315871

VOLUME 20 • NUMBER 1 • 1987

HU ISSN 0017-6559

EMATOLOGIA

20
1987

International Quarterly of Haematology

EDITOR-IN-CHIEF:
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VNU SCIENCE PRESS
UTRECHT, THE NETHERLANDS

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.

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VNU SCIENCE PRESS
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Subscription price for volume 20

surface mail DM 312 incl.
air mail DM 330 incl.

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

VOLUME 20 · NUMBER 1

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VNU SCIENCE PRESS, UTRECHT, THE NETHERLANDS

AKADÉMIAI KIADÓ, BUDAPEST

1987

Printed in Hungary

Akadémiai Kiadó és Nyomda, Budapest

MAGYAR — TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

Haematologia 20 (1), pp. 3—14 (1987)

Platelet Membrane Components and Receptors*

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(Received January 7, 1986; accepted February 10, 1986)

Receptor-ligand interactions on the platelet membrane control a number of phenomena unique to platelets such as shape change, aggregation, release reaction of secretion, clot retraction and activation of clotting factors. Localization of receptor on the well-defined platelet membrane glycoproteins appears to be a major target of numerous studies in the field of platelet research. The author is reviewing methodological development in this field including surface labeling of the platelets, one and two dimensional electrophoresis, development of monoclonal and polyclonal antibodies to platelet membrane glycoproteins and receptors, immunoblotting, radiolabeled ligand studies and preparative techniques. Classification of membrane glycoproteins is briefly discussed. The current knowledge of the receptors for fibrinogen and other adhesive proteins, clotting factors, ADP and prostanoids is reviewed.

Keywords: platelet membranes, receptors, receptor-ligand interaction, membrane glycoproteins, platelet function

Introduction

Plasma membranes have an essential function in mammalian cells. Influences from the extracellular environment must be translated across them and all cytoplasmic products can only reach the outside of the cell by the same membrane-controlled mechanism. The principal components of plasma membrane are lipids, proteins and carbohydrates. Lipids constitute the structural framework of the biological membrane while biological specificity resides primarily in the proteins interacting with the lipid bilayer. It is now well established that membrane receptors are associated with specific proteins. The membrane protein can either be peripheral or integral. The former can be located on either the inner or the outer surface of the plasma membrane. The latter spans the lipid bilayers with part of the protein located outside the cell, a portion spanning the bilayer and residual part inside the cell. The outside portion of the integral protein is usually glycosylated and this is important in the process of cellular recognition. The part of the integral protein located inside the cell may interact with components of the cell

* Paper presented at the VIIIth Meeting of the European-African Division of the International Society of Haematology, Warsaw, Poland, September 1985

VNU Science Press, Utrecht
Akadémiai Kiadó, Budapest

cytoskeleton. The proteins that span the lipid bilayer have a defined orientation and contribute to the membrane asymmetry [1]. The fluid mosaic model of membrane structure proposed by Singer and Nicholson [2] allows for free diffusion of both lipids and proteins in the bilayer, implying a random arrangement of lipids and proteins. The phenomenon of membrane fluidity is dependent upon the composition of lipids constituting the membrane. Under physiological conditions, an increase in the cholesterol : phospholipid ratio results in an increase in membrane viscosity.

A number of membrane proteins have receptor functions. They contain sites for the well-defined ligands which bind to the cell surface with high affinity in a reversible and saturable manner. Ligand-receptor interaction controls almost all cell functions including the transport of small and large molecules, growth, metabolic processes, protein synthesis, secretion and adhesion. In a typical ligand-receptor interaction, the cell response correlates with the receptor occupancy.

The plasma membrane of platelets serves as a barrier between the platelet cytoplasm and the surrounding plasma. Membranes of platelets and of other cells share many common features, e.g., active transport of ions and metabolites. However, in view of the role of platelets in hemostasis and tissue repair, the platelet plasma membrane is critical in a number of reactions unique to platelets such as shape change, aggregation, adhesion, release reaction of secretion, clot retraction and activation of clotting factors. All these phenomena are controlled by ligand interactions with receptors occurring on the platelet surface [3, 4].

Over the last decade, significant progress has been made in characterization of platelet membranes components and receptors [4–6]. In this review, we will briefly discuss the methodology used and outline the state of knowledge in this area of investigation.

Methodology

A number of techniques previously developed in the course of research on other cells have been adapted to study platelet membrane components and receptors. Isolation and characterization of platelet subcellular fractions including platelet membranes had been accomplished during the late sixties and early seventies [7]. However, a milestone in the research on platelet glycoproteins was the introduction of high resolution electrophoretic techniques such as one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis [8], two-dimensional gel electrophoresis [9, 10] and surface labeling of the intact platelets. In typical experiments, intact platelets are labeled with ^{125}I in the presence of lactoperoxidase [11] or iodogen [12]. Under these experimental conditions, ^{125}I does not penetrate to the interior of platelets, but labels specifically the surface-exposed glycoproteins. Subsequently, detergent extracts of labeled platelets are analyzed by gel electrophoresis. The separated surface-labeled glycoproteins can be identified by autoradiography using Kodak X-ray films and intensifying screens. The ^{125}I is usually introduced into the protein moiety of glycoproteins.

Specific labeling techniques for surface-exposed carbohydrate moieties of platelet membrane glycoproteins have also been developed. Using these methods, the sugar residues are first oxidized and then reduced with tritiated borohydride to introduce the radioactive label [13]. After electrophoresis of detergent extract of ^{3}H-labeled platelets, the gels are impregnated with scintillating substances and separated glycoproteins are identified by means of fluorography. Another approach to detect carbohydrate residues of electrophoretically separated platelet membrane glycoproteins is to label them selectively with radioactive lectins [4, 14].

An important methodological advance was the development of a number of polyclonal and monoclonal antibodies against platelet membrane components. Development of monoclonal antibodies is particularly useful since antigenic material cannot usually be obtained in a highly purified state. Monoclonal antibodies were helpful to characterize glycoprotein Ib, IIb, IIIa and IIb/IIIa complex [15–18].

Application of antibodies was essential in developing several techniques to identify platelet membrane glycoproteins including cross immunoelectrophoresis [19], immunoprecipitation using a detergent extract of surface labeled proteins [20], immunoblotting [21], immunoaffinity purification [16] and measurement of the number of antigenic sites of glycoproteins on the platelet surface [16].

The platelet membrane fraction represents a good starting material for further purification of platelet membrane glycoproteins. The methods used include extraction of peripheral proteins with high ionic strength solutions [22] and extraction of integral proteins with detergents such as Triton X-114 [23]; gel filtration, sucrose gradient ultracentrifugation [24, 25] and HPLC in the presence of detergents [26]; lectin-agarose chromatography [27] and immunoaffinity chromatography using monoclonal antibodies complexed to resins [16]. Purification of undenatured platelet membrane glycoproteins to homogeneity encounters considerable difficulties and the knowledge of the structure of these components is very limited.

A number of techniques to measure radiolabeled ligand interaction with intact or activated platelets are based on the procedure of Feinberg et al. [28]. A mixture of platelets and ligands is centrifuged through sucrose or silicone oil and the radioactivity is counted in supernatant and in platelet pellets. After constructing binding isotherms, the number of binding sites and association constants are calculated by means of Scatchard plot or derived equations [29].

An important aim of the research is to define the association of the receptors with the individual membrane glycoproteins and ultimately with particular binding domains of proteins for the ligands. The approaches include the use of monoclonal or polyclonal antibodies which recognize glycoproteins or their fragments and block receptor-ligand interaction [16, 18, 19], photoaffinity-labeling and affinity cross-linking of ligands and their receptors [31, 32] and the study of receptor-ligand interactions on platelets with congenital deficiencies of membrane glycoproteins [33, 34].

Platelet membrane glycoproteins. Using two dimensional gel electrophoresis

with the technique of ^{3}H-borohydride-labeled washed platelets, it was possible to identify more than 30 glycoproteins on the human platelet plasma membrane even after allowance was made for glycoprotein microheterogeneity and probable glycoprotein subunit composition [3]. We will briefly discuss glycoproteins that have been relatively well characterized, i.e., glycoproteins Ib, IIb, IIIa, V, and IX.

Glycoprotein Ib has an apparent molecular weight of 170k daltons on SDS-polyacrylamide gels under non-reducing conditions and consists of two disulfide linked polypeptide chains of 143k and 22k called GPIbα and GPIβ. Glycoprotein Ib is poorly visualized in ^{125}I-surface labeled platelets subjected to SDS polyacrylamide gel electrophoresis; on the other hand GPIb band is quite prominent in platelets labeled with ^{3}H-sodium borohydride. The products of proteolytic degradation of GPIb are glycocalicin (M_r 148k daltons) and macroglycopeptide (M_r 45k daltons). Both products are well soluble in water, contain the bulk of GPIb carbohydrates and have been purified to homogeneity [35].

There is evidence that GPIb may span the whole width of lipid bilayer and interact with cytoskeletal structures at the inner surface of the membrane [36]. GPIb may serve as one of two receptors for von Willebrand factor (vWF) on the platelet surface [37]. GPIb deficiency occurs in Bernard-Soulier syndrome [38]; GPIb-deficient platelets can be made by treating normal platelets with proteolytic enzymes [39]. GPIb binds thrombin but is not hydrolyzed by this enzyme [40]. A monoclonal antibody to GPIb blocks thrombin-induced platelet aggregation [41]. Platelets of patients with Bernard-Soulier syndrome, a congenital bleeding disorder, are characterized by their very large size [5]. These platelets are not aggregated by ristocetin nor do they adhere to the subendothelium [5], and their sensitivity to thrombin is reduced [42]. These data suggest that GPIb might take part in the platelet interaction with thrombin, but it is not necessary for the functional response of platelets to this enzyme.

Glycoprotein V (M_r 82k) and glycoprotein IX (M_r 17k – 22k) are related to GPIb. GPV and GPIX are also absent in Bernard-Soulier syndrome [5]. They contain high percentages of carbohydrate and can best be visualized after sodium ^{3}H-borohydride labeling. GPV is easily hydrolyzed by chymotrypsin and thrombin [43]. Recent evidence indicates that GPIb and GPIX form a complex on the platelet surface [44].

Glycoprotein IIb and IIIa are the best characterized platelet membrane components [3 – 6, 10]. They are most prominent on the surface of ^{125}I-labeled platelets. GPIIb consists of two disulfide-linked subunits, GPIIbα (M_r = 116,000) and GPIIbβ (M_r = 23,000), and contains approximately 15% carbohydrate by weight. In contrast, GPIIIa consists of a single polypeptide chain with interchain disulfides. It also contains 15% carbohydrate. The apparent M_r of GPIIIa as determined from SDS gels is 96k in nonreduced and 108k in reduced systems [4, 10]. Both components are integral parts of platelet membranes; they can be solubilized only upon membrane disruption by detergents. They form a 1 : 1 complex on the membrane surface in the presence of calcium.

Platelet fibrinogen receptors: The following experimental evidence indicates that fibrinogen receptors are associated with the GPIIb/GPIIIa complex. 1) These glycoproteins are deficient in Glanzmann's thrombasthenia, a bleeding disorder characterized by an absence of both platelet aggregation and fibrinogen binding to platelets [5, 45]; 2) Formation of complexes between fibrinogen and GPIIIa have been shown in a solid phase system using enzyme-linked immunoassay techniques [46] or using fibrinogen modified with a photoreactive heterobifunctional crosslinking reagent [31]; 3) A number of polyclonal and monoclonal antibodies directed against GPIIIa or GPIIb/GPIIIa complex block fibrinogen-receptor-mediated platelet aggregation [16, 30]. 4) Most recently, two groups of investigators demonstrated specific binding of ^{125}I-fibrinogen to GPIIb/GPIIIa complex incorporated into phospholipid vesicles [47, 48].

Fibrinogen receptors are latent on the surface of intact platelets, but they can be exposed following platelet stimulation by ADP and other agonists [33, 34, 49–51] or by treating platelets with proteolytic enzymes such as chymotrypsin [52] pronase [52] or elastase [53]. The mechanism by which fibrinogen receptors are exposed on the platelet surface is not well understood. It has been suggested that this phenomenon may be related to the conformational changes resulting from the interaction between ADP and its receptors [54] or to limited proteolysis of the GPIIb/GPIIIa complex [30] or other components of platelet membranes. It is noteworthy that monoclonal antibodies which block most strongly fibrinogen-receptor-mediated platelet aggregation recognize GPIIb/GPIIIa complex but do not react with separated GPIIb and GPIIIa proteins [16]. Therefore, the fibrinogen binding site may reside on an epitope formed by amino acid sequences of both GPIIb and GPIIIa molecules.

