fruitful in clarfying the relationship between abnormal structure(s) and function(s) and finally the molecular mechanisms of hemolysis in HNSHA.

However, I must stress that it is the concensus of investigators in this field that pathogenesis in about 3/4 of HNSHA patients remain unknown even after adequate red cell enzyme studies as well as Carrell's isopropanol test for unstable hemoglobin have been made [96, 97]. We have to think about it and make efforts in order to unravel new red cell enzymopathies. A most recent report by Beutler et al. [98] on red cell glutathione S-transferase deficiency is regarded to be one of these examples.

Acknowledgements

Part of our studies cited in this manuscript was supported by Scientific Research Grants from the Ministry of Education. Science and Culture, Japan and by Research Grant from the Ministry of Health and Welfare, Japan. The author gratefully acknowledges the kindness of Dr. J. Rosa and Dr. A. Nakazawa for allowing him to use their recent results, and Dr. H. Fujii for helping in preparing this manuscript.

References

1. Carson, P. E., Flanagan, C. L., Ickes, C. E., Alving, A. S.: Enzymatic deficiency in primaquine-sensitive erythrocytes. *Science 124*, 484 (1956).
2. Valentine, W. N., Tanaka, K. R., Miwa, S.: A specific erythrocyte glycolytic enzyme defect (pyruvate kinase) in three subjects with congenital non-spherocytic hemolytic anemia. *Trans. Assoc. Amer. Physicians 74*, 100 (1961).

3. Beutler, E.: *Hemolytic Anemia in Disorders of Red Cell Metabolism.* Plenum Publishing Corp., New York 1978.
4. Fujii, H., Miwa, S.: Red Cell Enzymes. In: *CRC Handbook Series in Clinical Laboratory Science. Section I: Hematology. Vol. IV.* R. M. Schmidt, V. F. Fairbanks (eds). CRC Press, Boca Raton 1986, p. 307.
5. Huang, I.-Y., Rubinfien, E., Yoshida, A.: A complete amino acid sequence of human phosphoglycerate kinase. Isolation and amino acid sequence of tryptic peptides. *J. Biol. Chem. 255*, 6408 (1980).
6. Huang, I.-Y., Welch, C. D., Yoshida, A.: Complete amino acid sequence of human phosphoglycerate kinase. Cyanogen bromide peptides and complete amino acid sequence. *J. Biol. Chem. 255*, 6412 (1980).
7. von Zabern, I., Wittman-Liebold, B., Untucht-Grau, R., Schirmer, R., Oaum E. F.: Primary and tertiary structure of the principal human adenylate kinase. *Eur. J. Biochem. 68*, 281 (1976).
8. Lu, H. S., Yuan, P. M., Gracy, R. W.: Primary structure of human triosephosphate isomerase. *J. Biol. Chem. 259*, 11958 (1984).
9. Haggarty, N. W., Dunbar, B., Fothergil, L. A.: The complete amino acid sequence of human erythrocyte diphosphoglycerate mutase. *EMBO J. 2*, 1213 (1983).
10. Yoshida, A., Watanabe, S., Chen, S.-H., Giblett, E. R., Malcolm, L. A.: Human phosphoglycerate kinase. II. Structure of a variant enzyme. *J. Biol. Chem. 247*, 446 (1972).
11. Fujii, H., Krietsch, W. K. G., Yoshida, A.: A single amino acid substitution (Asp→Asn) in a phosphoglycerate kinase variant (PGK München) associated with enzyme deficiency. *J. Biol. Chem. 255*, 6421 (1980).
12. Fujii, H., Yoshida, A.: Molecular abnormality of phosphoglycerate kinase-Uppsala associated with chronic nonspherocytic hemolytic anemia. *Proc. Natl. Acad. Sci. U. S. A. 77*, 5461 (1980).
13. Fujii, H., Chen, S.-H., Akatsuka, J., Miwa, S., Yoshida, A.: Use of cultured lymphoblastoid cells for the study of abnormal enzymes: molecular abnormality of a phosphoglycerate kinase variant associated with hemolytic anemia. *Proc. Natl. Acad. Sci. U. S. A. 78*, 2587 (1981).
14. Mennecier, F., Daegelen, D., Schweighoffer, F., Levin, M., Kahn, A.: Expression of aldolase A messenger RNAs in human adult and foetal tissues and in hepatoma. *Biochem. Biophys. Res. Commun. 134*, 1093 (1986).
15. Michelson, A. M., Alexander, F. M., Orkin, S. H.: Isolation and DNA sequence of a full-length cDNA clone for human X chromosome-encoded phosphoglycerate kinase. *Proc. Natl. Acad. Sci. U. S. A. 80*, 472 (1983).
16. Takizawa, T., Huang, I.-Y., Ikuta, T., Yoshida, A.: Human glucose-6-phosphate dehydrogenase: primary structure and cDNA cloning. *Proc. Natl. Acad. Sci. U. S. A. 83*, 4157 (1986).
17. Persico, M. G., Viglietto, G., Martini, G., Toniolo, D., Paonessa, G., Moscatelli, C., Dono, R., Vulliamy, T., Luzzatto, L., D'Urso, M.: Isolation of human glucose-6--phosphate dehydrogenase (G6PD) cDNA clones: primary structure of the protein and unusual 5-prime non-coding region. *Nucleic Acids Res. 14*, 2511 (1986).
18. Orkin, S. H., Daddona, E. P., Shebach, D. S., Markham, A. F., Bruns, G. A., Goff, S. C., Kelley, W. N.: Molecular cloning of human adenosine deaminase gene sequences. *J. Biol. Chem. 258*, 12753 (1983).
19. Valerio, D., Duyvesteyn, M. G. C., Khan, P., van Kessel, A. G., de Waard, A., van der Eb, A. J.: Isolation of cDNA clones for human adenosine deaminase. *Gene 25*, 231 (1983).
20. Wiginton, D. A., Adrian, G. S., Friedman, R. L., Suttle, D. P., Hutton, J. J.: Cloning of cDNA sequences of human adenosine deaminase. *Proc. Natl. Acad. Sci. U. S. A. 80*, 7481 (1983).
21. Nakajima, H., Noguchi, T., Gamasaki, T., Kono, N., Tanaka, T., Tarui, S.: Cloning of human muscle phosphofructokinase cDNA. *FEBS Lett. 223*, 113 (1987).

22. Levanon, D., Danciger, E., Dafni, N., Groner, Y.: Genomic clones of the human liver-type phosphofructokinase. *Biochem. Biophys. Res. Commun. 141*, 374 (1986).
23. Maquat, L. E., Chilcote, R., Ryan, P. M.: Human triosephosphate isomerase cDNA and protein structure. *J. Biol. Chem. 260*, 3748 (1985).
24. Joulin, V., Peduzzi, J., Romeo, P.-H., Rosa, R., Valentin, C., Dubart, A., Lapeynre, B., Blouquit, Y., Garel, M.-C., Goosens, M.-C., Rosa, J., Cohen-Solal, M.: Molecular cloning and sequencing of the human erythrocyte 2, 3-bisphosphoglycerate mutase cDNA: revised amino acid sequence. *EMBO J. 5*, 2275 (1986).
25. Tani, K., Fujii, H., Nagata, S., Miwa, S.: Human liver type pyruvate kinase: complete amino acid sequence and the expression in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A. 85*, 1792 (1988).
26. Daar, I. O., Artymiuk, P. J., Phillips, D. C., Maquat, L. E.: Human triose-phosphate isomerase deficiency: a single amino acid substitution results in a thermolabile enzyme. *Proc. Natl. Acad. Sci. U. S. A. 83*, 7903 (1986).
27. Kishi, H., Mukai, T., Hirono, A., Fujii, H., Miwa, S., Hori, K.: Human aldolase A deficiency associated with a hemolytic anemia: thermolabile aldolase due to a single base mutation. *Proc. Natl. Acad. Sci. U. S. A. 84*, 8623 (1987).
28. Rosa, J.: Personal communication.
29. Takizawa, T., Yoneyama, Y., Miwa., S., Yoshida, A.: Single nucleotide base transition is the basis of the common human glucose-6-phosphate dehydrogenase variant A(+). *Genomics 1*, 228 (1987).
30. Hirono, A., Beutler, E.: Molecular cloning and nucleotide sequence of cDNA for human glucose-6-phosphate dehydrogenase variant A(−). *Proc. Natl. Acad. Sci. U. S. A. 85*, 3951 (1988).
31. Vulliamy, T. J., D'Urso, M., Battistuzzi, G., Estrada, M., Foulkes, N. S., Martini, G., Calabro, V., Poggi, V., Giordano, R., Town, M., Luzzatto, L., Persico, M. G.: Diverse point mutations in the human glucose-6-phosphate dehydrogenase gene cause enzyme deficiency and mild or severe hemolytic anemia. *Proc. Natl. Acad. Sci. U. S. A. 85*, 5171 (1988).
32. Matsuura, S., Igarashi, M., Kishi, F., Kajii, T., Fujii, H., Miwa, S., Sakurai, M., Nakazawa, A.: in preparation.
33. Miwa, S., Fujii, H.: Molecular aspects of erythroenzymopathies associated with hereditary hemolytic anemia. *Am. J. Hematol. 19*; 293 (1985).
34. Magnani, M., Dallapiccola, B.: Regional mapping of the locus for hexokinase-1 (HK 1). *Hum. Genet. 62*, 181 (1982).
35. Rijksen, G., Staal, G. E. J.: Human erythrocyte hexokinase deficiency. characterization of a mutant enzyme with abnormal regulatory properties. *J. Clin. Invest. 62*, 294 (1978).
36. Rijksen, G., Akkerman, J. W. N., van den Wall Bake, A. W. L., Hofstede, D. P., Staal, G. E. J.: Generalized hexokinase deficiency in the blood cells of a patient with non-spherocytic hemolytic anemia. *Blood 61*, 12 (1983).
37. McMorris, F. S., Chen, T. R., Ricciuti, F., Tischfield, J., Greagan, R., Ruddle, F.: Chromosome assignments in man of the gene for two hexose phosphate isomerase. *Science 179*, 1129 (1973).
38. Welch, S. G.: Qualitative and quantitative variants of human phosphoglucose isomerase. *Hum. Hered. 21*, 467 (1971).
39. Arnold, H., Hasslinger, K., Witt, I.: Glucosephosphate-isomerase type Kayserslautern. A new variant causing congenital nonspherocytic hemolytic anemia. *Blut 46*, 271 (1983).
40. Hopkinson, D. A.: The investigation of reactive sulfhydryls in enzymes and their variants by starch-gel electrophoresis: Studies on the human phosphohexose isomerase variant PHI 5−1. *Ann. Hum. Genet. 34*, 79 (1970).
41. Kahn, A., Meienhofer, M.-C., Cottreau, D., Lagrange, J.-L., Dreyfus, J.-C.: Phosphofructokinase (PFK) isozymes in man. I. Studies of adult human tissues. *Hum. Genet. 48*, 93 (1979).