GPIIb/GPIIIa complex also expresses binding sites for calcium [55], and for such proteins as fibronectin [56], and von Willebrand's factor [37]. Therefore, GPIIb/GPIIIa complex appears to play a major role in platelet adhesion and aggregation.

Both fibrin and GPIIb/GPIIIa glycoproteins are incorporated into cytoskeleton of thrombin activated platelets [57]. The term "cytoskeleton" has been used to describe the complex network of intracellular fibrils which support the plasma membranes. Several cytoskeletal and contractile proteins have been identified which may be involved in such phenomena as the change of platelet shape, projection of filopodia, clot retraction and consolidation of hemostatic plug with fibrin strands. Tuszynski et al. [58] suggested that association of fibrin with platelet cytoskeleton may be mediated by GPIIb/GPIIIa complex and that this mechanism may play an important role in clot retraction induced by thrombin. Most recently Fox [59] identified actin binding protein (500k dalton) as the protein linking the cytoskeleton to glycoproteins on platelet plasma membranes.

Clotting factors receptors: As described above, GPIb and GPIIb/GPIIIa express binding sites for the adhesive proteins. A number of other receptors on platelet surface have been identified. Equilibrium binding to platelets of a number of plasma and platelet clotting factors including thrombin [60], factor Va [61],

factor Xa [62], factor XIa [63], high molecular weight kininogen [64], factor XIIIa [65] and platelet factor 4 [66] have been characterized.

It is generally accepted that the biological effects exerted by thrombin on platelets depend on the binding of this enzyme to specific receptors and on the hydrolysis of substrates on the platelet membrane [3]. Both low and high affinity thrombin binding sites on the platelets have been identified [60]. The nature of these binding sites is not well known. For instance, Larsen and Simons [32] using a photoreactive thrombin analogue determined that the high affinity binding site for thrombin has a molecular weight of 160k daltons. In a more recent study, Harmon and Jamieson [67] suggested that high affinity sites for thrombin-binding to platelets may involve a multimolecular complex of membrane components (M_r 900,000 daltons). Thrombin binding sites are resistant to chymotryspin treatment although chymotrypsin greatly decreases platelet response to thrombin as measured by aggregation and secretion. These results indicate that binding of thrombin and generation of signals are separate processes [68]. It is well known that thrombin causes release of the contents of dense granules, α-granules, lysosomes, and it causes shape change, aggregation and clot retraction. The other biological effects of thrombin on platelets include release of arachidonic acid, stimulation of phosphatidyl inositol turnover [69], increase of cytoplasmic calcium and phosphorylation of myosin light chain and 47,000 daltons M_r component [70–72]. Activation of platelets with thrombin also leads to the alteration of platelet cytoplasmic structural proteins such as actin and myosin and to the association of these proteins with platelet membrane glycoproteins and α-granules proteins [56, 58, 72]. Myosin phosphorylation appears to be critical for tension production since actomyosin threads from platelets can exert as much tension as muscle actomyosin only if myosin has been phosphorylated [73]. A number of researchers have unraveled the biochemical processes occurring during platelet activation by thrombin; these investigations have been recently summarized in an excellent review by Zucker and Nachmias [72]. However, the relationship between thrombin interaction with its receptors and platelet biological response to this enzyme requires further studies.

The factor Va binding sites are of special importance on the platelet surface because factor Va bound to the platelets serves as a receptor for factor Xa [62]. The bound factor Xa catalyzes the activation of prothrombin on the platelet surface at a rate which is 300,000-fold faster than the rate of activation induced by factor Xa in solution [62]. A patient with a moderately severe bleeding disorder and a congenital deficiency of factor Va binding sites has been described [74]. This report points out the significance of the platelet surface for the effectiveness of the intrinsic coagulation pathway.

High molecular weight kininogen (HMWK) binds to stimulated platelets in the presence of $ZnCl_2$ in a specific and saturable manner [64]. HMWK bound to platelets may serve as a receptor for factor XI and as a cofactor of factor XI activation on the platelet surface [63].

ADP and prostanoid receptors: Adenosine diphosphate (ADP) is one of the

most potent and specific platelet agonists. It has two distinguishable effects. It induces the shape change leading to fibrinogen receptor exposure and platelet aggregation and it inhibits the adenylate cyclase of intact platelets and of broken cell preparations. These two effects of ADP may be mediated through two different receptors. There is evidence that an ADP analogue, 2-methylthioadenosine (β-^{32}P) diphosphosphate is an agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in intact blood platelets [75], whereas 5'-p-fluorosulfonylbenzoyl adenosine covalently modifies ADP receptor controlling shape change and fibrinogen receptor exposure [54, 76]. Daniel et al. [77] demonstrated that phosphorylation of myosin 20k dalton light chain, mediated by calmodulin and myosin light chain kinase, is the earliest effect of ADP, slightly preceding the shape change response. We found that low concentrations of stable prostaglandin analogue, U46619 (50 – 100 nM), acting on receptors different from those affected by ADP produce the same effect on shape change and myosin light chain phosphorylation. In contrast to ADP, low concentrations of U46619 did not cause fibrinogen receptor exposure. Higher concentrations of U46619 caused fibrinogen receptor exposure and platelet aggregation but these phenomena were mediated through the released ADP [78, 79]. On the basis of these data, we propose that platelet shape change and myosin light chain phosphorylation are not sufficient to cause fibrinogen receptor exposure and that this event is specifically produced by ADP acting on platelet membrane receptors.

Accumulation of cyclic AMP in platelets results in the inhibition of platelet function. Activation of adenylcyclase is mediated by prostaglandin receptors [80] and inhibition of cyclic AMP phosphodiesterase regulate the cyclic AMP level. Inhibition of adenylcyclase activity in platelets is also mediated through ADP receptors (see above) and through α-adrenergic receptors [81].

The pharmacological studies on possible platelet membrane receptors for various drugs and neurotransmitters are beyond the scope of this review.

Platelet membrane fluidity: Although platelet membrane receptors are associated primarily with protein components, the lipid layer may significantly influence platelet function by modulating membrane fluidity and receptor movement. The mechanism whereby lipoproteins influence platelet function is most likely related to the transfer of cholesterol to the platelet membranes. Platelets enriched with cholesterol become more responsive than normal to platelet aggregating agents such as epinephrine and ADP and have decreased membrane fluidity. In contrast, incubation of platelets with cholesterol-free phospholipid dispersions results in a decrease in platelet membrane cholesterol and this is associated with an increase in membrane fluidity and a decrease in responsiveness of these platelets to epinephrine [82, 83]. Most recently, Tandon et al. [84] demonstrated that modification of platelet membrane viscosity results in changes in the number and affinity of both high and low affinity thrombin receptors. On the other hand, such phenomena as exposure of fibrinogen receptors on the platelet surface by proteolytic enzymes do not cause changes in membrane fluidity [85].

Conclusion

Receptor-ligand interactions on the platelet membrane control a number of phenomena unique to platelets such as shape change, aggregation, release reaction of secretion, clot retraction and activation of clotting factors. Studies on receptor-ligand interaction may have a number of clinical implications. Absence or abnormality of platelet membrane receptors cause such bleeding disorders as Glanzmann's thrombasthenia, Bernard-Soulier syndrome and thrombopathia (deficiency of factor Va receptor). Autoantibodies to platelet membrane glycoproteins and receptors may contribute to hemostatic abnormalities in idiopathic thrombocytopenic purpura and other disorders [86, 87]. The effect of a number of drugs on platelet function in vivo such as dipyridamole [88], prostaglandin analogues [80] and ticlopidine [89] may be mediated by ligand-receptor interactions on the platelet surface.

Acknowledgement

The author wishes to acknowledge the support of National Institutes of Health Bethesda, MD, USA (grants HL 15226 and HL 14217) for his research.

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Haematologia 20 (1), pp. 15–23 (1987)

Self-renewal Capacity of Mobilized Murine Haemopoietic Stem Cells

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(Received 6 December, 1985; accepted 16 December, 1985)

Self-renewal capacity of stem cells (9 day CFU-S) circulating in the blood and that of CFU-S mobilized into the circulation by various agents was compared in BDF_1 mice. For CFU-S mobilization 1.5 mg trypsin, 150 μg endotoxin, 1 mg Zymosan per mouse or 5 mg/kg methylprednisolone were injected i.v. and the blood CFU-S level was measured 15 min to several days after provocation. Self-renewal capacity of the mobilized CFU-S showed a 4 to 14-fold increase if compared to that of normal blood CFU-S. This, however, proved to be lower than that of bone marrow CFU-S. This increase in self-renewal capacity was independent of the type of mobilizer, suggesting that CFU-S mobilizing agents, in general, release the same subpopulation of CFU-S. The fairly good correlation between the amount and self-renewal capacity of CFU-S in the circulation supported the assumption that, up to a certain limit, the more CFU-S are mobilized, the better quality stem cells can be obtained for transplantation purposes.

Keywords: experimental bone marrow transplantation, mobilization of CFU-S, self-renewal of CFU-S

Introduction

Since haemopoietic stem cells (HSC) are the ultimate source of both the haemopoietic and lymphoid cell lines, it is generally accepted that haemopoietic reconstruction by successful bone marrow transplantation starts from the replication of HSC. Theoretically, any tissue that contains HSC (bone marrow, fetal liver and blood) is suitable for grafting, as it has been experimentally proved in animals. In clinical practice, however, apart from a few exceptions, only bone marrow cells were used as HSC source, because fetal liver is hardly accessible and the HSC content of the blood is very low, 28–46 HSC per ml in man [1, 2], therefore an enormous amount of blood would be needed for a successful transplantation. E.g. for allogeneic transplantation, HSC from 80–160 liters of blood would substitute a graft of 3.5×10^{10} bone marrow cells, the optimal graft for a 70 kg man.

Properties of blood and bone marrow HSC, however, are not completely identical. HSC are known to be capable of producing identical stem cells (self-renewal capacity) and of generating differentiated progenies (differentiation capacity). In mice, however, self-renewal capacity of blood HSC proved to be lower than that of bone marrow HSC [3], consequently blood HSC, if transplanted in equal amounts, will produce less identical stem cells than bone marrow HSC. This lower self-renewal capacity of blood HSC may influence the haematological regeneration

VNU Science Press, Utrecht
Akadémiai Kiadó, Budapest

kinetics of the recipient, therefore, for transplantation, blood stem cells are regarded to be less valuable than bone marrow HSC. Since, however, blood HSC can be collected in excess providing a better donor selection, recent advances in leukapheresis and storage techniques have aroused interest in peripheral blood cell transplantation [4, 5, 6, 7].

Murine experiments clearly indicated that the amount of HSC in the blood can easily be increased to 20–50-fold of the original level by various treatments [8, 9]. If mobilization does not reduce the self-renewal capacity of the HSC population, it may offer a suitable amount of HSC for transplantation purposes from a realistic amount of blood.

Therefore, in the present experiments the self-renewal capacity of blood and mobilized HSC was compared in mice, and the relationship between blood HSC level and self-renewal capacity of blood HSC was studied. Since a murine system was used, 9-day spleen colony forming cells (CFU-S) were regarded as HSC.

Materials and Methods

Male 12–14 week old C57Bl × $DBA/_2/F_1$ hybrid (BDF_1) mice were used as donors and female BDF_1 mice as recipients. The number of haemopoietic stem cells (CFU-S) was assayed by the spleen colony method of Till and McCulloch [10]. Recipients were irradiated with 9 Gy from a ^{60}Co gamma source, and spleen colonies were counted 9 days after transplantation. Bone marrow cells were obtained from the femur. For blood CFU-S assay donor mice were heparinized (60 I.U. i.p.) 10–15 min before aseptic exsanguination from the axillary vessels. According to the expected CFU-S content of the sample, blood was injected undiluted, or diluted with saline. When a low blood CFU-S level was expected, mononuclear cells were isolated by the usual density cut method [11]. Spleen colony count was referred to cell count per femur or per ml blood.

As stem cell mobilizer agents 1.5 mg trypsin (Bacto Trypsin, Difco Laboratories, Detroit, MI, USA), 150 μg endotoxin (Difco Laboratories), 1 mg Zymosan A (Sigma, St. Louis, MO, USA), or 5 mg/kg methyl-prednisolone (Depersolon, Richter Works, Budapest, Hungary) were injected i.v.

Self-renewal capacity (SRC) of blood and bone marrow CFU-S was studied by two methods:

1. CFU-S content of whole spleens of primary recipient was assayed 10 days after transplantation by the spleen retransplantation method according to Worton et al. [12]. In each experiment the amount of injected CFU-S was determined in 10 recipients and the CFU-S content of 5 pooled spleens was tested in 10 secondary recipients. Self-renewal capacity was calculated according to Hodgson [13]:

$SRC = \frac{C_t}{C_i}$; i.e. CFU-S recovered in the spleens of secondary recipients per CFU-S injected into the primary recipients.