42. Vora, S., Seaman, C., Durham, S., Piomelli, S.: Isozymes of human phosphofructokinase: Identification and subunit structural characterization of a new system. *Proc. Natl. Acad. Sci. U. S. A. 77*, 62 (1980).
43. Davidson, M., Miranda, A. F., Bender, A. N., DiMauro, S.: Muscle phosphofructokinase deficiency. Biochemical and immunological studies of phosphofructokinase isozymes in muscle culture. *J. Clin. Invest. 72*, 545 (1983).
44. Etiemble, J., Simeon, J., Buc, H. A., Picat, C., Boulard, M., Boivin, P.: A liver-type mutation in a case of pronounced erythrocyte phosphofructokinase deficiency without clinical expression. *Biochim. Biophys. Acta 759*, 236 (1983).
45. Chadefaux, B., Rethore, M. O., Allard, D.: Regional mapping of liver-type 6-phosphofructokinase isoenzyme on chromosome 21. *Hum. Genet. 68*, 136 (1984).
46. Vora, S., Durham, S., de Martinville, B., Gerge, D. L., Francke, U.: Assignment of the human gene for muscle-type phosphofructokinase (PFKM) to chromosome 1 (Region cen — q 32) using somatic cell hybrids and monoclonal anti-M antibody. *Somatic Cell Genet. 8*, 95 (1982).
47. Rottman, W. H., Tolan, D. R., Penhoet, E. E.: Complete amino acid sequence for human aldolase B derived from cDNA and genomic clones. *Proc. Natl. Acad. Sci. U. S. A. 81*, 2738 (1984).
48. Sakakibara, M., Mukai, T., Hori, K.: Nucleotide sequence of a cDNA clone for human aldolase A messenger RNA in the liver. *Biochem. Biophys. Res. Commun. 131*, 413 (1985).
49. Sakakibara, M., Mukai, T., Yatsuki, H., Hori, K.: Human aldolase isozyme gene: the structure of multispecies aldolase B mRNAs. *Nucleic Acids Res. 13*, 5055 (1985).
50. Beutler, E., Scott, S., Bishop, A., Margolis, N., Matsumoto, F., Kuhl, W.: Red cell aldolase deficiency and hemolytic anemia: a new syndrome. *Trans. Assoc. Am. Physicians 76*, 154 (1973).
51. Miwa, S., Fujii, H., Tani, K., Takahashi, K., Takegawa, S., Fujinami, N., Sakurai, M., Kubo, M., Tanimoto, Y., Kato, T., Matsumoto, N.: Two cases of red cell aldolase deficiency associated with hereditary hemolytic anemia in a Japanese family. *Am. J. Hematol. 11*, 425 (1981).
52. Sygusch, J., Beaudry, D., Allaire, M.: Molecular architecture of rabbit skeletal muscle aldolase at 2.7-Å resolution. *Proc. Natl. Acad. Sci. U. S. A. 84*, 7846 (1987).
53. Eber, S. W., Krietch, W. K. G.: The isolation and characterization of the multiple forms of human skeletal muscle triosephosphate isomerase. *Biochim. Biophys. Acta 614*, 173 (1980).
54. Decker, R. S., Mohrenweiser, H. W.: Origin of the triosephosphate isomerase isozymes in humans: genetic evidence for the expression of a single structural locus. *Am. J. Hum. Genet. 33*, 683 (1981).
55. Michelson, A. M., Markham, A. F., Orkin, S. H.: Isolation and DNA sequence of a full-length cDNA clone for human X chromosome-encoded phosphoglycerate kinase. *Proc. Natl. Acad. Sci. U. S. A. 80*, 472 (1983).
56. Banks, R. D., Blake, C. C. F., Evans, P. R., Haser, R., Rice, D. W., Merrett, M., Phillips, A. W.: Sequence, structure and activity of phosphoglycerate kinase: a possible hinge-bending enzyme. *Nature 279*, 773 (1979).
57. Yoshida, A., Watanabe, S., Chen, S.-H., Giblett, E. R., Malcolm, L. A.: Human phosphoglycerate kinase. II. Structure of a variant enzyme. *J. Biol. Chem. 247*, 446 (1972).
58. Saheki, S., Saheki, K., Tanaka, T.: Peptide structures of pyruvate kinase isozymes. 1. Comparison of the four pyruvate kinase isozymes of the rat. *Biochim. Biophys. Acta 524*, 327 (1978).
59. Noguchi, T., Inoue, H., Tanaka, T.: The M_1- and M_2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative splicing. *J. Biol. Chem. 261*, 13807 (1986).
60. Noguchi, T., Yamada, K., Inoue, H., Matsuda, T., Tanaka, T.: The L- and R-type iso-

zymes of rat pyruvate kinase are produced from a single gene by use of different promotors. *J. Biol. Chem. 262*, 14366 (1987).
61. Lonberg, N., Gilbert, W.: Primary structure of chicken muscle pyruvate kinase mRNA. *Proc. Natl. Acad. Sci. U. S. A. 80*, 3661 (1983).
62. Lonberg, N., Gilbert, W.: Intron/exon structure of the chicken pyruvate kinase gene. *Cell 40*, 81 (1985).
63. Inoue, H., Noguchi, T., Tanaka, T.: Complete amino acid sequence of rat L-type pyruvate kinase deduced from the cDNA sequence. *Eur. J. Biochem. 154*, 465 (1986).
64. Lone, Y.-C., Simon, M.-P., Kahn, A., Marie, J.: Complete nucleotide and deduced amino acid sequences of rat L-type pyruvate kinase. *FEBS Lett. 195*, 97 (1986).
65. Burke, R. L., Tekemp-Olaon, P., Najarian, R.: The isolation, characterization and sequence of the pyruvate kinase of *Saccharomyces cervisiae*. *J. Biol. Chem. 258*, 2193 (1983).
66. Cognet, M., Lone, Y.-C., Vaulont, S., Kahn, A., Marie, J.: Structure of the rat L-type pyruvate kinase gene. *J. Mol. Biol. 196*, 11 (1987).
67. Muirhead, H., Clayden, D. A., Barford, D., Lorimer, C. G., Fothergill-Gilmore, L. A., Schiltz, E., Schmitt, W.: The structure of cat muscle pyruvate kinase. *EMBO J. 5*, 475 (1986).
68. Tani, K., Fujii, H., Tsutsumi, H., Sukegawa, J., Toyoshima, K., Yoshida, M. C., Noguchi, T., Tanaka, T., Miwa, S.: Human liver type pyruvate kinase: cDNA cloning and chromosomal assignment. *Biochem. Biophys. Res. Commun. 143*, 431 (1987).
69. Satoh, H., Tani, K., Yoshida, M. C., Sasaki, M., Miwa, S., Fujii, H.: The human liver-type pyruvate kinase (PKL) gene is on chromosome 1 at band q21. *Cytogenet. Cell. Genet. 47*, 132 (1988).
70. Takegawa, S., Fujii, H., Miwa, S.: Change of pyruvate kinase isozymes from M_2- to L-type during development of the red cell. *Brit. J. Haematol. 54*, 467 (1983).
71. Takegawa, S., Miwa, S.: Change of pyruvate kinase (PK) isozymes in classical type PK deficiency and other PK deficiency cases during red cell maturation. *Am. J. Hematol. 16*, 53 (1984).
72. Takegawa, S., Shinohara, T., Miwa, S.: Hemin-induced conversion of pyruvate kinase isozymes in K562 cells. *Blood 64*, 754 (1984).
73. Miwa, S., Boivin, P., Blume, K. G., Arnold, H., Black, J. A., Kahn, A., Staal, G. E. J., Nakashima, K., Tanaka, K. R., Paglia, D. E., Valentine, W. N., Yoshida, A., Beutler, E.: International Committee for Standardization in Haematology: Recommended methods for the characterization of red cell pyruvate kinase variants. *Brit. J. Haematol. 43*, 275 (1979).
74. Zanella, A., Colombo, M. B., Miniero, R., Perroni, L., Melon, T., Sirchia, G.: Erythrocyte pyruvate kinase deficiency: 11 new cases. *Brit. J. Haematol. 69*, 399 (1988).
75. Tani, K., Yoshida, M. C., Satoh, H., Mitamura, K., Noguchi, T., Fujii, H., Miwa, S.: Human M_2-type pyruvate kinase: cDNA cloning, chromosomal assignment and expression in hepatoma cells. *Gene 73*, 509 (1988).
76. Schröter, W.: Kongenitale nichtsphärocytäre Hämolytische Anämie bei 2, 3-Diphosphoglyceratmutase-mangel der Erythrocyten im frühen Säuglingsalter. *Klin. Wschr. 43*, 1147 (1965).
77. Rosa, R., Prehu, M.-U., Beuzard, Y., Rosa, J.: The first case of a complete deficiency of diphosphoglycerate mutase in human erythrocytes. *J. Clin. Invest. 62*, 907 (1978).
78. Yoshida, A.: Glucose 6-phosphate dehydrogenase of human erythrocytes. I. Purification and characterization of normal (B+) enzyme. *J. Biol. Chem. 241*, 4966 (1966).
79. Yoshida, A.: A single amino acid substitution — asparagine to aspartic acid — between normal (B+) and the common Negro variant (A+) of human glucose-6-phosphate dehydrogenase. *Proc. Natl. Acad. Sci. U. S. A. 57*, 835 (1967).
80. Yoshida, A.: Amino acid substitution (histidine to tyrosine) in a glucose-6-phosphate dehydrogenase variant (G6PD Hektoen) associated with overproduction. *J. Mol. Biol. 52*, 483 (1970).

81. Miwa, S., Fujii, H., Tani, K., Takahashi, K., Takizawa, T., Igarashi, T.: Red cell adenylate kinase deficiency associated with hereditary nonspherocytic hemolytic anemia: clinical and biochemical studies. *Am. J. Hematol. 14*, 325 (1983).
82. Beutler, E., Carson, D., Dannawi, H., Forman, L., Kuhl, W., West, C., Westwood, B.: Metabolic compensation for profound erythrocyte adenylate kinase deficiency: a hereditary enzyme defect without hemolytic anemia. *J. Clin. Invest. 72*, 648 (1983).
83. Siciliano, M. J., Bordelon, M. E., Kohler, P. O.: Expression of human adenosine deaminase after fusion of adenosine deaminase-deficient cells with mouse fibroblasts. *Proc. Natl. Acad. Sci. U. S. A. 75*, 3876 (1978).
84. Giblett, E. R., Anderson, J. E., Cohen, F., Pollara, B., Meuwissen, H. H.: Adenosine deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet I*, 1067 (1972).
85. Valentine, W. N., Paglia, D. E., Tartaglia, A. P., Gilsantz, F.: Hereditary hemolytic anemia with increased red cell adenosine deaminase (45- to 70-fold) and decreased adenosine triphosphate. *Science 195*, 783 (1977).
86. Miwa, S., Fujii, H., Matsumoto, N., Nakatsuji, T., Oda, S., Asano, S., Miura, Y.: A case of red-cell adenosine deaminase overproduction associated with hereditary hemolytic anemia found in Japan. *Am. J. Hematol. 5*, 107 (1978).
87. Pérignon, J.-L., Hamet, M., Buc, H. A., Cartier, P. H., Derycke, M.: Biochemical study of a case of hemolytic anemia with increased (85-fold) red cell adenosine deaminase. *Clin. Chim. Acta 124*, 205 (1982).
88. Kanno, H., Tani, K., Fujii, H., Iguchi-Ariga, S. M. M., Ariga, H., Kozaki, T., Miwa, S.: Adenosine deaminase (ADA) overproduction associated with congenital hemolytic anemia: case report and molecular analysis. *Japan. J. Exp. Med. 58*, 1 (1988).
89. Fujii, H., Miwa, S., Suzuki, K.: Purification and properties of adenosine deaminase in normal and hereditary hemolytic anemia with increased red cell activity. *Hemoglobin 4*, 693 (1980).
90. Fujii, H., Miwa, S., Tani, K., Fujinami, N., Asano, H.: Overproduction of structurally normal enzyme in man: hereditary haemolytic anaemia with increased red cell adenosine deaminase activity. *Brit. J. Haematol. 51*, 427 (1982).
91. Chottiner, E. G., Cloft, H. J., Tartaglia, A. P., Mitchell, B. S.: Elevated adenosine deaminase activity and hereditary hemolytic anemia; evidence for abnormal translational control of protein synthesis. *J. Clin. Invest. 79*, 1001 (1987).
92. Chottiner, E. G., Ginsberg, D. S., Mitchell, B. S.: Hereditary red cell adenosine deaminase overproduction: association with increased levels of structurally normal ADA mRNA (Abstract). *Pediat. Res. 24*, 111 (1988).
93. Swallow, D. M., Aziz, I., Hopkinson, D. A., Miwa, S.: Analysis of human erythrocyte 5'-nucleotidase in healthy individuals and a patient in pyrimidine 5'-nucleotidase. *Ann. Hum. Genet. 47*, 19 (1983).
94. Paglia, D. E., Valentine, W. N., Brockway, R. A.: Identification of thymidine nucleotidase and deoxyribonucleotidase activities among normal isozymes of 5'-nucleotidase in human erythrocytes. *Proc. Natl. Acad. Sci. U. S. A. 81*, 588 (1984).
95. Hirono, A., Fujii, H., Natori, H., Kurokawa, I., Miwa, S.: Chromatographic analysis of human erythrocyte pyrimidine 5'-nucleotidase from five patients with pyrimidine 5'-nucleotidase deficiency. *Brit. J. Haematol. 65*, 35 (1987).
96. Beutler, E., Bonaventura, C., Huisman, T. H. J., Minakami, S., Miwa, S., Nakao, M., Schneider, R. G., Schroeder, W. A., Tanaka, K. R., Tashian, R. E., Valentine, W. N., Yoshida, A.: Round table; enzymatic deficiencies of the red cell. *Hemoglobin 4*, 805 (1980).
97. Hirono, A., Forman, L., Beutler, E.: Enzymatic diagnosis in non-spherocytic hemolytic anemia. *Medicine 67*, 110 (1988).
98. Beutler, E., Dunning, D., Dabe, I. B., Forman, L., Erythrocyte glutathione S-transferase deficiency and hemolytic anemia. *Blood 72*, 73 (1988).

99. Cohen-Haguenauer, O., Van Cong, N., Mennecier, F., Kahn, A., Frezal, J.: The human aldolase A gene on chromosome 16 (Abstract). *Cytogenet. Cell Genet. 40*, 605 (1985).
100. Barichard, F., Joulin, V., Henry, I., Garel, M.-C., Valentin, C., Rosa, R., Cohen-Solal, M., Junien, C.: Chromosomal assignment of the human 2, 3-bisphosphoglycerate mutase gene (BPGM) to region 7q34—7q22. *Hum. Genet. 77*, 283 (1987).
101. Sukegawa, Y., Ishida, K., Takeda, T., Takagi, K.: cDNA sequence coding for human glutathione peroxidase. *Nucleic Acids Res. 15*, 7178 (1987).
102. Johannsmann, R., Helkuhl, B., Grzeschik, K.-H.: Regional mapping of human chromosome 3: assignment of a glutathione peroxidase-1 gene to 3p13—3q12. *Hum. Genet. 56*, 361 (1981).

Haematologia 22 (4), pp. 233–238 (1989)

The Effect of Human Recombinant Granulocyte-Macrophage Colony Stimulating Factor on the Proliferation and Differentiation of Myeloid Progenitors in Congenital Agranulocytosis Marrow Cells

H. T. HASSAN, J. K. H. REES

Department of Haematological Research, University of Cambridge
Clinical School, MRC Building, Hills Road, Cambridge CB2 2QL, England, UK

(Received 28 July, 1988; accepted 14 October, 1988)

Congenital agranulocytosis is a rare and frequently fatal infantile disease characterized by recurrent bacterial infections, persistent absence of neutrophils in the peripheral blood and an arrest of myeloid maturation at the promyelocyte/myelocyte stage. The effect of human recombinant Granulocyte-Macrophage colony stimulating factor (GM-CSF) alone and in combination with retinoic acid, dimethylsulphoxide or actinomycin-D on the proliferation and differentiation of bone marrow cells from a child with congenital agranulocytosis was studied. Cells were treated at a concentration of 1×10^5 per ml in in-vitro culture with GM-CSF alone and in combination with retinoic acid, dimethylsulphoxide or actinomycin-D for 7 days at 37 °C in humidified incubator containing 5 % CO_2 in air. GM-CSF showed a profound stimulatory effect on the proliferation of myeloid progenitors from the child bone marrow and restored colony numbers in the retinoic acid-, dimethylsulphoxide- and actinomycin-D-inhibited cultures.

Keywords: cell differentiation, cell proliferation, congenital agranulocytosis, congenital neutropenia, dimethylsulphoxide, colony-stimulating factor, Kostmann syndrome, retinoic acid

Introduction

Human recombinant Granulocyte-Macrophage colony stimulating factor (rHu GM-CSF) has been reported to enhance proliferation and differentiation of normal and leukaemic myeloid bone marrow cells [13, 14, 15]. Partial differentiation of the human myeloid leukaemia cell line KG-1 has been induced by rHu GM-CSF [16]. It has also been reported to restore the growth inhibition caused by dimethylsulphoxide in human promyelocytic leukaemia cell line HL-60 [17].

In the present study, the effect of human recombinant GM-CSF alone and in combination with retinoic acid, dimethylsulphoxide and actinomycin-D on the proliferation and differentiation of bone marrow myeloid progenitors from a

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

child with congenital agranulocytosis has been studied. Retinoic acid, dimethylsulphoxide and actinomycin-D induce differentiation of cells from several myeloid leukaemia cell lines [18] and from patients with acute myeloid leukaemia [19].

Materials and Methods

Preparation of bone marrow cells

Bone marrow was obtained by aspiration from the posterior iliac crest of a 6-months female child with congenital agranulocytosis into a sterile syringe containing preservative-free heparin after an informed consent from her parents. Her blood count showed: Hb 100 g/L, platelets 800×10^9/L, nucleated cells 10×10^9/L, eosinophils 0.8×10^9/L, monocytes 1.8×10^9/L and complete absence of neutrophils. Her bone marrow aspirate showed normal cellularity with normal erythropoiesis and megakaryocytes and a maturation arrest at an early myelocyte stage. Non-adherent monomuclear marrow cells were prepared as described before [20].

Preparation of human recombinant GM-CSF

rHu GM-CSF was generously provided by Dr. R. E. Marcus from the Department of Haematology of Addenbrooke's Hospital, Cambridge, at a concentration of 1×10^5 units per ml in medium containing 0.1% bovine serum albumin (BSA). It was diluted to a final concentration of 100 Units/ml before addition to culture.

Preparation of retinoic acid

A stock solution of 10^{-2}M retinoic acid was prepared as described before [20]. Before use, it was serially diluted to provide final concentrations of 10^{-9}M, 10^{-8}M, 10^{-7}M, and 10^{-6}M. All tubes were covered with aluminium foil and the manipulations were carried out in subdued light.

Bone marrow culture

Non-adherent mononuclear bone marrow cells were plated at a concentration of 1×10^5 cells/ml in 35 mm Petri dishes containing 0.8% methylcellulose, RPMI 1640 culture medium 30% fetal calf serum, 1% bovine serum albumin and 10^{-4}M 2-mercaptoethanol in a total volume of 1 ml, rHu GM-CSF was added at a concentration of 100 Units/ml. Retinoic acid was added at the concentrations of 10^{-9}M, 10^{-8}M, 10^{-7}M and 10^{-6}M. Dimethylsulphoxide was added at a concentration of 100mM and actinomycin-D at a concentration of 5nM. A control plate containing neither rHu GM-CSF nor any differentiating agent was also set up in

conditions otherwise identical. Triplicate plates of each of the culture conditions were set up in a humidified incubator containing 5% CO_2 in air at 37 °C for 7 days. After days of incubation, colonies of 40 or more cells were counted using an inverted microscope.

Assessment of differentiation at day 7

On day 7, cells from each 35 mm Petri dish were resuspended to produce a mononuclear suspension. Morphological maturation was assessed on cytocentrifuge smears using Hyel-Romanowsky stain. Functionally, the differentiation was estimated by the nitroblue tetrazolium dye reduction test which was performed as described before [20].

Results

The mean number of CFU-GM per 1×10^5 cells in control cultures with no additions was 39 ± 12. Human recombinant GM-CSF at a concentration of 100 Units/ml markedly increased the CFU-GM in the congenital agranulocytosis bone marrow cells by more than five times the control level (Table 1). Moreover, it was able to restore the growth inhibition caused by the three differentiating agents: retinoic acid, dimethylsulphoxide and actinomycin-D by 33%, 45% and 35%, respectively. The enhancement of proliferation of the progenitor cells was

Table 1

Effect of human recombinant GM–CSF alone and in combination with differentiating agents on the colony formation of congenital agranulocytosis bone marrow cells

Differentiating agent	CFU-GM on Day 7*	
	without rHu GM-CSF	with rHu GM-CSF
Control	39± 12**	202.8± 5.7
Retinoic acid (10^{-6} M)	46.8± 5.9	205.1± 4.8
Retinoic acid (10^{-7} M)	44.8± 5.9	202.8± 5.7
Retinoic acid (10^{-8} M)	23.4± 8.6	200.2± 6.2
Retinoic acid (10^{-6} M)	23.9± 9.2	179.4± 7.1
Actinomycin-D (5 nM)	20.8± 6.5	182.0± 9.2
Dimethylsulphoxide (100 mM)	15.6± 6.1	175.5± 9.4

Cells were cultured at a concentration of 1×10^5/ml for 7 days in 35 mm Petri culture dishes. All experiments were performed in triplicate.