2. Distribution of CFU-S among spleen colonies can give the characteristic profile of SRC, allowing to calculate the self-renewal probability of CFU-S [14]. Primary recipients were transplanted with a dose of blood or bone marrow that resulted in 4–6 colonies per spleen. On day 9 individual colonies were dissected, suspended and injected individually into secondary recipients irradiated with 9 Gy. CFU-S content of 35 to 50 individual colonies were studied in each experiment and the distribution of frequency was calculated. Probability of self-renewal was calculated by Vogel's formula [14]:

$$v^2 = \left(\frac{2 - 2p}{2p - 1}\right) + \frac{1}{M'}$$

where v is the variation coefficient of CFU-S per colony in the primary recipient, p is the probability of self-renewal and M' is the mean CFU-S per colony.

Results

Comparative CFU-S mobilizing effect of various agents

In the first experiments the CFU-S mobilizing effect of various agents known to induce granulocyte and/or CFU-S mobilization was compared. Doses applied were those known to induce granulocytosis.

In the dose of 5 mg/kg body weight Depersolon did not induce CFU-S mobilization. Zymosan, though inducing rapid mobilization of CFU-S was found to be highly toxic in the effective dose, while 1.5 mg trypsin induced rapid mobilization of CFU-S without producing toxic effects. Mobilization after trypsin injection proceeded in a single wave, the blood CFU-S level returned to normal 6 h after trypsin injection [9]. Endotoxin is known to induce a prolonged increase in the

Table 1

Mobilization of CFU-S after the injection of various mobilizing agents

Mobilizer	Time of blood sampling	CFU-S/ml blood*
5 mg/kg Depersolon	30 min	26.0 ± 13.0
1 mg Zymosan	60 min	309.4 ± 42.2
1.5 mg trypsin	15 min	313.7 ± 25.3
150 μg endotoxin	30 min	282.0 ± 26.0
	3 days	215.0 ± 71.0
	6 days	432.0 ± 73.0
	21 days	51.0 ± 39.0
Control level	–	20.6 ± 4.0

* Mean ± S.E. of 3–8 experiments, each on 4 donor and 10 recipient mice

blood CFU-S level [8]. In our experiments the maximum level of blood CFU-S was found 6 days after injection, and the blood CFU-S level approached the control 21 days after injection (Table 1).

Self-renewal capacity (SRC) of mobilized CFU-S

SRC of CFU-S mobilized into the circulation by various agents was characterized by the amount of newly formed CFU-S 10 days after transplantation. SRC was calculated by referring the amount of CFU-S in the spleen of the secondary recipients (corrected for injected spleen fraction) to the amount of injected CFU-S. For comparison, SRC of normal bone marrow CFU-S was also studied (Table 2). SRC for normal bone marrow CFU-S was found to be 40 times higher than that of normal blood CFU-S. Depersolon injection resulted in a slight increase, Zymosan induced a 10-fold increase in SRC as compared to the SRC of normal blood CFU-S. After endotoxin injection the SRC increased; its highest value was observed 3 days after injection.

Table 2

Self-renewal capacity of mobilized CFU-S

Source of CFU-S[a]	Amount of injected CFU-S[b]	Retrans-planted fraction of spleen	CFU-S recovered[c]	SRC[d]	SRC increase factor[e]
Bone marrow[f]	17.4	1/20	557.5	32.0	—
Normal blood	76.9 ± 5.6	1/2.5	55.5 ± 2.8	0.72	—
	61.6 ± 5.1	1/2.5	56.0 ± 9.8	0.91	—
Blood, after trypsin injection[f]	80.2	1/25	807.2	11.57	14.1
Blood, after Depersolon injection	23.4 ± 1.8	3/10	45.6 ± 6.2	1.95	2.4
Blood, 30 min after	16.3 ± 2.3	1/15	78.0 ± 11.8	4.80	5.9
endotoxin injection	13.5 ± 1.6	1/8	62.4 ± 6.7	4.60	5.7
Blood, 3 days after	26.6 ± 4.2	1/15	243.7 ± 10.6	9.15	11.3
endotoxin injection	16.5 ± 2.2	1/10	86.0 ± 9.7	5.21	6.4
Blood, 6 days after	48.4 ± 6.1	1/15	132.8 ± 22.0	3.06	3.9
endotoxin injection	38.1 ± 2.2	1/10	134.3 ± 7.5	3.52	4.3
Blood, 21 days after endotoxin injection	23.7 ± 2.8	1/5	73.5 ± 6.1	3.1	3.8

[a] Blood was obtained from 3—10 donors

[b] Calculated from colony count of primary recipients. Mean ± S.E. of minimum 10 spleens

[c] Calculated from spleen colony count in secondary recipients, corrected for injected spleen fraction

[d] Self-renewal capacity (SRC): CFU-S recovered per CFU-S injected

[e] Related to SRC value of normal blood CFU-S (0.815)

[f] Mean values obtained from Fehér and Gidáli [9] [19]

Self-renewal profile of mobilized CFU-S

SRC of CFU-S is a result of stochastic process [15]. Table 2 shows the mean SRC of various CFU-S populations. Self-renewal profile, however, indicates the frequency distribution of CFU-S with various self-renewal capacity within a given CFU-S population.

41% of the bone marrow CFU-S did not produce new stem cells within the observation period, but 10% of the population consisted of high reproducer CFU-S forming more than 20 new CFU-S within 9 days. Blood CFU-S did not contain high reproducer CFU-S and 62% of the CFU-S did not reproduce themselves at all. Self-renewal profile of mobilized CFU-S from the blood of endotoxin injected mice fell between that of the bone marrow and blood CFU-S: less non-reproducer CFU-S were found than in blood CFU-S population and a few per cent "high reproducer" CFU-S occurred (Table 3).

Table 3

Profile of self-renewal capacity of blood borne, mobilized and bone marrow CFU-S

Origin of primary spleen colonies	CFU-S per colony (mean)	Distribution of CFU-S per colony (%)					
		0	1–5	6–10	11–15	16–20	>20
Normal blood (91)*	1.0	62.6	30.8	5.5	0	1.1	0
Blood 4 days after endotoxin injection (51)	3.7	31.0	54.0	8.0	5.0	0	2.0
Blood 7 days after endotoxin injection (58)	3.8	59.1	34.0	3.3	2.2	0	1.1
Bone marrow (133)	7.0	41.4	28.6	9.8	6.8	3.7	9.7

* Numbers in brackets indicate number of colonies tested

Self-renewal probability of mobilized CFU-S

Probability of self-renewal for CFU-S derived from bone marrow cells or blood cells from control or endotoxin injected mice were calculated from the CFU-S content of individual colonies 9 days after transplantation [14].

A significant difference was found in the mean CFU-S content of colonies derived from bone marrow, normal blood or blood from endotoxin injected mice (Table 3); due to the limitations of the analysis [16, 17], however, the calculated self-renewal probabilities: 0.656 for bone marrow, 0.610 for normal blood and 0.650 for mobilized CFU-S did not reflect these differences (Table 4).

Table 4

Self-renewal probability of bone marrow, blood and mobilized CFU-S

Origin of primary spleen colonies	Self-renewal probability[b] (p)
Bone marrow (133)[a]	0.656 ± 0.039
Normal blood (91)	0.610 ± 0.027
Blood from endotoxin injected donors (109)	0.650 ± 0.011

[a] Numbers in brackets indicate number of colonies

[b] Calculation according to Vogel's formula (see Methods) was carried out from detailed data of Table 3. Data for endotoxin injected donors are compiled data obtained 4 and 7 days after endotoxin injection since no difference was found between these groups

Correlation between SRC of blood CFU-S and blood CFU-S level

To study the possible correlation between SRC of blood CFU-S and the actual level of circulating CFU-S, data were collected from experiments where both parameters were assayed parallel. Data plotted on Fig. 1 were obtained from control, Depersolon, endotoxin, Zymosan or trypsin injected groups, samples were collected at times indicated in Table 1. A fairly good correlation (r = 0.955) between blood CFU-S level and their SRC was found up to about 300 CFU-S/ml blood. At higher blood CFU-S levels, however, though SRC was found to be higher than for normal blood CFU-S, this correlation ceased to exist.

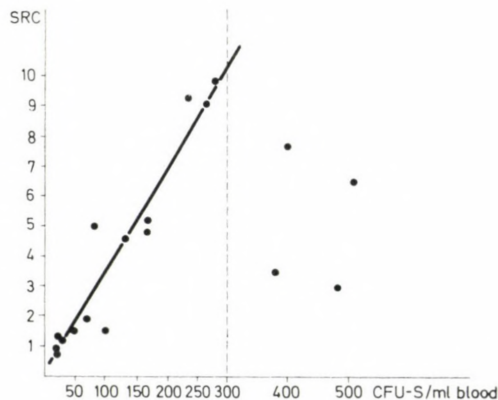

Fig. 1. Correlation between self-renewal capacity and level of circulating CFU-S before and after injecting various mobilizing agents. Each point represents the mean of 4 donor, 10 primary and 10 secondary recipient mice. Self-renewal capacity (SRC) was calculated according to Hodgson [13] as described in Methods. Curve was fitted to a linear regression equation: $y = 0.103 + 0.03x$, where x is for mean CFU-S/ml blood. r = 0.955

Discussion

Mononuclear blood cells have already been successfully used to restore haemopoiesis in lethally irradiated dogs [18] and methods for collection and preservation of human blood mononuclear cells suitable for autologous or allogeneic transplantation have been developed [5, 6, 7]. Several problems, however, concerning the use of poor quality blood stem cells as well as the low level of stem cells in the circulation have not been solved. The quantity of circulating stem cells can be improved by mobilizing bone marrow stem cells into the circulation in mice [8, 9] and several authors claimed successful mobilization of granuloid progenitors in dogs and men [19, 20, 21, 22, 23]. However, relatively little is known about the quality of the mobilized stem cells. The low self-maintaining capacity of murine blood stem cells suggested that these cells are more mature than the pluripotent stem cells of the bone marrow, probably representing an aged population [3]. Mobilizable murine stem cells, however, seemed to represent an intermediate population between sessile bone marrow and blood stem cells [9], capable of better self-renewal than blood stem cells.

The present experiments were focused on the self-renewal capacity of mobilized stem cells in a murine system.

The CFU-S population is known to be heterogeneous, consisting of subpopulations of various self-renewal capacity. According to the age structure hypothesis of CFU-S [24, 25], the less divisions occurred in the life history of a CFU-S the younger the CFU-S was, having the higher self-renewal capacity. In this context blood CFU-S probably belong to the more mature subpopulation of the HSC pool. Blood CFU-S have a higher mitotic activity [26], lower SRC [3] than bone marrow CFU-S, and they also have a special feature: mobility. This latter means that they are able to leave the bone marrow matrix under normal physiological conditions.

In our previous studies [9] we showed that the SRC of CFU-S fraction that does not leave the bone marrow spontaneously, but can be mobilized by certain mobilizing agents is different from both bone marrow and blood CFU-S. The fact that the mobilizable CFU-S pool could be exhausted, and a definite time was needed for the replacement of the pool induced us to assume that this "mobilizable CFU-S fraction" represents a special subset of the pluripotent bone marrow CFU-S population.

The present data clearly proved that SRC of mobilized CFU-S increased irrespective of the mobilizer injected, suggesting that all mobilizers studied affect the same subpopulation of CFU-S with a similar self-renewal capacity. Although neither SRC nor the self-renewal profile of mobilized CFU-S had reached those of normal bone marrow CFU-S, mobilized CFU-S were of better quality for transplantation purposes than blood CFU-S. Calculated probability of self-renewal which expresses the probability of the formation of new CFU-S in transplantation systems [14], was found to be similar for mobilized and bone marrow CFU-S, while the self-renewal probability was lower for blood CFU-S.

This finding suggests that a lower portion of mobilized CFU-S is differentiat-

ing (i.e. following a suicide pathway) than that of blood CFU-S. The positive correlation between level and SRC of blood CFU-S suggested that, up to a certain limit, the more CFU-S are mobilized, the better quality stem cells are obtained for transplantation purposes.

As this correlation was not influenced by the mobilizing agent, i.e. SRC was increased after the injection of agents inducing rapid mobilization (Zymosan or trypsin) or producing a long term or biphasic effect (endotoxin), one could assume that mobilizers inducing a prolonged increase in blood CFU-S level can also be considered for obtaining large quantity of adequate HSC for transplantation. Presently, however, mobilizers can only be used in experimental systems as they are active only in near-toxic doses. Clinical applications must therefore await future advances in finding other, less toxic mobilizers.

Acknowledgement

This work was supported by the Scientific Research Council, Hungarian Ministry of Health (08/6-16/281). The skillful technical assistance of Mrs. I. Blasko and K. Erdősi is highly appreciated.

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Haematologia 20 (1), pp. 25–30 (1987)

Particular Interest of HLA Typing for Genetic Counselling in Families with Congenital Adrenal Hyperplasia (21-OH Deficiency)

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(Received 11 December, 1985; accepted 14 January, 1986)

The close genetic linkage between 21 hydroxylase deficiency and HLA loci considerably enlarges the possibilities of genetic counselling. Two 21-OH deficient complex families are reported, illustrating several aspects of this genetic counselling: detection of carriers, determination of the actual risk to related couples, suspicion of 21-OH deficiency by an HLA specificity association, and prenatal diagnosis.

Keywords: 21-hydroxylase deficiency, HLA typing, congenital adrenal hyperplasia

Introduction

A number of studies have confirmed the observation of Dupont et al. [1] concerning the close genetic linkage between HLA and congenital adrenal hyperplasia (CAH) due to 21-hydroxylase (21-OH) deficiency.

More recently Migeon et al. [2] suggested that late onset adrenal hyperplasia (LAH) due to 21-OH deficiency is also linked with HLA loci, and that the responsible gene may be allelic to the CAH gene. These findings were confirmed by subsequent studies which also demonstrated a strong association between LAH and the HLA antigen B14, and the whole haplotype Aw33C–B14 DRw1 [3].

For CAH, the main clinical applications of these data were the detection of heterozygous CAH carriers, detection of 21-OH homozygous-deficient but apparently clinically normal subjects [4, 5], and prenatal diagnosis of fetuses at risk in affected families [6–14].

For LAH, HLA antigens may also be used as markers for following the segregation of the deficient 21-OH alleles in affected families, and in addition, the strong association with B14 may be an additional element in the diagnosis of 21-OH deficiency among hirsutic women.

These findings considerably widen the possibilities of genetic counselling of families with 21-OH deficiency [8, 15]. Here we describe two complex families in which several aspects of this genetic counselling are illustrated.

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Case Reports

Family P.D.C. (Figure 1)

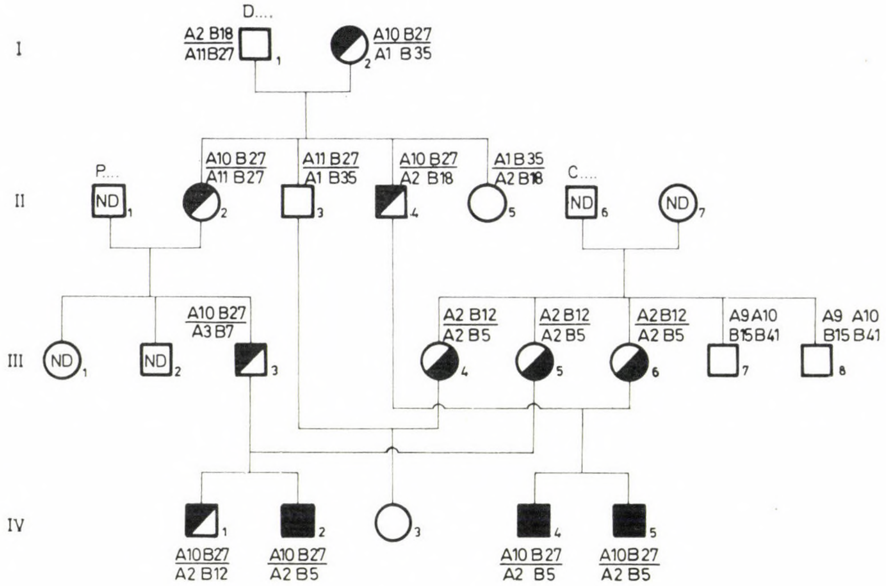

Fig. 1. Pedigree of family P.D.C.

The couple (III-3 and III-5) with one affected CAH child (IV-2) and one apparently normal child (IV-1) was referred for genetic counselling about the possibility of a prenatal diagnosis in a further pregnancy. It appeared that two cousins of the affected child were also suffering from CAH and indeed the whole family had a rather complex pedigree resulting from three intermarriages between two families.

As shown in Fig. 1, two sisters (III-4 and III-6) married two brothers (II-3 and II-4), whereas the third sister (III-5) married a nephew (III-3) of the brothers. Two of these couples had affected children, the third couple (II-3, III-4) had a healthy daughter and the parents were anxious to know whether a further pregnancy could lead to an affected child. We tried to determine if this third couple was at risk.

Family B.P.G.F. (Figure 2)

Family P included four married sisters. Although the two first sisters (II-2 and II-3) were married to non-related men (II-1 and II-4) from families B and G, both couples had a CAH affected child. They were referred for genetic counselling

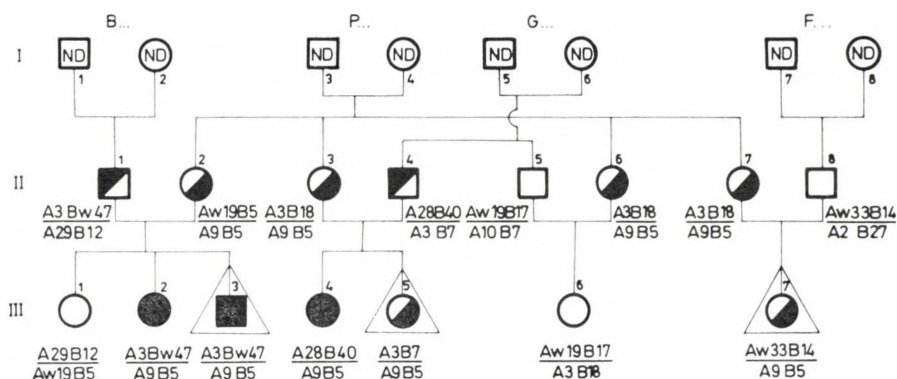

Fig. 2. Pedigree of Family B.P.G.F. △ = prenatal diagnosis

and a prenatal diagnosis was made in the subsequent pregnancy (III-3 and III-5). The third sister (II-6) married the second son of family G (II-5, brother of II-4). Although they had a healthy girl, this couple was theoretically at risk and anxious to plan a further pregnancy. The fourth sister (II-7) married a non-related man (II-8) from family F. Anxious because of familial precedents, she also consulted us for genetic counselling.

Methods

Taking into account the close genetic linkage between HLA loci and the CAH 21-OH trait, the segregation of 21-OH deficiency "genes" was established using HLA antigens as markers. HLA typing was carried out using a lymphomicrocytotoxicity test adapted from the method of Mittal et al. [16].

Results

Family P.D.C.

HLA typing showed that the three affected children were identical for HLA-A, B and C loci. The genes responsible for the 21-OH deficiency are linked respectively to the HLA haplotypes A26 (10) Cw1 B27, but the third husband (II-3) had two different haplotypes, A1 C-B35, A11 C-B27, and consequently had not inherited the 21-OH deficiency gene. The hypothesis that this haplotype, A11 C-B27, had originated from the original 21-OH deficiency linked haplotype A26 (10) Cw1 B27 by a recombination between C and B, was ruled out by studying all the sibs (II-2, 4, 5) and the parents (I-1, I-2).

Family B.P.F.G.

HLA typing of couples 1 and 2 (II-1/II-2 and II-3/II-4) revealed three 21-OH deficient alleles carried by families P, B and G which were linked to HLA haplotypes A9B5 (sister-mothers II-2 and II-3), A3 Bw47 (father II-1) and A28 B40 (father II-4), respectively.

The four sisters of family P carried the HLA haplotype A9 B5, linked to 21-OH deficiency, and consequently the mothers in couples 3 and 4 were also carriers.

In the third couple (II-5/II-6) the husband (II-5, brother of the carrier II-4) did not inherit the 21-OH deficiency-linked HLA haplotype of family G and consequently the risk for this couple could be excluded.

In the fourth couple (II-7/II-8) the father was not related to P, B and G families but as the mother was carrier, a prenatal diagnosis based on biochemical investigation was proposed. HLA typing revealed that the father carried the haplotype Aw33 B14, frequently associated with LAH. The fetus inherited this haplotype and one of his mother's haplotypes which was linked to CAH, but in the amniotic fluid, the 17-hydroxyprogesterone (17 OHP) level (measured by M. G. Forest) was within the normal range.

Discussion

The results obtained from the study of these complex families illustrate the possibilities offered by HLA typing in improving genetic counselling for 21-OH deficiency.

Based on the calculations, without taking into account the HLA genotypes, in cases of intermarriages like couple II-3/III-4 from family P.D.C. and couple II-5/II-6 from family B.P.G.F., where fathers and mothers were brothers and sisters of carriers, they had a one in four chance of being at risk and a one in sixteen chance of having a CAH affected child. On the basis of hormonal tests, due to numerous negative results, no definite conclusion could be reached as to whether or not the parents were normal or heterozygous for the CAH gene [17, 18]. On the other hand, HLA genotyping does provide a considerable level of accuracy in predicting the real risk of transmitting the CAH gene. In the two intermarriages described above the risk was excluded for each couple at risk. The genetic linkage between CAH and HLA has now been confirmed by the observing of several hundred families; therefore, the risk of error caused by genetic recombinations seems particularly low.

The case of the first two couples of family B.P.G.F. illustrates the high incidence of 21-OH deficiency. In France the frequency of heterozygous carriers was estimated at 1/76 by epidemiological investigations [19] and up to 1/43 by neonatal screening (Limal, 1984, personal communication). Consequently, the risk for a married carrier to have an affected child can be estimated at about 0.5% ($1/50 \times 1/4$), which is low, but not negligible. Since it is also possible to make a

reliable prenatal diagnosis of 21-OH deficiency through biochemical studies of the amniotic fluid [12, 14, 20, 21] it would be of interest to use HLA typing in affected families to obtain a precise indication of the carriers.

This same process was followed for the fourth couple of family B.P.G.F. in which the mother was shown to be a carrier. HLA typing revealed that the fetus had inherited the 21-OH deficient CAH from its mother's alleles, but biochemical investigations ruled out the risk of CAH homozygosity.

This case illustrates the contribution of genetic counselling provided by HLA antigen associations. Two HLA haplotypes have been demonstrated to be associated with 21-OH deficiencies:

– A3 Cw6 Bw47, associated with one of the CAH alleles [22], but the relatively low incidence of this allele in France: 8% of the CAH patients [23] reduces its potential use. For more than 90% of CAH cases, HLA antigen distribution is random. In contrast, B14 and the haplotype Aw33 Cw8 B14 are frequently associated with LAH. In France (Couillin et al. [23] 68.2% of LAH patients are B14 (relative risk: ×16.2) and 36.4% carry the haplotype Aw33 B14 (relative risk: ×81.2).

The fetus of the fourth couple of family B.P.G.F. inherited a CAH linked haplotype from its mother and the haplotype Aw33 B14 from its father. He could be suspected of being affected with 21-OH deficiency.

Prenatal investigation excluded a CAH affected fetus, but could not completely rule out the hypothesis of a LAH affected fetus (genotypically LAH/CAH), since it appears that in cases of LAH, no significant amniotic 17-OHP elevation can be observed (Forest et al. [14]). This last eventuality might be excluded (or confirmed) in early childhood by ACTH stimulation and plasmatic 17-OHP dosage.

Acknowledgement

This work was supported by a grant from I.N.S.E.R.M. (contract ATP 78-79-110).

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Haematologia 20 (1), pp. 31–39 (1987)

Heterogeneity of Chronic T-Cell Lymphocytosis: Immunological and Clinical Aspects

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(Received 29 August, 1986; accepted 30 December, 1985)

The clinical and laboratory findings as well as the immunological cell phenotype of nine patients with chronic T-cell lymphocytosis (T-CL) are presented. The clinical course was stable in most patients and only one patient died. Splenic enlargement was the main clinical finding and in contrast to B-CLL the age at presentation was lower; T-CL predominated in females and lymphadenopathy was rare. The lymphocyte count was moderate (range $4.1-23.8 \times 10^9/L$). Six patients displayed neutropenia; in contrast, anaemia was only observed in one patient and thrombopenia in another. The immunological cell phenotype was heterogeneous: four cases exhibited a cytotoxic/suppressor phenotype, two a helper phenotype and in the remaining three cases mixed OKT4+ and OKT8+ lymphocytes were observed; these results suggest that T-CL may originate from different T-cell subsets.