* represents the number of colonies on day 7, each colony contained 40 or more cells;

** Mean ± Standard Deviation

Table 2

Effect of Human Recombinant GM-CSF alone and in combination with differentiating agents on the differentiation of congenital agranulocytosis bone marrow cells

Differentiating agent	Morphology (% of cells)				% of nitro-bluetetrazolium
	Immature[a]	Intermediate[b]	Mature[c]	Others[d]	Reducing cells
Control*	81.5 ± 2.4**	11.0 ± 4.5	5.5 ± 2.1	4.0 ± 1.2	4.5 ± 3.5
GM-CSF alone (100 U/ml)	8.0 ± 1.9	31.0 ± 2.7	29.0 ± 2.1	22.5 ± 2.4	46.0 ± 4.2
GM-CSF + RA 10^{-9}M	3.0 ± 1.2	17.0 ± 1.4	81.5 ± 2.5	7.0 ± 3.5	80.0 ± 1.2
GM-CSF + RA 10^{-8}M	2.5 ± 1.7	16.5 ± 1.2	83.5 ± 4.6	2.5 ± 1.9	81.5 ± 2.9
GM-CSF + RA 10^{-7}M	1.5 ± 2.1	15.5 ± 1.6	84.5 ± 3.8	2.0 ± 1.2	82.5 ± 2.5
GM-CSF + RA 10^{-6}M	1.0 ± 1.2	14.0 ± 1.2	84.5 ± 3.2	1.0 ± 1.3	82.5 ± 2.7
GM-CSF + DMSO 100 mM	1.0 ± 1.4	35.5 ± 5.9	55.5 ± 2.4	9.0 ± 1.4	53.5 ± 3.5
GM-CSF + Act-D 5 nM	1.0 ± 1.6	44.0 ± 4.2	34.5 ± 1.7	20.5 ± 2.9	49.5 ± 2.4

Cells were cultured at a concentration of 1×10^5/ml for 7 days. All experiments were performed in triplicate.

RA: Retionic acid, DMSO: dimethylsulphoxide, Act-D: actinomycin-D; * Contains neither GM-CSF nor any agent; ** Mean ± Standard Deviation. a: myeloblasts and promyelocytes, b: myelocytes, c: metamyelocytes and mature granulocytes, d: eosinophils and monocytes.

accompanied by the partial differentiation when rHu GM-CSF was used alone (Tables 1, 2).

Morphological examination of cytocentrifuge smears of cells from treated cultures revealed an increase in the number of mature granulocytes from 5.5% in the controls to 29% with rHu GM-CSF (Table 2). The addition of dimethylsulphoxide and actinomycin-D to rHu GM-CSF cultures increased the percentage of mature granulocytes to 54% and 55% respectively (Table 2). When the marrow was incubated with rHu GM-CSF and retinoic acid, over 80% of the resulting cells on day 7 were metamyelocytes and mature granulocytes (Table 2).

We studied the ability of human recombinant GM-CSF alone and in combination with retinoic acid, dimethylsulphoxide or actinomycin-D to induce a functional differentiation in the cultured cells by assessing their ability to reduce the nitroblue tetrazolium dye. While only 4.5% of cells from the control cultures were able to reduce the dye, 46% of those from rHu GM-CSF treated cultures, reduced it (Table 2). The addition of retinoic acid to rHu GM-CSF treated cultures increased the number of nitroblue tetrazolium reducing cells to more than 80% (Table 2).

Discussion

The pathological basis for congenital agranulocytosis remains obscure. The condition affects only neutrophils with platelets, red cells and more significantly eosinophil production remaining normal. No immunological basis either humoral or cellular has been consistently shown to be responsible for the failure of maturation observed.

Our findings show that rHu GM-CSF profoundly enhanced the proliferation of myeloid progenitors derived from the bone marrow of a child with congenital agranulocytosis and restored the growth inhibition caused by retinoic acid, dimethylsulphoxide and actinomycin-D.

Also, we have shown little myeloid differentiation in the bone marrow colonies on day 7 from a child with congenital agranulocytosis in the absence of rHu GM-CSF. On incubation with rHu GM-CSF, however, a partial maturation of these colonies took place showing that myeloid progenitors in this case were sensitive to the differentiating effect of this human haemopoietic growth factor. This effect was markedly enhanced on the addition of the differentiating agent: retinoic acid.

Differentiation of myeloid progenitor cells in in-vitro culture by agents such as retinoic acid, dimethylsulphoxide and actinomycin-D is usually at the expense of proliferation [19]. We have shown that the combined incubation of human recombinant GM-CSF with the above differentiating agents restores the inhibited growth. The block in differentiation observed in this child may be overcome by a combination of human recombinant GM-CSF and retinoic acid. Endogenous GM-CSF levels were not assessed in the patient. Antigen density of GM-CSF receptors in the myeloid progenitors similarly could not be established. Nevertheless we have demonstrated the ability of pharmacological dosages of a combination of human recombinant CM-CSF and retinoic acid to differentiate the myeloid progenitors in this case. This combination may be effective in the treatment of children suffering from congenital agranulocytosis.

Acknowledgements

H. T. Hassan was supported by a scholarship from the Medical Research Institute. J. K. H. Rees is the coordinator of the 9th and 10th MRC AML U. K. clinical trials.

References

1. Kostman, R.: Infantile genetic agranulocytosis: a review with presentation of ten new cases. *Acta Pediatr. Scan. 64*, 362 (1975).
2. Parmley, R. T., Crist, W. M.: Childhood neutropenia. *Ala J. Med. Sci. 19*, 249 (1982).
3. Rich, K., Falk, P. M., Stiehm, E. R., Feig, S., Golde, D. W., Cline, M. J.: An abnormal in-vitro granulopoiesis in phenotypically normal parents of some children with congenital agranulocytosis. *Pediatr. 59*, 396 (1977).

4. L'Esperance, P., Brunning, R., Deinard, A. S., Park, B. H., Bigger, W. D., Good, R. A.: Congenital neutropenia: impaired maturation with a diminished stem cell input. *Birth Defects 11*, 59 (1975).
5. Coulombel, L., Morardet, N., Veber, F., Leroy, C., Mielot, F., Fisher, A., Thiebaud, F. T., Tchernia, G., Criscelli, C., Parmentier, C.: Granulopoietic differentiation in long term bone marriow cultures from children with congenital neutropenia. *Am. J. Haematol. 27*, 93 (1988).
6. Olofsson, T., Olsson, I., Kostmann, R., Malmstrom, S., Thelen, A.: Granulopoiesis in infantile genetic agranulocytosis in in-vitro cloning of marrow cells in agar cultures. *Scan. J. Haematol 16*, 18 (1976).
7. Falk, P., Rich, K., Feig, S., Steihn, R., Golde, D., Cline, M.: Evaluation of congenital neutropenic disorders by in vitro bone marrow cultures. *Pediatr. 59*, 739 (1977).
8. Parmley, R. T., Ogawa, M., Darby, C. P., Spicer, S.S.: Congenital neutropenia neutrophil proliferation with abnormal maturation. *Blood 46*, 723 (1975).
9. Weisenburger, DD., Jackson, J. D., Gnarra, D. J., Crouse, D. A., Sharp, J. G.: Characterization of a defect in a patient with congenital neutropenia. *Blood 66*, 81A (1985).
10. Barak, Y., Paran, M., Levin, S.: In vitro induction of myeloid proliferation and differentiation in infantile genetic agranulocytosis. *Blood 38*, 74—80 (1971).
11. Kawagushi, Y., Kobayashi, M., Tanabe, A., Hara, M., Nishi, Y., Usui, T., Nagal, S., Nishibayashi, Y., Nagao, K., Yokoro, K.: Granulopoiesis in patients with congenital neutropenia. *Am. J. Haematol 20*, 223 (1985).
12. Chusid, M. J., Pisciotta, A. V., Duquesnoy, R. J., Camitta, B. M., Tomasulo, P. A.: Congenital neutropenia: studies of pathogenesis. *Am. J. Haematol. 8*, 315 (1980).
13. Vellenga, E., Ostapovicz, D., O'Rourke, B., Griffin, J. D.: Effects of recombinant IL-3, GM-CSF and G-CSF on the proliferation of leukaemic clonogenic cells in short-term and long-term cultures. *Leukaemia 1*, 584 (1987).
14. Miyauchi, J., Kelleher, C. A., Yang, Y. C., Wong, G. G., Clark, S. C., Minden, M. D., Minkin, S., McCulloch, E. A.: The effects of three recombinant growth factors IL-3, GM-CSF and G-CSF on the blasts of acute myeloblastic leukaemia maintained in short-term culture. *Blood 70*, 657 (1987).
15. Strife, A., Lambek, C., Wisniewski, D., Gulati, S., Gasson, J. C., Golde, D. W., Welte, K., Iove, J. L., Clarkson, B.: Activities of four purified growth factors on highly enriched human haematopoietic progenitor cells. *Blood 69*, 1508 (1987).
16. Hegewisch, S., Bressler, J., Haimi, J., Souza, L., Welte, K., Andreeff, M.: Induction of differentiation of human rGM-CSF and rG-CSF with cytosine arabinoside in the human myeloid leukaemic cell line KG-1. *Cancer Res. 48*, 4049A (1988).
17. Brennan, J. K., Lee, K. S., Abboud, C. N., Erickson-Miller, C. L., Keng, P. C.: Interactions of dimethylsulphoxide and GM-CSFs on the growth and maturation of HL-60 cells. *J. Cell Physiology 132*, 246 (1987).
18. Hassan, H. T.: Differentiation inducers of human myeloid [leukaemia cell] lines. *Folia Haematol. 115*, 6 (1988).
19. Hassan, H. T.: Diffffferentiation induction therapy of acute myelogenous leukaemias. *Haematologia 21*, 141 (1988).
20. Hassan, H. T., Rees, J. K. H.: Retinoic acid alone and in combination with cytosine arabinoside induces differentiation of human acute myelomonocytic and monoblastic leukaemia cells. *Haematol. Oncol. 6*, 39 (1988).

Haematologia 22 (4), pp. 239–247 (1989)

Clinical Analysis of Patients with Non-Hodgkin's Lymphomas (1965–1985)

K. PÁLÓCZI[1], E. BERÉNYI[2], Á. ILLÉS[2], Z. NEMES[3], G. KRASZNAI[4], G. KRAJCZÁR,[5] GY. SZEGEDI[2]

[1]National Institute of Haematology and Blood Transfusion, 1113 Budapest, Daróczi ut 24., Hungary, [2]Third Department of Medicine and [3]Institute of Pathology, University Medical School Debrecen, [4]Department of Pathology of Géza Hetényi County Hospital, Szolnok, [5]Department of Pathology of Gyula Kenézy County Hospital, Debrecen, Hungary

(Received 5 October, 1988; accepted 15 November, 1988)

Clinical and histopathological data of 307 patients with non-Hodgkin's lymphoma (NHL) was reviewed applying the Kiel histopathologic classification scheme and the Ann Arbor clinical and pathologic staging system.

The first group included 74 patients who were diagnosed as having lympho- and reticulosarcomas during the 1965–1974 period. The case histories and histological diagnoses of these patients were retrospectively reevaluated. These patients were given orthovoltic irradiation and single agent chemotherapy. The second group included 233 patients who were entered into a prospective study from 1975 onwards. These patients were given combined polychemotherapeutical regimes. The comparative investigation has shown that the proportion of the low and high grade NHLs were the same in the groups. The administration of the new chemotherapeutical regimes did not influence significantly the survival of NHL patients.