Although the question of the benign or neoplastic nature of the T-CL remains open, some of the characteristics of the immunological phenotype could provide additional evidence to demonstrate the malignant condition of the process: in contrast to normal lymphocytes (CD5+, CD7+) the lymphocytes in T-CL were generally CD5±, CD7−, and one patient showed an aberrant phenotype (OKT3+, OKT6+), an occasional finding in other T lymphoproliferative disorders.

Keywords: T-CL, clinical findings, surface markers

Introduction

Chronic lymphocytic leukaemia of T-cell origin (T-CLL) is a rare disease which accounts for approximately 2% to 5% of all CLL [1, 2, 3, 4].

T-CLL shows a variable clinical picture since whereas some patients display an aggressive course [5, 6] others show a relatively benign evolution with a long survival rate; these latter cases are usually associated with neutropenia and the malignant nature of the condition has been questioned by several authors [7, 8, 9] who prefer a term such as chronic lymphocytosis of T-cells for these patients.

Within the group of B-CLL there are also indolent forms characterized by their stability, both clinical and biological [10]. Moreover, Caligaris-Cappio et al. [11] have identified a characteristic subgroup of B-CLL with a stable clinical picture and good prognosis which presents a characteristic membrane-phenotype.

The present paper describes in 9 patients with chronic T-cell lymphocytosis

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(T-CL) the clinical and morphological findings as well as the surface markers defined by classical markers and a panel of monoclonal antibodies (McAb) in an attempt to better characterize this clinical entity.

Materials and Methods

Patients: 9 patients with a persistent increase of mature T-lymphocytes in peripheral blood (PB) and bone marrow (BM) were studied. All these cases were classified according to Rai's staging system [12]. Other chronic T-lymphoproliferative disorders, such as prolymphocytic leukaemia (PLL), adult T-cell leukaemia lymphoma (ATLL) and the Sezary syndrome were excluded using both morphological and marker studies.

Morphology and cytochemistry: May-Grunwald/Giemsa stained smears of PB and BM were examined by light microscopy. Acid phosphatase including an incubation with tartaric acid, B-glucuronidase and the periodic acid Schiff (PAS) stainings were carried out by standard techniques.

Immunologic markers: Mononuclear cells were isolated from heparinized peripheral blood by means of Ficoll-Isopaque density gradient centrifugation. E-rosette-forming cells were detected using AET-treated sheep red blood cells [13]. Lymphocyte forming rosettes with mouse erythrocytes (MRBC-rosettes) were determined by the method of Stathopoulos and Elliot [14]. The expression of surface immunoglobulins (SIg) was analyzed by direct immunofluorescence with FITC rabbit antiserum ($F(ab')_2$ fragment) against human immunoglobulins (Behring Lab.).

The binding of McAb was assessed by indirect immunoflurescence. Briefly, 10^6 cells were incubated for 30 min at 4 °C with McAb and then stained with a FITC goat antimouse Ig (Meloy Lab.). The characteristics of the McAb used were as follows: OKT3 (CD3, p19) mature T-cells, OKT4 (CD4, p55) helper/inducer T-cells, OKT8 (CD8, p32) cytotoxic/suppressor T-cells, OKT6 (CD1, p45) common thymocyte, OKT9, transferrin receptor-positive cells, OKT10, immature thymocyte and replicating cells [15]; OKT11 (CD2, p45) E-rosette cells [16]; WT1 and 3A1 (CD7, p41) pan-T lymphocytes [17]; Leu-1 and Cris-1 (CD5, p67) peripheral and thymic T-cells and B-CLL [18]; B1 (CD20, p35) B lymphocytes [19]; OKIal and GRB1 [20] Ia-like antigen; OKM1 [21] monocytes, granulocytes and null cells with natural killer functions.

Terminal deoxynucleotidyl-transferase (TdT) was determined by the indirect immunoflurescence technique on cytocentrifuged preparations. Rabbit anti-TdT (Bethesda Research Lab.) was used as a first layer and a goat anti-rabbit antiserum coupled to FITC as the second layer.

Results

Clinical data: The clinical data at presentation of the nine patients included in the study are shown in Table 1. Ages ranged from 45 to 79 years, with a clear predominance of females (6/3). Their distribution according to the Rai et al. [12] staging system was: 3 patients in clinical stage 0, 4 in stage II, 1 in stage III and 1 in stage IV.

Table 1

Clinical features of T-CLL patients at diagnosis and course

Case	Age	Sex	Rai-stage	Lymph-adeno-pathy	Spleno-megaly	Hepato-megaly	Skin	Treatment	Survival after diagnosis (months)
1	50	M	0	–	–	–	+	None	32
2	48	F	0	–	–	–	+	None	42
3	55	M	II	–	+	+	–	None	54
4	45	F	IV	+	+	+	–	Splenectomy	45
5	75	F	III	–	+	+	–	None	3
6	75	F	II	–	+	–	–	Splenectomy	11
7	62	F	II	–	+	+	–	Chlorambucil-	13
								Prednisone	13
8	56	F	II	–	+	–	–	None	114
9	79	M	0	–	–	–	+	None	1

* All the patients except No. 6 survived up to the moment of this analysis

The spleen was palpable in six cases while hepatomegaly was seen in 4 patients and enlarged lymph nodes in only one. Three patients had skin lesions but no lymphocyte infiltration was evidenced by biopsy in any case.

Four patients (Case Nos. 3, 4, 6, 7) showed repeated infections – pulmonary and urinary – and another had staphylococcal skin abcesses (Case No. 8).

Laboratory data: The patients displayed a moderate lymphocyte count, ranging between 4.1 and $23.8 \times 10^9/l$. Seven patients displayed neutropenia which was moderate in five (between 1 and $3 \times 10^9/l$) and severe in another two ($< 1 \times 10^9/l$) (Table 2). In contrast, anaemia was only observed in one patient (Case No. 5) and thrombopenia in another (Case No. 4).

Five patients exhibited alterations in the levels of serum immunoglobulins: one showed a polyclonal – IgG, IgA, IgM – increase, two a polyclonal IgG increase, one a slight decrease in IgA and another a slight hypogammaglobulinaemia (Table 2). Rheumatoid factor was detected at a level of 1 : 128 in one (Case No. 8) of the 7 patients analyzed.

Morphology and cytochemistry: Lymphocytes were classified according to the morphological criteria proposed by Huhn et al. [22]; only 3 of the 5 subtypes described were found. Four cases corresponded to the small lymphocyte subtype with scant cytoplasm and no granules; in the other five the lymphocytes had

Table 2

Laboratory and morphological features of T-CLL patients

Case	Lymphocytes $\times 10^9/l$	Neutrophils $\times 10^9/l$	Bone marrow percentage lymphocytes	Morphologic subtype	Serum immunoglobulins
1	11.4 (79%)	2.4 (17%)	34	Lymphocytic, abundant cytoplasm and granules	Normal
2	15.5 (84%)	2.5 (14%)	42	Lymphocytic, abundant cytoplasm	Slightly elevated IgG level
3	23.8 (85%)	3.9 (14%)	49	Lymphocytic, small	Normal
4	12.8 (80%)	2.8 (18%)	39	Lymphocytic, abundant cytoplasm	Moderate diffuse decrease
5	4.2 (70%)	1.8 (30%)	40	Lymphocytic, small	Normal
6	6.4 (57%)	3.0 (27%)	28	Lymphocytic, abundant cytoplasm and granules	Slightly elevated IgA level
7	9.6 (91%)	0.4 (4%)	68	Lymphocytic, small	Slightly elevated IgG level
8	4.1 (83%)	0.3 (6%)	70	Lymphocytic, abundant cytoplasm and granules	Moderate diffuse increase (polyclonal)
9	13.2 (63%)	7.9 (37%)	46	Lymphocytic, small	Normal

abundant cytoplasm, three of them with azurophilic granules and the other two without them (Table 2).

Bone marrow biopsy revealed an interstitial pattern in 6 cases (Nos. 1, 2, 3, 4, 5, 6 and 9), a mixed – nodular-interstitial – in one case (No. 4) and diffuse in another (No. 7) case.

All cases exhibited localized acid phosphatase activity sensitive to tartaric acid in a high percentage of the lymphocytes (70–90%). B-glucuronidase showed a similar positivity, while PAS was generally negative with a weak granular diffuse pattern in <10%.

Surface markers. The immunological phenotypes are shown in Table 3. In four cases (Nos. 2, 3, 7 and 8) there was a predominance of cells with the cytotoxic/suppressor phenotype (OKT8+), in two (Nos. 1, 9) a predominance of helper cells (OKT4+) and in three cases (Nos. 4, 5 and 7) there was a heterogeneous proliferation of helper and suppressor cells (mixed cases). The McAb OKT11 and OKT3 were positive in all the patients. In contrast, the reactivity of other pan-T McAbs (CD5, CD7) was lower: the CD5 (Cris-1/Leu-1) was positive in four cases (Nos. 1, 3, 6 and 9) and the CD7 (WT1/3A1) in two (Nos. 2 and 6).

All cases tested were consistently negative for TdT, OKT9, OKT10 and OKT6 except case No. 6 studied in the progressive phase of the disease who displayed 30% of cells reactive with the McAb OKT6. B-cell markers – SIg, B1, mouse rosettes – as well as OKIa-1 and OKM1 were negative in all patients.

Table 3

Membrane markers in T-CLL patients

Case	E. Ro-settes	OKT11	OKT3	OKT4	OKT8	Cris-I/Leu 1	WT1/3A1	OKTIal/ /GRB1	% positive cells		
									SIg	MRBC-rosettes	B-I
1	41	98	90	76	16	62/	1/	18/14	0	2	0
2	40	76	92	38	75	nd	94/	10/14	8	4	14
3	39	50	86	32	62	85/	5/	14/16	0	2	20
4	40	52	64	18	30	5/	0/	12/10	12	1	6
5	40	71	78	40	18	12/	10/	16/10	2	1	10
6	80	80	53	14	36	/55	/84	nd	6	—	—
7	75	77	90	5	83	/13	/21	8/	8	—	8
8	71	nd	53	3	65	/10	/16	3/	6	—	—
9	85	30	90	85	7	/80	/30	/12	4	6	4

Clinical course of the disease and treatment. The patients were followed up from 1 to 114 months and only one patient died (Case No. 6). Six patients showed a stable course without therapy; however, spontaneous regression was never observed. Of the other three patients, two underwent splenectomy (Case Nos. 4 and 6) and the other (Case No. 7) received chemotherapy. In Case No. 4, the spleen was removed because of thrombopenia and the patient has remained stable with normal platelets. Patient No. 6, after 10 months with stable disease, displayed progressive cytopenia; splenectomy was performed without response and the patient died one month later of pneumococcal sepsis. Post-mortem examination revealed generalized lymphoid infiltration. Case No. 7 – owing to progression after six month with stable disease – received treatment with chlorambucil and prednisone, which led to partial remission.

Discussion

The present paper describes the clinical and laboratory findings as well as the immunological characteristics of 9 patients with T-CL. The immunological phenotype of our patients was heterogeneous: four cases exhibited a cytotoxic/suppressor phenotype; two a helper phenotype and in the remaining three cases a mixed proliferation of OKT4+ and OKT8+ lymphocytes was observed. This phenotypic heterogeneity is in accordance with other series [2, 23, 24, 25] though it differs from the series reported by Newland et al. [4] who described an almost constant cytotoxic/suppressor phenotype. The immunological heterogeneity of our patients could be due to the inclusion of other T-lymphoproliferative disorders different from T-CL, such as T-PLL, Sezary syndrome and ATLL, but this was specifically excluded by clinical and morphological features. The reactivity of the McAbs CD5 and CD7 was variable in the OKT8+ and mixed (OKT4+, OKT8+)

patients, in contrast to the OKT4+ cases who displayed uniform reactivity for these markers (CD5+, CD7−). Pandolfi et al. [2], however, found a positive reaction for CD7 in two out of four T-CLL of helper phenotype.

The clinical features of these patients differed from classical B-CLL in their lower age at presentation, the predominance of females and the absence of enlarged lymph nodes accompanying a severe degree of splenomegaly. As in the other series [2, 4, 26] neutropenia was a frequent feature; a possible role of suppressor T-lymphocytes in its genesis has been suggested based on: 1) the inhibitory effect of cytotoxic/suppressor lymphocytes on the CFU-GM cultures [26]; and 2) the increase in T-suppressor cells in patients with BM aplasia [27]. It should be noted that of the two patients with helper phenotype one had high neutrophil counts and the other was in the lower normal limit.

Bone marrow infiltration was generally moderate and like B-CLL [27] the interstitial and mixed patterns were usually associated with a stable clinical picture. Lymphocyte morphology was variable and, as in the other series, no correlation was detected with the immunological phenotype [22, 29]. Accordingly, the presence of lymphocytes with abundant cytoplasm and azurophilic granules was neither constant nor exclusive of the OKT8+ cases [30].