Keywords: chemotherapy, non-Hodgkin lymphoma, survival probability

Introduction

Histopathology of lymphoma has changed during the last 20 years [1, 2, 3 4]. Modern classifications, such as the Kiel diagnostic scheme, were introduced into our practice 13 years ago [5, 6, 7, 8, 9]. Around the same time new polychemotherapeutic regimes have also been introduced. While the new classification, helped evaluate the lymphomatous process in a more meaningful way [2, 4, 10, 11], it became more difficult to compare the earlier single drug therapy and the new polychemotherapy regimes. For this reason we decided to perform a retrospective analysis and reclassify the early cases by the modern Kiel classification.

In this paper we describe the comparative analysis of the two different therapeutic approaches on the basis of the unified diagnostic and clinical criteria.

VSP, Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Patients and Methods

The histological revision and reclassification of biopsy sections from 307 patients initially diagnosed as lymphosarcoma and reticulosarcoma was carried out in 1976. Questionnaires were sent to the 152 patients with obtainable address. More detailed information was gained for 74 cases from several clinics files, from the patients and their relatives. 43 of our questionnaires remained unanswered: we suppose some of these patients had alredy deceased by that time. The reexamination of the ten patients still alive has been performed at our clinics in 1976. The main modality of treatment in the first period was orthovoltic irradiation while Degranol, Cyclophosphamide, Leukeran, Vinblastine, Prednisone were used mostly as single agent therapy. Attempts to learn exact dosage were unsuccesful.

In the second ten year period, between January 1, 1975 and December 31, 1984, 233 NHL – patients were examined at the 3rd Department of Medicine University Medical School of Debrecen. The diagnosis was based in all cases on the detailed histologic study of the biopsy sample applying the Kiel scheme for the classification [2].

Following clinical staging, patients were classified according to the Ann Arbor criteria [12]. Patients were treated on the basis of published literature, as well as comprehensive studies [13, 14, 15] and concepts described in the "Manual of Cancer Chemotherapy" [16]. Patients in the low grade malignancy group were treated by the COP (Cyclophosphamide, Oncovine, Prednisone) protocol, with the exception of CLL (chronic lymphocytic leukaemia) patients where the combination of Prednisone and Leukeran or Prednisone monotherapy was applied. In the first five years of the second ten-year period patients with high grade NHLs were also treated by the COP scheme. In the case of unfavorable clinical effects we changed to BACOP (Bleomycin, Adriamycin, Cyclophosphamide, Oncovine, Prednisone) [17] or CHOP (Cyclophosphamide, Adriablastine Oncovine, Prednisone) [18] polychemotherapy. In the second five-year period patients with high grade malignancy lymphomas were treated primarily according to the CHOP or BACOP scheme, individual modifications were used as second treatment. Radiotherapy was applied only in the I–II clinical stages or as palliation, mostly on vital indication to decrease the tumorous mass. Irradiation was administered at the Clinic of Radiology, Cobalt Therapy Ward, with 40 Gy per region cumulative dose, giving 2 Gy daily.

At the assessment of therapeutic effects we observed suggestions issued by the "World Health Organisation" in 1979.

Survival and follow-up data were obtained on 194 patients during the second 10 year period. We have no information on 39 patients; they were supposedly rehospitalized elsewhere.

Survival data were processed by the method of Kaplan and Meier, with a computer.

Results

Among the NHL patients diagnosed between 1965 and 1974 we gained evaluable clinical data in 74 cases. 46 were males (62.8%) and 28 females (37.8%). The male – female ratio was 1.64 : 1. Mean age at diagnosis was 58.1 years (22–79). Distribution of patients according to diagnosis is shown in Table 1. 69% of the patients belonged to the group with low-grade NHL, while 31% were in the group with high-grade malignancy. In this group of patients no case of T-cell lymphoma was found. Though available data were not sufficient to evaluate clinical stage and judgement of remission, nevertheless we were successful in determining the survival time from diagnosis. At the time of the study 10 patients were still alive, all of them belonged to the group with low-grade lymphomas.

In the course of the next 10 years we examined altogether 233 NHL-patients. Among them 148 (63.5%) were male and 85 (36.5%) female. The male-female ratio was 1.75 : 1. The mean age of the patients was 58.47 (18–89) years.

Table 1

Distribution of histologic type of NHLs diagnosed between 1965 and 1974

Histological type of NHLs	Number of patients	%
Low-grade malignancy		
CLL	24	32.45
Centrocytic	7	9.45
Centrocytic-centroblastic	2	2.75
Immunocytic	18	24.35
	51	69.0
High-grade malignancy		
Centroblastic	16	21.62
Immunoblastic	5	6.75
Centroblastic — immunoblastic	2	2.70
	23	31.0

NHLs occured most frequently in old age. There was a difference in the age distribution of the groups of low-grade and high-grade NHLs (Fig. 1). High-grade lymphomas occured most frequently at an age 10 years earlier than low-grade NHLs. No difference was found in the sexes in the different age groups. Summarising the data obtained on histological evaluation (Table 2), 60% of the patients belonged to the group with low-grade and 31.7% to the group with high-grade lymphomas.

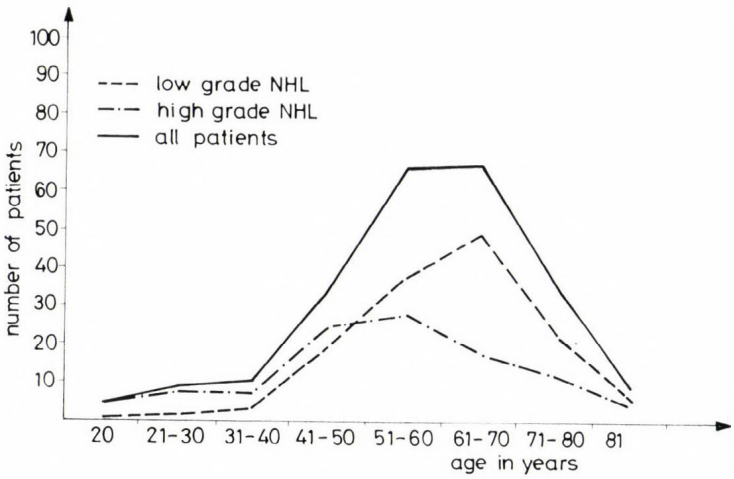


Fig. 1. Age specific distibution of low and high grade NHLs

Table 2

Classifications of NHLs between 1975 and 1985

Histological type of NHLs	Number of patients	%
Low-grade malignancy		
Lymphocytic type		
CLL	56	24.0
Mycosis f., Sezary sy.	3	1.2
Hairy-cell leukaemia	2	0.9
Immunocytic	26	11.2
Plasmocytic	3	1.3
Centrocytic	24	10.3
Centrocytic — centroblastic	26	11.2
	140	60.0
High-grade malignancy		
Centroblastic	35	15.02
Immunoblastic	17	7.29
Lymphoblastic	17	7,29
Unclassified	5	2.14
	74	31.74
Others	19	8.16

Table 3

Distribution by clinical stage (1975–1985)

Stage	Number of patients	%
I.	7	3.9
II.	21	11.8
III.	47	26.8
IV.	99	55.9
Unknown	3	1.6
All patients	177	100.0
+ CLL	56	
	233	
Systemic symptoms (without CLL)		
"A"	16	9.0
"B"	124	70.1
Unknown	37	20.9

It was not possible to classify the biopsy specimens of 19 patients (8.16%).

From the immunological point of view 198 NHL cases (84.9%) were of B-cell type and only 10 cases (4.3%) were of T-cell origin.

82.7% of our patients were diagnosed in progressive (III–IV) clinical stage of the disease (Table 3) and the illness was accompanied by so called B symptoms in 70.1% of the patients at diagnosis. The results of therapy could be evaluated according to histological diagnosis and clinical stage in 158 patients. CLL patients were not evaluated from this point of view as the concept of remission cannot be applied to them.

Table 4

Comparison of survival data of the two main groups (mean ± SE)

Diagnosis	Mean survival time (months)		p<
	1965–1974	1975–1985	
NLH (with CLL) with low-grade malignancy	25.5±5.4 n = 51	31.01±2.82 n = 120	not significant
NHL with high-grade malignancy	12.8±2.4 n = 23	14.09±2.01 n = 74	not significant
CLL (alone)	36.8±8.4 n = 24	39.7±5.46 n = 47	not significant

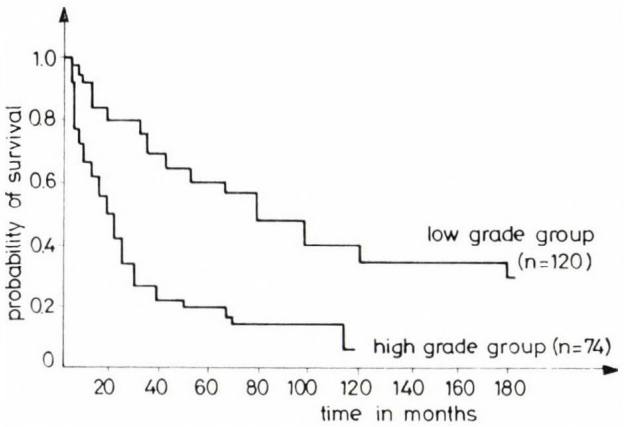


Fig. 2. Comparison of survival of patients with low and high grade lymphomas (1975—1985)

Following the first treatment, 40.9% of the patients with low-grade malignancy obtained complete remission, the overall response rate was 73.2%. Among patients with high-grade NHLs complete remission was proven in 43.8% and the overall response rate was 63.87%. Mean time of remission was 14.5 months in the group with low-grade and 8.37 months in the group of high-grade malignancy.

Mean survival time of patients during the second 10 year period was 25.5 ± ± 5.4 months in the low-grade and 12.8 ± 2.4 months in the high-grade malignancy group. Looking at the CLL patients, their mean survival time was 36.85 ± ± 8.4 months (Table 4). Comparing mean survival times in the two 10 year periods we found no statistically significant differences, but the mean survival time of patients with low-grade lymphomas proved longer during the second 10 year

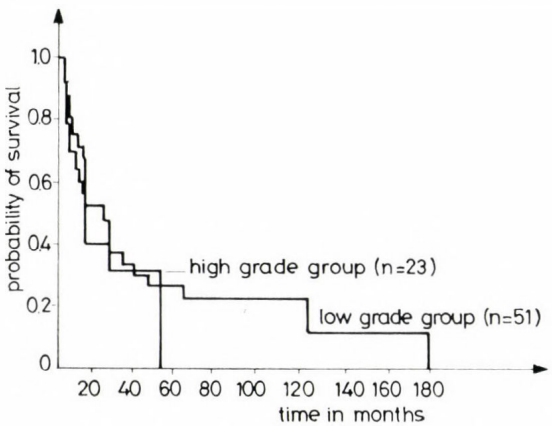


Fig. 3. Comparison of survival of low and high grade NHLs between 1965 and 1974

period. Treatment results of the second 10 year period were compared even in 5 year terms, but no important differences were found. Survival curves of patients treated between 1975 and 1985 differ greatly according to histological type in favour of the low-grade malignancy group (Fig. 2). These diferences are less apparent in the first 10 year period (Fig. 3).