The existence of clinical and biological differences in T-CL patients due to the immunological phenotype is difficult to establish because of the rarity of patients with the helper phenotype. Nevertheless, the two OKT4+ patients displayed distinct features: stage 0 and a lower incidence of neutropenia. Further series, however, are needed to confirm such a correlation. At present it is not known whether a specific membrane phenotype could differentiate patients with aggressive disease from the rest. One of the two cases with aggressive evolution showed an aberrant phenotype – OKT3+, OKT6+ (Case No. 6). This could suggest a possible relationship between these phenotypic anomalies and the clinical course of the disease.

In contrast to the B-lymphoproliferative syndromes, where the easy determination of the light chain isotype (kappa/lambda relationship) permits the assessment of the reactive or malignant nature of the proliferation, in T-lymphoproliferations the tests which allow one to establish the monoclonality of the process – chromosomal studies, T-cell receptor gene rearrangement – are not readily available [31, 32]. In T-CL the uniformity of cell morphology together with tissue infiltration and the absence of spontaneuos regressions are suggestive of, but do not ensure, a malignant process. Some of the characteristics of the immunological phenotype could provide additional evidence to demonstrate the clonal nature of the process: 1) lymphocytes of T-CL are generally CD5±, CD7−, in contrast to the normal lymphocytes, both helper and cytotoxic/suppressor, which are CD5+, CD7+ [17, 18]; 2) T-CL patients usually display a well defined phenotype, either OKT8+ or, more rarely, OKT4+, suggesting that one deals with a single cell clone. The cases of mixed proliferations (OKT4+, OKT8+), in contrast, would support the notion of a reactive condition. These cases, however, could either be explained by a dilution of the neoplastic cells among normal residual

cells or by the existence of a more undifferentiated target cell with the ability to differentiate into either helper or suppressor lymphocytes. The existence of T-CLL with coexpression of the same cells of both OKT4+ and OKT8+ markers [33, 34] and the presence of cells with an immature phenotype (OKT6+) as in case No. 6, would support the latter hypothesis.

Taking all these results together, it could be assumed that chronic T-cell lymphocytosis is a heterogeneous disorder which includes processes of stable clinical course [7, 8, 35] together with others of aggressive nature [5, 6]. In view of such findings, a description of the clinical, biological and immunological characteristics of new cases of T-CL is needed to demarcate the different clinical pictures.

Acknowledgement

We are grateful to Dra. Mª T. Gallart for the gift of the Cris-1 McAb and to Dr. W. Tax for providing the WT1 McAb.

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Haematologia 20 (1), pp. 41–47 (1987)

Spinal Fluid Procoagulant Activity in Leukaemic Patients Treated with Intrathecal Methotrexate

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(Received 17 October, 1985; accepted 20 February, 1986)

A procoagulant activity was found in cerebrospinal fluid (CSF) of patients with myelo- or lymphoproliferative diseases on intrathecal therapy with methotrexate, independently of leukaemic CNS involvement. This activity did not correlate with the cell count in CSF and disappeared on storage at −40 °C or after filtration with 0.22 nm filters. Dosage of coagulation factors revealed a strong increase in Factor V activity (F. V : C), an increase in Factor VIII procoagulant activity (F. VIII : C) without a correspondent increase in Factor VIII related antigen (F. VIII R : Ag), and an inconstant increase in Factor IX activity (F. IX : C). These activities all disappeared after filtration with 0.22 μm filters but not with 1.2 μm filters. It is concluded that complexes formed by membrane phospholipids and Factor V were responsible for the procoagulant activity lost after storage. The F. VIII and F. IX.-like procoagulant activity was not lost after storage; it was considered unspecific and attributed to thromboplastin-like substances.

Keywords: cerebrospinal fluid, Factor V, intrathecal methotrexate, myelo-lymphoproliferative diseases.

Introduction

Investigations concerning the origin of the procoagulant activity found in cerebrospinal fluid (CSF) of patients with neurological diseases were inconclusive [1–8]. In early studies the procoagulant activity was attributed to the presence of a labile factor, probably Factor V [5] or Factor VIII [4]; more recently it was ascribed to a phospholipid derived from the damaged CNS [7] or a cell-associated tissue factor [8] in patients with acute leukaemia who had undergone intrathecal methotrexate prophylaxis. The presence of a procoagulant effect in CSF seems, however, to be an important finding possibly reflecting a CNS damage [7, 8], as in normal controls the CSF shows a slight anticoagulant activity [1–3]. The aim of the present study was to investigate the characteristics of CSF procoagulant activity in subjects affected by myelo-lymphoproliferative diseases.

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Akadémiai Kiadó, Budapest

Materials and Methods

Seventy-three CSF samples were collected by lumbar puncture in 32 subjects: 11 subjects (6 males and 5 females, aged 35 – 72) suffering from sciatalgia without signs of medullary compression, and 21 patients (10 males and 11 females, aged 20 – 65) affected by myelo-lymphoproliferative diseases (10 ALL, 7 ANLL, 4 lymphoblastic lymphomas).

CSF samples belonging to the patients with myelo-lymphoproliferative diseases were grouped as follows: 8 CSF samples were collected from 8 patients without CNS involvement before intrathecal prophylaxis (group 1); 30 CSF samples were repeatedly obtained from 9 patients without CNS involvement after 3 – 8 intrathecal injections of methotrexate (12 mg) (group 2); 24 CSF samples were collected from 4 patients with CNS involvement after 3 – 10 intrathecal injections of methotrexate (12 mg) (group 3).

The interval between lumbar puncture and laboratory investigations never exceeded 2 hours. CSF examination routinely included: cell count, cytocentrifugation, quantitation of total proteins and glucose. Contamination with red blood cells was less than 200 cells/mmc in all samples tested. The assay for measurement of procoagulant activity was a modification of the recalcification time test (R.T.) according to Komp et al. [8]. A platelet poor plasma pool (PPPP) was obtained from venous blood collected in 0.129 M sodium citrate (ratio 1 : 10) from 20 healthy donors and centrifuged at 3000 *g* for 15 min. The plasma pool was then stored in small aliquots in plastic tubes at – 70 °C. 0.1 ml of PPPP and 0.1 ml of physiological sodium chloride solution were incubated separately at 37 °C for 2 min, mixed and incubated at 37 °C for additional 3 min; 0.1 ml of 0.025 M $CaCl_2$ was then added. The end point was obtained by tilting the tubes in a regular manner and observing the formation of first fibrin threads. Time was expressed in seconds. In the test, 0.1 ml of CSF replaced sodium chloride. The presence of procoagulant activity or anticoagulant was established when the R.T. of the mixture plasma plus CSF was out of the double standard deviation of 15 consecutive determinations of R.T. of the mixture plasma plus saline. All CSF samples were examined before and after filtration with 0.22 μm filter (Millipore, Bedford, MA, USA). Five CSF samples were tested before and after filtration with different filters (0.22 μm, 0.45 μm, 1.2 μm) on PPPP and on PPPP filtered with 0.22 μm filter.

Plasmas deficient in Factor II, V, VII, VIII, IX and X were purchased from General Diagnostics (Morris Plains, NJ, USA). Factor V level in CSF samples was measured measuring the prothrombin time (P.T.) using Simplastin-plus (General Diagnostics) on a mixture of CSF and Factor V deficient plasma according to Babson et al. [9]. Factor VIII : C and Factor IX were measured by measuring aPTT in a mixture of CSF plus F.VIII or F.IX deficient plasma respectively, according to Hardisty et al. [10]. CSF samples were tested undiluted and as 1 : 2, 1 : 4, 1 : 8 dilutions with imidazole 0.05 M buffer (pH 7.8), whereas reference PPPP was tested in 1 : 10, 1 : 20, 1 : 40, 1 : 80, 1 : 160, 1 : 320 dilutions defined as 100%, 50%, 25%, 12.5%, 6.2% and 3.1%, respectively. CSF Factor V, VIII and IX levels

were calculated by interpolation with the reference plasma line on semilogarithmic paper.

Factor VIII antigen (F. VIII : RAg) was measured by Laurell's method [11] using specific Behringwerke antiserum, in 4–8 times concentrated ultrafiltered CSF (membranes NMWL 10,000 D Millipore).

Statistical methods

Results were analysed by using Students' *t* test for unpaired data and Pearson's correlation coefficient (r). Student's *t* test for paired data was used when appropriate.

Results

Fresh CSF of normal subjects or untreated patients did not significantly influence the R.T. of PPPP (Table 1). The R.T. was significantly shortened by fresh CSF of patients on CNS prophylaxis (group 2; $p < 0.001$) or on CNS

Table 1

The effect of fresh CSF of healthy subjects and differently treated patients on the recalcification time of PPPP

	Sample number	Recalcification time s (mean ± SD)	
		Fresh whole samples	Fresh filtered samples
Saline	15	136 ± 13	138 ± 12
Healthy subjects	11	122 ± 11	164 ± 16
Untreated patients	8	131 ± 17	170 ± 23
Treated patients without CNS involvement	30	83 ± 18	135 ± 35
Treated patients with CNS involvement	24	91 ± 32	138 ± 39

Table 2

Factor V, VIII and IX activity of fresh CSF of healthy subjects and patients

	Sample number	Fresh whole samples			Fresh filtered samples		
		F.V : C %	F.VIII : C %	F.IX : C %	F.V:C %	F.VIII:C %	F.IX:C %
Healthy subjects	10	2.4 ± 1.6	<2	<2	<2	<2	<2
Untreated patients	5	2.2 ± 1.2	<2	<2	<2	<2	<2
Treated patients without CNS involvement	12	10.1 ± 5.3	8.3 ± 6.2	6.7 ± 6.2	<2	<2	<2
Treated patients with CNS involvement	16	12.0 ± 8.3	5.9 ± 4.2	6.5 ± 5.8	<2	<2	<2

Table 3

The effect of storage on the

	Sample number	Fresh whole samples			
		R.T. s	F.V : C %	F.VIII : C %	F.VIII : RAg %
Untreated subjects	7	119 ± 6	2 ± 1	<2	<2
Treated subjects	10	81 ± 13	11 ± 7	7 ± 5	<2

therapy (group 3; $p < 0.001$) (Table 1). Thus, a significant difference was found between untreated and treated patients with ($p < 0.01$) or without ($p < 0.001$) CNS involvement (Table 1).

F.VIII : C and F.IX were below 2% in most of CSF samples of normal and untreated subjects, F.V. ranged from 2% to 5% (Table 2). A significant increase ($p < 0.01$) of a F.V. and F.VIII : C was found in the fresh CSF of patients during intrathecal treatment (Table 2) independently of leukaemic CNS involvement. The increase in F.IX was not significant. No significant correlation was found between any coagulant property and CSF proteins, glucose or cell count. A positive correlation was found between F.V and F.VIII : C levels ($r = 0.53$, $p < 0.001$). A strict correlation was found between F.V level and corrected R.T. ($r = 0.75$, $p < 0.001$). F.VIII : RAg was below 2% in all samples (Table 3).

To exclude that the procoagulant activity was due to thrombin or tissue thromboplastin-like substances, the active CSF was added to plasmas deficient in Factor II, V, VII, VIII, IX, X and plasma recalcification time was estimated. Table 4 presents the average of four active CSF. Shortening was seen in F.V and F.VII deficient plasmas but not in those deficient in F.II and F.X.

Table 4

The effect of fresh and stored CSF on the recalcification time of different deficient plasmas

Substrate plasma	Plasma recalcification time (s)		
	Saline	CSF (fresh)	CSF (stored at −40 °C)
Normal	138	75	120
II Deficient	360	360	360
V Deficient	360	60	120
VII Deficient	165	70	110
X Deficient	360	250	300
VIII Deficient	360	140	140
IX Deficient	360	160	160

factor V and VIII level of CSF

Samples stored at −40 °C			Filtered stored samples		
R.T. s	F.V : C %	F.VIII : C %	R.T. s	F.V : C %	F.VIII : C %
143 ± 7	<2	<2	188 ± 14	<2	<2
114 ± 18	<2	7 ± 4	184 ± 25	<2	<2

Effect of Filtration

Filtration with 0.22 μm filters induced a strong reduction of procoagulant activity (Table 1). Filtered CSF of normal and untreated subjects constantly induced a significant prolongation of R.T. of PPPP ($p < 0.02$ and $p < 0.01$ respectively). Filtered fresh CSF of treated patients had a variable effect on PPPP R.T: 36 out of 54 samples did not significantly influence the PPPP R.T., some [5] induced a shortening, others [13] a prolongation. Factor V, VIII : C and IX were no longer detectable in CSF samples after filtration (Table 2).

Five CSF samples showing high procoagulant activity and belonging to patients of groups 2 and 3 did not lose their procoagulant properties after filtration with 1.2 μm filters, but not with 0.45 μm filters. In these samples, however, the procoagulant activity, expressed as a shortening of R.T. in PPPP, was lost after incubation at 37 °C for 4 hours.

Effect of Storage

CSF procoagulant activity in PPPP R.T. was strongly reduced by storage at −40 °C (Table 3).

Filtration further reduced the procoagulant activity and revealed a significant anticoagulant activity in CSF of untreated or treated subjects (Table 3).

Factor V was no longer detectable in CSF samples at −40 °C or after 4 hours incubation at 37 °C. Factor VIII : C level was not affected by storage at −40 °C (Table 3).

Discussion

A procoagulant activity has been demonstrated in patients with CNS damage for many years; it is easily revealed by shortening of the recalcification time of normal plasma after addition of the test CSF [1–3]. It has been found in inflammatory as well as in neoplastic meningitis [7, 8] and also in leukaemic patients

without CNS involvement after intrathecal methotrexate prophylaxis [7, 8]; its origin and significance remains controversial. In leukaemic patients treated with intrathecal methotrexate Graeber and Stuart [7] attributed most of the procoagulant activity present in CSF to an active, not cell-associated substance containing lipids probably derived from damaged brain tissue. More recently Komp et al. [8] postulated that CSF procoagulant activity is cell-associated, behaves like a tissue factor and cannot be demonstrated in a platelet poor system. The levels of single clotting factors have rarely been investigated in CSF: an old, but accurate study of Niewiarowski et al. [5] showed no cefalin-like activity in normal or pathological CSF, but a high activity of Factor V and a variable level of Factor VIII : C. An increase of Factor VIII : RAg was found in bacterial meningitis but not in lymphoproliferative diseases with or without CNS involvement [12].

Our study has confirmed that the increase in CSF procoagulant activity in patients with leukaemia or lymphoma was strictly correlated to the intrathecal therapy and not to the cell number. It was abolished by filtration with 0.22 μm filters and strongly reduced by storage at −40 °C as well as by storage at 37 °C. Furthermore, an anticoagulant activity, previously reported by many authors in normal CSF [1 – 3] was evidenced by filtration, in accordance to Komp et al. [8].

Factor V-like activity was found in fresh, but not in stored CSF: a strict correlation was found between the F.V level and the corrected R.T. Factor VIII : C was increased too, but its level was not affected by storage and did not correlate to corrected R.T.

Factor V and Factor VIII-like activities were both removed by filtration with 0.22 μm and 0.45 μm filters, but not by filtration with 1.2 μm filters. Factor VIII : RAg was always below 2%.

These data permitted to draw some conclusions on CSF procoagulant activity developing during intrathecal methotrexate treatment: it is bound to a corpusculate material smaller than 1.2 μm, most of it is thermolabile, it possesses Factor V-like activity and is able to reduce the R.T. of plasmas deficient in Factor V, VII, VIII and IX, but not of those deficient in Factor II and F.X. These properties are extremely different from those of the procoagulant tissue factor present in monocytes, which, being a tissue thromboplastin-like substance, does not accelerate the plasma recalcification time of F.VII deficient plasma and is stable at temperatures below 56 °C [13].

Our study stresses the importance of Factor V: it was the factor more and more frequently increased while its concentration paralleled the CSF procoagulant activity. Graeber and Stuart showed that CSF material, with procoagulant activity, contains lipids and is inactivated by heating [7]. It is noteworthy that Factor V and lipids tend to form complexes and the phospholipid portion of the membrane provides a locus for the binding of protein [14] and the procoagulant activity of these complexes is lost after storage [14]. In our study we tested F.V with a one-stage assay: consequently the net Factor V activity reflects the relative activity of a given phospholipid-Factor V complex and the amount of that complex [15]. Thus, in CSF of treated patients phospholipid membranes are probably present;

they seem to originate from damaged CNS which binds the Factor V normally present in CSF, increasing the F.V coagulant activity.

After heating [7] or storage, the CSF loses partially its procoagulant effect because the protein (F.V) of the lipoprotein complex decays [15]. The presence of a Factor VIII : C-like activity in the absence of Factor VIII : RAg probably reflects the action of thromboplastin-like material which may simulate F.VIII : C when a one-stage assay is used [16]. The frequent, even if not significant, increase of Factor IX-like activity might further strengthen this hypothesis.

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Haematologia 20 (1), pp. 49—50 (1987)

Immunological Phenotype of the Microgranular Variant of Acute Promyelocytic Leukaemia

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(Received 5 November, 1985; accepted 13 February, 1986)

Acute promyelocytic leukaemia (APL) is a well defined type of acute myeloid leukaemia (AML) frequently associated with disseminated intravascular coagulation (DIC) and characterized by coarse dark cytoplasmic granules, folded nuclei and a 15, 17 chromosomal translocation [1]. Recently, a morphologic variant in which the granules are smaller — microgranular — and frequently fewer — hypogranular — has been recognized [2]. The finding of this microgranular variant raises two questions: 1) Whether the abnormal morphological appearance of the cells is accompanied by alterations in the membrane phenotype which will permit its differentiations from classical hypergranular APL and 2) the possible confusion of the micrografnular caseş of APL with the monocytic forms of AML (M4 & M5) owing to the reniform nuclei associated with an absence or paucity of granules.

PM, a 35 year old woman presented with clinical and laboratory evidence of DIC, and an otherwise normal physical examination. A blood count revealed: normal haemoglobin (14.8/dl), marked thrombocytopenia (11×10^9/l) and 6.7×10^9 WBC/l with 50% of blast cells. The bone marrow showed 90% of blast cells with bilobed and reniform nuclei and a fine or absent granulation, giving the cells a monocytoid appearance. All blast cells displayed strong peroxidase activity and stained for non-specific esterase though the reaction was fluoride resistant. The immunological phenotype of peripheral blood cells showed the absence of lymphoid — TdT, J5 (CD10), B1 —, erythroid — Glycophorin — and megakaryocytic — J15(GPII/IIIa), FMC25(GPI) — markers. 40% of the blast cells were My7+ (CDw13) but were negative for other specific monocytic — FMC17 & FMC33 (CDw14) —; granulocytic — FMC10 & VIMD6 (CDw15) —; and granulo-monocytic (OKM1, VIM2) antibodies. In addition, the cells were completely negative for OKIa while 95% of them expressed the FMC8 (CD9) antigen. This phenotype (CDw13+, CDw14$^-$, CDw15$^-$, Ia$^-$, CD9+) has also been found in 14 hypergranular APL cases and is clearly different from that of other 45 monocytic leukaemias (M4 and M5) tested simultaneously, which together with Ia, expressed other myelomonocytic marker antigens (CDw13+, CDw14+, CDw15±, Ia+, CD9+) [3].

Our results shows that the immunological phenotype of microgranular APL

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together with cytochemical findings may help in differential diagnosis with monocytic leukaemias thus confirming the recent report of Schnitzer et al. [4] concerning a case of the microgranular variant diagnosed by flow cytometry. Furthermore these cells had the same membrane phenotype as hypergranular promyelocytes showing that the abnormal morphological appearance of micrin granular APL does not imply an aberrant membrane phenotype. Recognition of such variants of AML may help towards a better understanding of cell biology and to better define the prognosis and therapeutic strategy for these patients.

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Haematologia 20 (1), pp. 51–55 (1987)

Erythrocyte PK Deficiency: Biochemical Characterization of a Patient with Haemolytic Anemia

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(Received 6 November, 1985; accepted 26 February, 1986)

Erythrocyte pyruvate kinase deficiency was detected in a Cuban girl with congenital nonspherocytic haemolytic anemia. Kinetic and immunochemical studies showed that the case was different from those hitherto reported. The variant(s) was tentatively designated PK "Alquizar", following the recommendations of the International Committee for Standardization in Hematology.

Keywords: double heterozygote, haemolytic anemia, PK deficiency.

Introduction

Hereditary erythrocyte pyruvate kinase (PK) deficiency is the most common, although not frequent, glycolytic enzyme defect associated with haemolytic anemia [1]. Since 1961 more than 300 cases have been reported [2], and these studies indicated that in most cases PK deficiency was due to structural mutations which produce a great variety of clinical and haematological pictures. The allosteric model distinguishes deficient PKs with increased PEP affinity and no activation by fructose 1–6 diphosphate (FDP) from the deficient enzymes showing increased affinity for PEP and sensitivity to FDP activation and ATP inhibition [3]. A complete characterization of PK variants requires, however, other studies such as electrophoresis and immunochemical tests [4]. Unfortunately, variations in the techniques and inadequate characterization make it difficult to compare many of the PK variants reported. The publication of Recommended Methods for the Characterization of Red Cells PK variants by the International Committee for Standardization in Haematology [5] offers a uniform methodology allowing a valid comparison among PK variants. We used these techniques to characterize a case of PK deficiency observed in Cuba.

Materials and Methods

Venous blood collected in heparinized test tubes was processed immediately after sampling. Leukocytes and most of the platelets were eliminated by filtration

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through cellulose column. PK activity was determined according to the method of Beutler et al. [6]. Activity was expressed as IU/gHb at 30 °C.

Partial purification of the enzyme, PEP kinetics, $K_{0.5(s)}$ ADP thermostability test at 53 °C on crude haemolysates and in partially purified preparations, adenosine triphosphate (ATP) inhibition test, fructose 1–6 diphosphate (FDP) activation and pH curves were performed by the Recommended Methods for the Characterization of Red Cell PK Variants [5]. $K_{0.5}$ PEP and Hill coefficient (n) were calculated from a Hill plot and $K_{0.5(S)}$ ADP from a Lineweaver-Burk plot.

Urea stability test was performed by the method of Miwa et al. [7]. Thin layer polyacrylamide gel electrophoresis was carried out on partially purified samples with a 10 mM Tris-HCl, 5 mM $MgSO_4$, 0.5 mM FDP, 50 mM 2-mercaptoethanol buffer pH 8.3 at 14 V/cm for 4 hours [5].

Neutralization test by anti-human red-cell PK serum was performed by the method of Miwa et al. [7]. Anti human red cell PK serum was obtained from rabbits after immunization with purified human red cell PK and Freund's complete adjuvant. Antiserum was diluted with 0.5% BSA to 5, 10, 20, 40, 160 and 320 times the original volume and mixed with the same volume of the haemolysate. PK activity was assayed and the activity of the haemolysate mixed with the same volume of 0.5% BSA was taken as 100%.

Case Report

The patient R.L.V., a 17 year-old white girl had been icteric and anemic since birth. She had experienced neonatal hyperbilirubinemia and was admitted several times to hospital because of a moderate chronic anemia (Hb 7 to 10 g/dl). The liver and the spleen were palpable 3 and 1 cm below the respective costal margins. Bone marrow showed red cell hyperplasia. Reticulocyte count was 8.2%, Coombs' test was negative. Serum bilirubin was 3.5 mg/100 ml of which 2.3 mg was reacting indirectly. Serum iron was 140 μg/100 ml. Osmotic fragility was normal. Red cell PK activity was 2.05 IU/g Hb (mean $\pm$ SD of 20 normal controls, 5.5 $\pm$ 1.5). Glucose 6 phosphate dehydrogenase (G6PD) was of the B type with normal activity. Abnormal Hb was not detected. The parents were asymptomatic and not consanguineous. They showed intermediate levels of red cell PK activity.

Results

The biochemical data of the patient are summarized in Table 1.

In the semipurified preparations the activation by FDP and the inhibition test for ATP were normal. The affinity for the substrate PEP was also normal, but the curve of enzymatic activity versus PEP concentration did not exhibit a simple sigmoidal shape (Fig. 1). The $K_{0.5(S)}$ (ADP) was increased to 50%. The thermostability test at 53 °C gave a residual activity of 66% in haemolysate and 60% in partially purified samples compared to 90% and 92% respectively in controls (Fig.