Discussion

Investigation of NHLs focuses on three major problems: histopathological classification, determination of the clinical stage and therapy depending on both.

We evaluated the data of our own patients using the Kiel classification. In accordance with the literature, we found that the appearence of NHLs means an increasing risk for the elderly population (mean age 58 years) and occurs more often in men than in women. Evaluating the histological diagnoses, the number of centrum cell tumors was extremly high, higher as could be awaited on the basis of international [19, 20] and national [8] data. The frequency of cases with low-grade and high-grade malignacy is in accordance with the results of international and national centres [2, 3, 8, 19, 20]. Immunological evaluation pointed to the predominance of B-cell tumors.

The clinical staging system is the same for Hodgkin's and non-Hodgkin's lymphomas. In Hodgkin's disease, however, it is of greater importance from the point of view of both therapy and prognosis. The reason for this is, that in NHLs in over 80% of patients (in our cases 82.8%) the disease is generalised already at first presentation [21] as it is rapidly progressive and certain forms are prone to leukaemic transformation [13, 23, 24, 25]. On the other hand, the heterogeneity of NHLs necessitated the modification of the staging system of mycosis fungoides [21], gastrointestinal lymphomas [21] and last but not least the staging of CLL [26] differ from the Ann Arbor criteria. It is important that mainly the high-grade NHLs often are accompanied by central nervous system manifestations. This makes prophylactic therapy for the protection of the central nervous system necessary. In our patients treatment of lymphoblastic (mainly T-cell) lymphoma has been combined automatically with BCNU and/or intrathecal Methotrexate.

Overall response rate and complete remission rate of our patients does not reach published results based on the Kiel classification [19, 20]. A possible explanation of this is that we treated patients at an advanced stage of the disease.

It was possible to compare results of the two 10 year periods from the point of view of survival (Table 4). Though we used more modern diagnostic and therapeutic regimens, survival times of patients did not increase compared to the former 10 year period, when treatment consisted of radiotherapy and single agent chemotherapy.

In the case of NHLs there is essential difference between cases with low-grade or high-grade malignancy. In the latter case, using aggressive therapy, the rate of complete remission is certainly higher, the duration of remission is longer

[22]. On the other hand low-grade NHLs remain stable without treatment and even spontaneous regression may occur [22, 27], but they can also transform to more aggressive disease. According to the observation of Smith et al., the duration of complete remission is short and monoclonal B lymphocytes may remain in the circulation even after treatment [23]. Presumably, the disease can almost never be eradicated completely, only macroscopic signs are missing. This is the reason why the principle of watchful waiting seems to be valid even today in the therapy of low grade NHLs [22, 28, 29], all the more because the proliferative activity of these tumours is low [22]. The high-grade lymphomas being tumours of high proliferative rate respond well to the first therapy. In the case of complete remission no monoclonal B-cell population can be found in the circulation so there is hope for complete recovery. One of the real possibilities is autologous bone-marrow transplantation that has been introduced at the National Institute of Haematology and Blood Transfusion.

Although watchful waiting, the frequent, systematic checkup of patients is very important, it should be kept in mind that the majority of patients with low grade NHL also die from the disease. We should make efforts to integrate the results of immunology, cytogenetics and molecular biology not only into the diagnostics, but also into the therapy of NHLs so that the chances of therapy should improve. The therapy should not become by all means more aggressive, but more specific in order to make complete cure of patients a reality.

References

1. Rappaport, H.: Tumors of the hematopoetic system. In: *Atlas of tumor pathology, sect. 3.* Faside 8. Armed Forces Institute of Pathology Washington. DC. 1966, pp. 9.
2. Lennert, K., Mohri, N., Stein, H., Kaiserling, E.: The histopathology of malignant lymphoma. *Br. J. Haematol. 31 (suppl)*, 193 (1975).
3. Lennert, K., Collins, R. D., Lukes, R. J.: Concordance of the Kiel and Lukes—Collins clasification of non-Hodgkin's lymphomas. *Histopathology 7*, 549 (1983).
4. Lukes, R. J., Collins, R. D.: Immunologic characterisation of human malignant lymphomas. *Cancer 34*, 1488 (1974).
5. Kelényi, G.: Nicht-Hodgkin-Lymphome hoher malignität. Pathologische Diagnose und Differentialdiagnose. *Arch. Geschwulstforsch. 49*, 648 (1979).
6. Kelényi, G., Várbiró, M.: Malignant Lymphome Reference Center — Hungary 1978. *Virchows Arch. A. 388*, 213 (1980).
7. Kelényi, G., Mioduszewska, O., Rüdiger, K-D.: Malignant lymphoma reference centers: GDR, Hungary and Poland 1979. *Zbl. allg. Pathol. u. pathol. Anat. 125*, 122 (1981).
8. Varga, Gy., Borbényi, Z., István, L., Szabó, K., Nagy, I., Kelényi, G., Krizsa, F., Cserháti, I.: Die Rolle der Splenektomie in der Behandlung von Lymphomen niedrigen Malignität. *Dtsch. Med. Wochenschr. 110*, 328 (1985).
9. Rák, K., Pecze, K., Kiss, A., Telek, B.: Study of cell surface markers in chronic lymphocytic leukaemia (CLL). *Folia Haematol. 111*, 294 (1984).
10. Reinherz, E., L., Schlossmann, S. F.: The differentiation and function of human T-lymphocytes. *Cell 19*, 822 (1980).
11. Waldmann, T. A., Davis, M. M., Bongiovanni, K. F., Korsmeyer, S. J.: Rearrangement of genes for the antigen receptor gene on T cells as markers of lineage and clonality in human lymphoid neoplasms. *N. Engl. J. Med. 313*, 776 (1985).

12. Carbone, P. P., Caplan, H. S., Musshoff, K., Smithers, D. W., Tubiana, M.: Report of the comittee on Hodgkin's disease staging clasification. *Cancer Res. 31*, 1060 (1971).
13. Bonadonna, G., Lattuada, A., Banfi, A.: Recent trends in the treatment of non-Hodkin's lymphomas. *Eur. J. Cancer 12*, 661 (1976).
14. Skarin, A. T., Canellos, G.: Chemotherapy of advanced non-Hodgkin's lymphoma. *Clinics in Haematology 8*, 667 (1979).
15. Parlier, Y., Gorin, N. C., Najman, A., Stachowiek, J., Dukamel, G.: Combination chemotherapy with Cyclophosphamide, Vincristine, Prednisone, and contribution of Adriamycin in the treatment adult non-Hodkin's lymphomas. A report of 131 cases. *Cancer 50*, 401 (1982).
16. Monfardini, S., Brunner, K., Crowther, D., Olive, D., MacDonald, J., Eckhardt, S., Whitehouse, J., Reed, D.: *Manual of cancer chemotherapy (3rd edition)*. UICC Technical Report Series, Geneva 1981, p. 56.
17. Schein, P. S., De Vita, V., Canellos, G., Chabner, B., Young, R.: A new combination chemotherapy program for diffuse histiocytic (DHL) and mixed (DML) non-Hodgkin's lymphoma. *Proc. Amer. Ass. Cancer Res. 16*, 248 (1975).
18. Armitage, J. O., Dick, F. R., Corder, M. P., Garneau, S. C., Platz, C. E., Slywen, D. J.: Predicting therapeutic outcome in patients with diffuse histiocytic lymphoma treated with cyclophosphamide, adriamycin, vincristine and prednisone (CHOP). *Cancer 50*, 1695 (1982).
19. Brittinger, G., Bartels, H., Common, H. et al.: Clinical and prognostic relevance of the Kiel Classification of non-Hodgkin lymphomas: Results of a prospective multicenter study by the Kiel Lymphoma Study Group. *Hematological Oncology 2*, 269 (1984).
20. Heinz, R., Hanak, H., Stacher, A.: Progress in the management of high risk non-Hodgkin's lymphomas. *Klin. Wochenschr. 63*, 615 (1985).
21. Crowther, D., Rankin, E. M.: Clinical annotation staging patients with non-Hodgkin's lymphoma. *Br. J. Haematol. 52*, 357 (1982).
22. Gaynor, E. R., Ultman, J. E.: Non-Hodgkin's lymphoma: management strategies. *N. Engl. J. Med. 311*, 1506 (1984).
23. Smith, B. R., Weinberg, D. S., Robert, N. J., Towler, M., Luther, E., Pinkus, G. S., Ault, K. A.: Circulating monoclonal B-lymphocytes in non-Hodgkin's lymphoma. *N. Engl. J. Med. 311*, 1476 (1984).
24. Pálóczi, K., Berényi, E., Surányi, P., Kiss, S. I., Szegedi, Gy.: Lymphocyte subpopulation in peripheral blood of patients with non-Hodgkin lymphoma. *Acta. Med. Hung. 44*, 231 (1987).
25. Stewart, M. L., Felman, I. E., Nichols, P. W., Pagnini-Hile, Lukes, R. J., Levine, A. M.: Large non-cleaved follicular center cell lymphoma. Clinical features in 53 patients. *Cancer 57*, 288 (1986).
26. Rai, K. R., Sawitsky, A., Cronkite, E. P., Chanana, A. D., Levy, R. N., Pasternack, B. S.: Clinical staging in chronic lymphocytic leukaemia. *Blood 46*, 219 (1975).
27. Krikorian, J. G., Portlock, C. S., Cooney, P., Rosenberg, S. A.: Spontaneous regression of non-Hodgkin's lymphoma: a report of nine cases. *Cancer 46*, 2093 (1980).
28. Portlock, C. S.: Management of the indolent non-Hodgkin's lymphomas. *Sem. Oncol. 77*, 292 (1980).
29. Portlock, C. S.: "Good risk" non-Hodkin lymphomas: approaches to management *Sem. Haematol. 20*, 25 (1983).

Abstracts

Uptake of ferritin and iron bound to ferritin by rat hepatocytes: modulation by apotransferrin, iron chelators and chloroquine. J. C. Sibille, H. Kondo and P. Aisen (Department of Physiology and Biophysics, and Medicine, Albert Einstein College of Medicine, New York, N. Y., USA). *Biochim. Biophys. Acta 1010*, 204 (1989).

Rat liver ferritin is an effective donor of iron to rat hepatocytes. Uptake of iron from ferritin by the cells is partially inhibited by including apotransferrin in the culture medium, but not by inclusion of diferric transferrin. This inhibition is dependent on the concentration of apotransferrin, with a 30% depression in iron incorporation in the cells detected at apotransferrin concentrations above 40 μg/ml. However, apotransferrin does not interfere with uptake of ^{125}I-labeled ferritin, suggesting that apotransferrin decreases retention of iron taken up from ferritin by hepatocytes by sequestering a portion of released iron before it has entered the metabolic pathway of the cells. The iron chelators desferrioxamine (100 μM), citrate (10 mM) and diethylenetriaminepentaacetate (100 μM) reduce iron uptake by the cells by 35, 25 and 8%, respectively. In contrast, 1 mM ascorbate increases iron accumulation by 20%. At a subtoxic concentration of 100 μM, chloroquine depresses ferritin and iron uptake by hepatocytes by more than 50% after 3 h incubation. Chloroquine presumably acts by retarding lysosomal degradation of ferritin and recycling of ferritin receptors.

A. Egyed

Role of phosphate-containing compounds in the transfer of indium-111 and gallium-67 from transferrin to ferritin. R. E. Weiner (Division of Nuclear Medicine, Department of Radiology, Indiana University Medical Center, Indianapolis, Indiana, USA). *J. Nucl. Med. 29*, 70 (1989).

Physiologic concentrations of ATP stimulate the translocation of gallium-67 (^{67}Ga) from human transferrin (TF) to horse ferritin (HoFE). The mechanism of this translocation was examined. One millimolar ATP did not speed the binding of ^{67}Ga or indium-111 (^{111}In) to HoFE. ATP and pyrophosphate (PP_i) at 1 mM, did not form high affinity complexes with ^{67}Ga or ^{111}In. ATP and PP_i interacted directly with the [^{67}Ga]TF complex and could within minutes increase the amount of nonprotein-bound ^{67}Ga. Serum HCO_3^- concentration, 30 mM, prevented the ATP-induced dissociation of ^{67}Ga from TF, whereas intracellular concentrations (0.4 and 5 mM) did not. Using a dialysis technique, ATP also stimulated the translocation of ^{111}In from TF to HoFE; however, this process was much slower than with ^{67}Ga. ATP caused an increase in the nonprotein-bound ^{111}In compared to the control. These results suggest the formation of nonprotein-bound nuclide by these phosphate-containing compounds in a kinetically labile form is important to the translocation mechanism.

A. Egyed

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Ca^{2+}-activated K^+ efflux limits complement-mediated lysis of human erythrocytes. J. A. Halperin, C. Brugnara and A. Nicholson-Weller (Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA, USA). *J. Clin. Invest. 83*, 1466 (1989).

The lytic effect of complement on human erythrocytes has been reported by others to increase when Na^+ is substituted for K^+ in the external medium. In this paper it is investigated the hypothesis that net loss of K^+ through a K^+ transport pathway protects erythrocytes from complement-induced colloidosmotic swelling and lysis. Antibody-sensitized human erythrocytes containing different intracellular cation concentrations (nystatin treatment) were exposed to low concentrations of guinea pig serum in media of different cation composition; complement lysis was assessed by the release of hemoglobin and the volume of the surviving cells estimated by their density distribution profles. Complement-dependent swelling and lysis of erythrocytes (a) were limited by the presence of an outwardly directed K^+ electrochemical gradient and (b) were enhanced by carbocyanine, a specific inhibitor of the Ca^{2+}-activated K^+ transport pathway, and by absence of Ca^{2+} in the external medium. It is proposed that during complement activation a rising cytosolic calcium triggers the Ca^{2+}-activated K^+ permeability pathway, the Gárdos effect, produces a net K^+, Cl^- and water loss, and thus limits the colloidosmotic swelling and lysis of erythrocytes.

Ilma Szász

Volume-sensitive, Cl-dependent K transport in resealed human erythrocyte ghosts. W. C. O'Neill (Department of Medicine, Emory University School of Medicine, Atlanta, Georgia, USA). *Am. J. Physiol. 256*, C81 (1989).

Potassium influx and efflux in Cl and NO_3 media were measured in resealed ghosts prepared from human red cells. Cl-dependent K influx was three times that in intact cells and, as in intact cells, was partially supported by Br but not by thiocyanate (SCN). In other properties, this flux differed from that in intacts cells: substitution of N-methylglucamine for Na did not decrease but rather increased Cl-dependent K influx, the affinity for external K was reduced, with a K_m of 21.3 ± 12.5 mM, and inhibition by furosemide and bumetanide was incomplete. Furosemide at 1 mM inhibited Cl-dependent influx by 26 and 51% at 4 and 20 mM K, respectively. Bumetanide inhibited Cl-dependent K influx by 0 and 55% at concentrations of 10 μM and 1 mM, respectively, in 4 mM K, with no further inhibiton at 20 mM K. Neither the magnitude nor the properties of the flux were altered by preparing ghosts in the presence of 1,4-dithiothreitol, indicating that sulfhydryl oxidation was not responsible for the altered flux in ghosts. Treatment with N-ethylmaleimide (NEM) either before or after ghost preparation did not increase Cl-dependent K influx. However, Cl-dependent influx in ghosts could be augmented by increasing ghost volume or ATP content. Resealed human erythrocyte ghosts thus exhibit a volume- and ATP-sensitive, Cl-dependent K flux that differs substantially from the putative Na-K-Cl cotransport in intact cells in that it is independent of Na, is relatively resistant to furosemide and bumetanide, and has a low affinity for K. Its properties are identical to those of the putative K-Cl cotransport seen in swollen or NEM-treated red cells, and the activation of this K-Cl cotransport in the resealed ghosts may be due to the initial cell swelling during ghost preparation or to changes related to the action of NEM in intact red cells.

Ilma Szász

Changes in the proteolytic activity of human erythrocyte membrane during red cell aging. M. Gaczynska (Laboratory of Biophysics of Development and Aging, Department of Biophysics, University of Lodz, Poland). *Biochim. Biophys. Acta 981*, 173 (1989).

The action of endogenous membrane proteinases in membranes isolated from human red cells of various ages was assayed by three groups of methods: (1) determina-

tion of the amount of protein fragments released to the acid-soluble fraction; (2) monitoring of changes in ESR spectra of maleimide spin-labeled erythrocyte membranes; (3) electrophoretic methods: a two-dimensional analysis of the activity inside SDS-PAGE gels. For all the methods the effects of proteinase action were highest in ghosts isolated from the erythrocytes of middle age.

G. Gárdos

A cAMP-regulated chloride channel in lymphocytes that is affected in cystic fibrosis. J. H. Chen, H. Schulman and P. Gardner (Department of Medicine, Stanford University, Stanford, CA, USA). *Science 243*, 657 (1989).

A defect in regulation of a chloride channel appears to be the molecular basis for cystic fibrosis (CF), a common lethal genetic disease. It is shown here that a chloride channel with kinetic and regulatory properties similar to those described for secretory epithelial cells is present in both T and B lymphocyte cell lines. The regulation of the channels by adenosine 3', 5'-monophosphate (cAMP)-dependent protein kinase in transformed B cells from CF patients is defective. Thus, lymphocytes may be an accessible source of CF tissue for study of this defect, for cloning of the chloride channel complex, and for diagnosis of the disease.

B. Sarkadi

Effects of sodium removal on calcium mobilization and dense granule secretion induced by thrombin in human platelets. M. T. Alonso, A. Sanchez and J. García-Sancho (Departamento de Bioquimica y Biologia Molecular y Fisiologia, Facultad de Medicina, Universidad de Valladolid, Valladolid, Spain). *Biochim. Biophys. Acta 981*, 367 (1989).

Removal of extracellular sodium decreased calcium mobilization from intracellular stores induced by thrombin in aspirin-treated human platelets. ATP and serotonin secretion were also significantly reduced. Secretion was positively correlated with calcium mobilization, but the presence or absence of sodium did not modify the slope of the regression line. Half-maximal secretion was reached when $[Ca^{2+}]_i$ was increased by about 0.1 μM. Calcium mobilization induced by the divalent cation ionophore ionomycin was not modified by sodium removal. Secretion induced by ionomycin was much smaller than the thrombin-induced one for the same increases of $[Ca^{2+}]_i$. These results suggest that the presence of external sodium is required for normal thrombin-induced calcium release from the intracellular stores and hence for dense granule secretion. However, secretion cannot by only attributed to the increase of cell $[Ca^{2+}]_i$ but also to other process(es) which are not affected by external sodium.

B. Sarkadi

Reversible electropermeabilisation of human and rat blood platelets: evaluation of morphological and functional integrity in vitro and in vivo. K. Hughes and N. Crawford (Department of Biochemistry and Cell Biology, Hunterian Institute, Royal College of Surgeons of England, London, UK). *Biochim. Biophys. Acta 981*, 277 (1989).

A high-voltage discharge procedure has been developed for permeabilising the plasma membranes of both human and rat blood platelets. The cells can be resealed by incubation at 37 °C, show < 4% loss of lactate dehydrogenase (LDH) implying minimal cell lysis and also have well maintained morphological and functional integrity. The prototype apparatus used at field strengths between 6 and 8 kV/cm produces membrane pores which allow free diffusion of low molecular weight substances such as adenine nucleotides, inositol phosphate and fluorescent dyes. Two properties, namely Ca^{2+}-induced secretion of granule stored 5-hydroxytryptamine (5HT) and inositol 1,4,5-trisphosphate (IP_3)-induced release of intracellularly sequestered ^{45}Ca, which are both well expressed immediately after permeabilisation, are essentially abolished after resealing. The efficiency of permeabilisation and resealing can be simply monitored by shifts in "apparent platelet volume" using a resis-

tive particle counter (Coulter). Permeabilised platelets show a shift in modal volumes from a control range 4–7 fl to 10–15 fl. Resealing restores these modal volumes to the original control range. Encapsulation of the fluorochrome, Lucifer yellow (M_r 550), during permeabilisation revealed that after resealing > 85% of rat platelets, and close to 100% human platelets, contained the encapsulated dye. The initial rates and % aggregation responses of both human and rat platelets to collagen, thrombin and the thromboxane A_2-mimetic U46619 remained essentially normal after permeabilisation and resealing further illustrating the maintenance of functional competence following treatment. Resealed rat platelets reinfused into the circulation after labelling with [^{111}In] indium oxine gave survival curves similar to those of control platelets. Therefore, this reversible permeabilisation procedure may allow the use of autologous or heterologous platelets as carrier vehicles for the delivery of drugs and other agents "in vivo".

G. Gárdos

Acknowledgement to the Reviewers

On behalf of the Editorial Board and the Editorial Office of Haematologia, we would like to express our sincere thanks to the many reviewers of the papers submitted to our journal. The reviews are of great value to the authors and, of course, indirectly to the readers of Haematologia.

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Index to Volume 22

CONTENTS

Mirčevova, L., Viktora, L., Řeháčková, H.: Dual effect of calcium on erythrocyte membrane contration 3
Czokalo, M., Tomasiak, M.: Alpha-fetoprotein inhibits aggregation of human platelets 11
Selypes, A., László, A., Virág, I.: Chromosome examinations on a six-hour culture of unstimulated peripheral blood from a patient with childhood erythroleukaemia 19
Phan, D. T., Mihalik, R., Benczur, M., Pálóczi, K., Gidáli, J., Fehér, I., Dömötöri, J., Kiss, Cs., Petrányi, G. Gy., Natonek, K., Hollán, S. R.: Human fetal liver as a valuable source of haemopoietic stem cells for allogeneic bone marrow transplantation 25
El-Hazmi, M. A. F., Warsy, A. S.: Glutathione reductase in the south-western province of Saudi-Arabia — Genetic variation vs. acquired deficiency 37
Berceanu, S., Roman, S., Butoianu, E., Leahu, S., Gabrielescu, E., Olinescu, A., Moldoveanu, E., Moraru, I.: A particular case of large granular lymphocytes lymphoma 43
Csató, E., Vass, E.: Frequency of Lewis-group substances in patients with cancer of stomach 55
Abstracts 61

Weitberg, A. B.: The effect of alkaline phosphatase on platelet aggregation 65
Brunori, M., Coletta, M., Bellelli, A., Evangelista, V., Benedetti, P. A., Brumen, M.: Microspectroscopy of red blood cells 69
Van Waarde, A., Lombarts, A., van den Thillart, G., Erkelens, C., Lugtenburg, J.: ^{31}P NMR studies on rejuvenation of outdated red blood cells: complete regeneration of ATP is accompanied by partial Mg-ATP recomplexation 79
Rácz, Z., Haskó, F.: Room temperature storage of pooled platelet concentrates in gas-permeable plastic bags for five days 89
Kramer, J., Thiry, E., Füst, G.: Rapid determination of the human complement factor B phenotypes 97
Gamba, G., Ippoliti, G., Invernizzi, R., Gatti, V., Zocchi, M. T., Perugini, O.: Cytochemical profile of lymphocytes in haemophilia patients at risk of AIDS 101
Maslanka, K., Lucas, G. F., Gronkowska, A., Davis, J. G., Zupanska, B.: A second case of neonatal alloimmune thrombocytopenia associated with anti-PI^{A2} (Zw^b) antibodies 109
Altankov, G., Smilenov, L.: Effect of plasma fibronectin on the adhesive properties of human peripheral lymphocytes 115

Abstracts 125

Announcements 131

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Petri, I. B., Gyódi, É., Kaiser, G., Kassai, M., Petrányi, G. Gy.: Dynamism of the development of "Fc receptor-blocking antibodies" after immunization with buffy coat 135
Invernizzi, R., Perugini, O., De Fazio, P., Bonetti, F., Nespoli, L.: Cytochemistry of dipeptidylaminopeptidase IV in T lymphoblastic leukaemia — lymphoma 143
Merétey, K., Jakab, Á., Szilassy, K., Medgyesi, G. A.: IgE levels in normal human sera and IgG preparations 151
Kopff, M., Zakrzewska, I., Klem, J., Strzelczyk, M.: Relationship between adenosine deaminase activity (ADA) and inosinemonophosphate (IMP) accumulation in fresh erythrocytes incubated with adenosine and dipyridamole 161
Hernández, P., Dorticós, E., Espinosa, E., González, X., Svarch, E.: Clinical features of hepatic sequestration in sickle cell anaemia 169
Tejuca, M., Martinez, G., Ferreira, R., Hernández, A., Ferrer, L., Goicochea, A., Hernández, A. C., Coyula, R., Wade, M., Colombo, B.: Alpha-thalassemia changes the cell density profile in sickle cell anaemia 175
Koerner, K.: Preparation of leukocyte-poor platelet concentrates for transfusion by filtration through a new cellulose acetate filter 181
Benny, A. G., Brimer, C. M.: Functional determination of C 1 esterase inhibitor in fractions derived from plasma 189
Marra, R., Stori, S., Pagano, L., Fioritoni, G., Rabitti, C., Sica, S., Leone, G., Torlontano, G., Bizzi, B.: Central nervous system acute promyelocytic leukaemia: a report of three cases 195
Abstracts

Bernard, J.: Epidemiology of leukaemia 201
Miwa, S.: Molecular basis of red cell enzymopathies associated with hereditary nonspherocytic hemolytic anemia 215
Hassan, H. T., Rees, J. K. H.: The effect of human recombinant granulocyte-macrophage colony stimulating factor on the proliferation and differentiation of myeloid progenitors in congenital agranulocytosis marrow cells 233
Pálóczi, K., Berényi, E., Illés, Á., Nemes, Z., Krasznai, G., Krajczár, G., Szegedi, Gy.: Clinical analysis of patients with non-Hodgkin's lymphomas (1965—1985) 239

Abstracts 249

Acknowledgement to the Reviewers 253

Contents of Volume 22 255

Author Index 257

Subject Index 259

Author Index

A

Altankov, G. 115

B

Bellelli, A. 69
Benczur, M. 25
Benny, A. G. 189
Berceanu, S. 43
Berényi, E. 239
Bernard, J. 201
Bizzi, B. 195
Bonetti, F. 143
Brimer, C. M. 189
Brumen, M. 69
Brunori, M. 69
Butoianu, E. 43

C

Coletta, M. 69
Colombo, B. 175
Coyula, R. 175
Csató, E. 55
Czokalo, M. 11

D

Davis, J. G. 109
De Fazio, P. 143
Dömötöri, J. 25
Dorticós, E. 169

E

El-Hazmi, M. A. F. 37
Erkelens, C. 79
Espinosa, E. 169
Evangelista,V. 69

F

Fehér, I. 25
Ferreira, R. 175
Ferrer, L. 175
Fioritoni, G. 195
Füst, G. 97

G

Gabrielescu, E. 43
Gamba, G. 101
Gatti, V. 101
Gidáli, J. 25
Goicochea, A. 175
Gonzalez, X. 169
Gronkowska, A. 109
Gyódi, É. 135

H

Haskó, F. 89
Hassan, H. T. 233
Hernández, A. 175
Hernández, A. C. 175
Hernández, P. 169
Hollán, S. R. 25

I

Illés, Á. 239
Invernizzi, R. 101, 143
Ippoliti, G 101

J

Jakab, Á. 151

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

K

Kaiser, G. 135
Kassai, M. 135
Kiss, Cs. 25
Klem, J. 161
Koerner, K. 181
Kopff, M. 161
Krajczár, G. 239
Kramer, J. 97
Krasznai, G. 239

L

László, A. 19
Leahu, S. 43
Leone, G. 195
Lombarts, A., 79
Lucas, G. F. 109
Lugtenburg, J. 79

M

Marra, R. 195
Martinez, G. 175
Maslanka, K. 109
Medgyesi, G. A. 151
Merétey, K. 151
Mihalik, R. 25
Mirčevová, L. 3
Miwa, S. 215
Moldoveanu, E. 43
Moraru, I. 43

N

Natonek, K. 25
Nemes, Z. 239
Nespoli, L. 143

O

Olinescu, A. 43

P

Pagano, L. 195
Pálóczi, K. 25, 239
Perugini, O. 101, 143
Petrányi, G. Gy. 25, 135
Petri, I. B. 135
Phan, D. T. 25

R

Rabitti, C. 195
Rácz, Z. 89
Řehačková, H. 3
Rees, J. K. H. 233
Roman, S. 43

S

Selypes, A. 19
Sica, S. 195
Smilenov, L. 115
Storti, S. 195
Strzelczyk, M. 161
Svarch, E. 169
Szegedi, Gy. 239
Szilassy, K. 151

T

Tejuca, M. 175
Thiry E 97
Tomasiak, M. 11
Torlontano, G. 195

V

Virág, I. 19
van den Thillart, G. 79
Van Waarde, A. 79
Vass, E. 55
Viktora, L. 3

W

Wade, M. 175
Warsy, A. S. 37
Weitberg, A. B. 65

Z

Zakrzewska, I. 161
Zocchi, M. T. 101
Zupanska, B. 109

Subject Index

acute promyelocytic leukaemia 195
adenosine deaminase activity 161
— diphosphate 65
adhesion 115
AIDS 101
aldolase deficiency 215
alkaline phosphatase 65
alpha-thalassaemia 175
anti-Pl^{A2} (Zw^b) antibodies 109
ATP regeneration 79

blood donors 151
— storage 79

C1 esterase inhibitor 189
calcium 3
cancer of stomach 55
cell density profile 175
— differentiation 233
— proliferation 233
central nervous system 195
chemotherapy 239
childhood erythroleukaemia 19
chromosome examination 19
clinical concentrates 189
collagen and fibronectin-coated surfaces 115
colony-stimulating factor 233
complement factor B phenotypes 97
congenital agranulocytosis 233
— neutropenia 233
contractile membrane protein 3
cryopreservation 25
cytochemistry 101, 143

dimethylsulphoxide 233
dipeptidylaminopeptidase IV 143
erythrocyte 69
— ghost shape 3
erythroenzymopathies 215

Fc receptor-blocking antibody 135
filtration 181
fresh erythrocytes 161
functional assay 189

glucose 6-phosphate dehydrogenase deficiency 215
glutathione reductase 37

haemophilia 101
HbF 175
hepatic crisis 169
— sequestration 169
hereditary nonspherocytic haemolytic anaemia 215
HLA-alloimmunization 181
human alpha-fetoprotein 11
— fetal liver cells 25
— platelets 11
hypogammaglobulinaemia 43

IgE 151
IgG 151
immunization 135
immunosuppression 135
inosine monophosphate accumulation 161

Kostmann syndrome 233

leukaemia 201
leukocyte-poor platelet concentrates 181
Lewis group substance 55
LGL lymphoma 43
life span 89
ligand diffusion 69
lineage infidelity 43
lymphocytes 101
— peripheral 115

VSP Utrecht, Tokyo
Akadémiai Kiadó, Budapest

Mg-ATP recomplexation 79
molecular basis 215
myeloid progenitors 25

neonatal alloimmune thrombocytopenia 109
non-Hodgkin lymphoma 239
normal level 151

painful crisis 169
phosphoglycerate kinase deficiency 215
physiologic membrane contraction 3
platelet aggregation 11, 65
— function 89, 181
^{31}P NMR spectroscopy 79
pyruvate kinase deficiency 215
rapid determination 97
red cell enzymopathies 215
retinoic acid 233
riboflavin 37
ristocetin 65

Saudi Arabia 37
serum 151
short term culture 19
sickle cell anaemia 169
— — disease 175
single cell microspectroscopy 69
storage 89
survival probability 239

T cells 25
—, mature and progenitor 25
thrombocytopathy 65
T lymphoblastic leukaemia 143
— — lymphoma 143

Printed in Hungary

Akadémiai Kiadó és Nyomda Vállalat, Budapest

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 brackets. 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.

Authors are entitled to 50 reprints free of charge.

HAEMATOLOGIA
VOLUME 22 · NUMBER 4 **1989**

Contents

Bernard, J.: Epidemiology of leukaemia 201

Miwa, S.: Molecular basis of red cell enzymopathies associated with hereditary non-spherocytic hemolytic anemia 215

Hassan, H. T., Rees, J. K. H.: The effect of human recombinant granulocyte-macrophage colony stimulating factor on the proliferation and differentiation of myeloid progenitors in congenital agranulocytosis marrow cells 233

Pálóczi, K., Berényi, E., Ellés, Á., Nemes, Z., Krasznai, G., Krajczár, G., Szegedi, Gy.: Clinical analysis of patients with non-Hodgkin's lymphomas (1965—1985) 239

Abstracts 249

Acknowledgement to the Reviewers 253

Index to Volume 22 255

Author Index 257

Subject Index 259

Index: 26.861