Table 1

Biochemical characterization of PK "Alquizar"

	R.L.V.	Normal controls (n = 8)
PK activity (% of normal)	38	100 ± 30
$K_{0.5(S)}$ (PEP)	0.75	0.86 ± 0.16
Hill coefficient (n)	1.13	1.25 ± 0.40
$K_{0.5(S)}$ (ADP) (mM)	0.48	0.19 ± 0.058
ATP inhibition (% of 1 mM ATP)	81	76.5 ± 5.0
F-1,6 DP activation (μm F-1,6 DP for 50% activation)	0.50	0.70 ± 0.20
Thermostability (% of activity remaining at 60 minutes):		
— Haemolysate (53 °C)	72–77	90 ± 8.0
— Partially purified sample (53 °C)	60	92
Optimum pH	6.5	6.5 – 7
Electrophoretic mobility (% of normal)	100	100

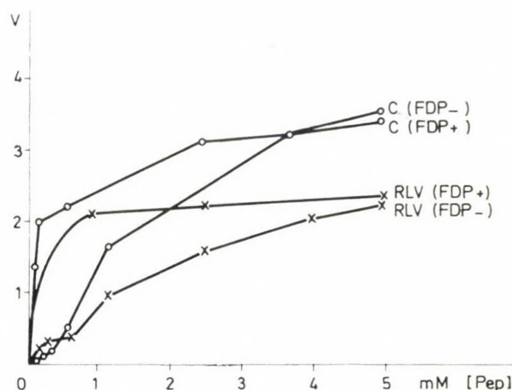

Fig. 1. $K_{0.5(S)}$(PEP)

2). The lability of the enzyme was supported also by the urea stability test which gave a residual activity of 39% compared to the normal 90%. The pH curve was normal with a peak of activity at pH 6.5. Red cell PK neutralization by antiserum was intermediate (Fig. 3).

Discussion

The biochemical findings showed that the residual activity of the enzyme was reduced while the kinetic properties were affected only slightly. Only the $K_{0.5(S)}$

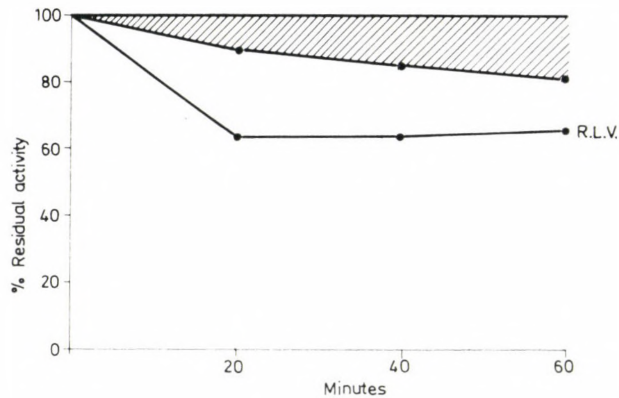

Fig. 2. PK thermostability at 53 °C in haemolysate

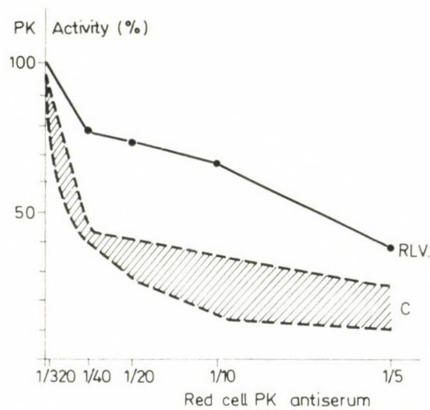

Fig. 3. Neutralization test by anti-human red-cell PK serum

ADP was increased, but the decrease of the affinity for ADP apparently did not determine an increase of ATP fixation to the ADP substrate site.

It has been observed that the haematological picture of PK deficiency correlates with residual activity, PEP affinity, decreased sensitivity to ATP inhibition and thermal instability. This case can therefore be placed in the group of PK deficiencies in which the basic defect is a reduced residual activity due to the presence of at least one structural variant, as indicated by the neutralization test with anti-human red cell PK. Moreover, a partial loss of allosteric behavior occurred, as indicated by the absence of the simple sigmoidal shape in the curve of enzymatic activity versus PEP concentration. Since two different components were present, kinetic data are of limited value. A comparison with previously reported cases of either double heterozygotes or homozygotes showed, however, some differences

and indicated that we may be faced with a new variant. For example: in most PK variant $K_{0.5(S)}$ ADP is normal [8–11]. PK Inglewood and PK Sanger [9] have increased $K_{0.5(S)}$ ADP, but immature bands appear in its electrophoretic patterns and PK Sanger has a normal stability. The Colmar variant [4] with a decreased affinity for ADP has an abnormal moving component. In the Amish PK [11] the $K_{0.5(S)}$ PEP and the Hill coefficient are also decreased.

The International Committee for Standardization in Haematology [5] recommended that the variant should be named for practical purpose even when it is almost certain that the study was carried out on a mixture of two PK deficient enzymes. We designated this variant(s) "PK Alquizar".

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Haematologia 20 (1), pp. 57–62 (1987)

Splenic Function in Haemophilia

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(Received 20 September, 1985; accepted 3 March, 1986)

Studies of splenic function were carried out on 17 haemophilic patients over 9 years of age, and 20 control patients. The clearance of autologous heat-damaged ^{99m}Tc-labelled erythrocytes from circulation and into the spleen was measured: the spleen area by scintillation scanning, and the enumeration of pitted erythrocytes by direct interference microscopy. Splenic enlargement was observed in 10 patients (59 %). On the basis of the clearance half-time, splenic function was normal in 3/13 (23 %) and hyperactive in 9/13 (69 %) patients. One heavily transfused patient had a hypoactive spleen with long clearance half-time, slow splenic uptake of radioactivity and high pit counts. These results demonstrate that the spleen of haemophiliacs is usually enlarged and functionally abnormal.

Keywords: spleen function, spleen scanning, erythrocyte pits, haemophilia

Introduction

Splenomegaly has been reported in a variable proportion of patients with haemophilia [1 – 5], usually on the basis of physical examination. The spleen size was evaluated by liver-spleen imaging with ^{99m}Tc sulfur colloid in a single study [5]. Thus far, however, no data have been reported on splenic function in haemophilia.

This paper reports the results of a study of splenic function in 17 patients with haemophilia of variable severity which are compared with 20 controls.

Patients and Methods

The investigations were performed on 17 patients with haemophilia (16 with haemophilia A and one with haemophilia B) of variable severity. The clinical data are given in Table 1. The patients were aged 9 – 47 years (median 19 years). For comparative purposes, 20 controls were also studied, including 10 subjects (6 males, 4 females), aged 13 – 68 years (median 23 years), with haemoglobin concentrations of 10.5 – 16.4 g/dl (median 13.1 g/dl), with apparently normal spleens, 6 who had undergone splenectomy (3 males, 3 females), aged 18 – 60 years (median 30.5 years), with haemoglobin 7.0 – 14.1 g/dl (median 12.0 g/dl) and four individuals with splenomegaly (one with hereditary spherocytosis and three with β-thalassemia), aged 8 – 28 years with haemoglobin 6.2 – 13.2 g/dl. Diagnosis was established in each case by clinical and laboratory studies. Patients with haemophilia were under

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Table 1

Clinical and laboratory data and the results of spleen function measurements in 17 patients with haemophilia

Patient	Age (years)	Replacement therapy*				Clearance T 1/2 (min)	Pits (%)	Q_{15}	Spleen area (cm^2)
		Procoagulant factor/kg/yr (Unit)	Cryo	Lyo	AHG				
01	11	2287	+	+	+	50.1	8.2	1.1	84
02	15	1347	+	+	—	9.6	0.5	3.1	72
03	9	1246	+	+	—	11.9	0.5	1.9	84
04	31	1219	+	+	+	2.0	0.3	4.2	165
05	22	1108	—	+	—	38.7	1.7	1.9	102
06	14	481	+	+	—	9.1	0.1	2.3	94
07	22	470	—	+	—	5.6	0.1	3.6	92
08	14	347	—	+	—	7.3	1.5	3.8	165
09	20	328	+	+	—	8.4	0.5	3.5	136
10	47	171	+	+	—	11.0	0.0	3.4	101
11	15	132	—	+	—	26.4	0.1	2.5	132
12	14	76	—	+	—	10.7	0.3	2.4	79
13**	9	52	—	—	—	30.7	0.1	1.9	67
14	23	2407***	+	+	—	—	0.2	2.8	167
15	19	465	+	+	—	—	0.6	1.9	87
16	26	382	+	+	+	—	0.2	3.3	87
17	21	339	+	+	+	—	1.2	2.4	113
Normal controls (range)						18.6—41.3	0.0—3.2	1.1—2.8	24—91
Splenomegaly (range)						4.8—15.7	0.8—3.7	1.7—4.8	92—206
Splenectomized (range)						67.8—180.3	28.8—63.0	0.8—1.0	—

* Dosage based on the records of the last two-year follow up; cryo = cryoprecipitate (single-donor), lyo = lyophilized pooled cryoprecipitate, AHG = lyophilized AHG concentrate.

** Haemophilia B: the patient was treated only with plasma.

*** Based on 87 060 units in a 7-month period.

regular follow up in a Haemophilia Centre. They were treated with cryoprecipitate (single donor), lyophilized cryoprecipitate (prepared from pooled plasma of 100 regional donors) or highly concentrated lyophilized AHG (Kryobulin[R], Immuno) when necessary. One patient (case 14) also employed self-therapy at home. On the basis of the previous two-year records patients with haemophilia A had received 3,180 to 96,340 (median 24,850) units/year, except for patient 14 who had received 87,060 units during a 7 month follow up period. The majority of patients had been treated in the past with whole blood or plasma transfusions. The single patient with haemophilia B (case 13) had received only small amounts fresh or fresh-frozen plasma. Total bilirubin, GOT and GPT were measured for 15 patients. Bilirubin was normal (below 0.9 mg/dl) for all cases. Slight elevations of one or both enzymes (less than twice the upper normal value) were observed in 4 patients (cases 1, 2, 4 and 7); patient 8 had GOT 100 IU/ml (normal up to 40 IU/ml) and GPT 87 IU/ml

(normal up to 36 IU/ml). Consent to carry out the splenic function study was obtained from all patients (and also from the parent of patients with less than sixteen years of age) after a full explanation of the procedure. No patient had clinical or laboratory manifestations of viral hepatitis, fever or any other sign of infection at the time of the study.

Spleen function was evaluated by the enumeration of erythrocytes with pits on direct interference microscopy [6], the clearance from circulation and accumulation into the spleen of heat-damaged autologous erythrocytes [7, 8], and the measurement of spleen size by scintillographic scanning.

One drop of freshly collected blood was added to 0.5 ml of phosphate-buffered saline, pH 7.4, containing 2% formaldehyde. Wet preparations were examined under direct-interference contrast microscopy (Zeiss microscope equipped with Nomarski optics) at ×1000 magnification. The percent of erythrocytes with one or more pits was determined by counting 500 cells, regardless of the size or number of pits per cell.

The techniques used to study the removal of autologous heat-damaged erythrocytes from circulation have been described previously [9]. Briefly, erythrocytes obtained from 10 ml of blood were heat-damaged by incubation at 50 ± 0.5 °C for 30 min, labelled with 1.5–2.0 mCi of ^{99m}Tc, resuspended in autologous plasma and re-injected. Radioactivity was measured in 2 ml samples of blood taken 3, 6, 10, 15, 20, 30 and 45 min after injection to calculate the clearance half-time (T 1/2 clearance). At the same time, the surface radioactivity over the spleen and the liver was measured simultaneously for 60 min by placing two collimated scintillation counters (Nucleopan-M, Siemens) over the organs. The Q_{15} coefficient was calculated as a measure of the uptake of radioactivity by the spleen. This coefficient represents the ratio of surface radioactivity over the spleen and the liver at 15 min divided by the same ratio obtained at the first min. Two hours after injection the area over the spleen was scanned with a rectilinear scintillation scanner (Scintimat-2, Siemens), in the left lateral position and the area was measured with a planimeter. The area of the lateral projection seems to provide a better correlation with the actual weight of the spleen than other projections [10].

Results

All measurements were performed in 13 patients with haemophilia, while in the four remaining patients (cases 14–17) only the percent of erythrocytes with pits and the spleen area were measured.

The results of spleen area measurements on lateral scans, clearance half-time (T 1/2) and Q_{15} are shown in Table 1. For normal controls the area projected by the spleen on lateral scans varied from 24 to 91 cm^2 (median 47 cm^2) while for controls with splenomegaly it varied from 92 to 206 cm^2. For the 17 patients with haemophilia the lateral projection of the spleen measured from 67 to 167 cm^2, and it was larger than 91 cm^2 for 10 patients (59%).

The clearance T 1/2 was 18.6 – 41.3 min (median 23.1 min) for normal controls, 4.8 – 15.7 min (median 6.9 min) for controls with splenomegaly and 67.8 – 180.3 (median 103.4 min) for the splenectomized individuals. Three patients with haemophilia had T 1/2 values of 26.4, 30.7 and 38.7, similar to the normal controls, while nine patients had rapid clearance, with T 1/2 varying from 2.0 to 11.9 min. For one patient (case 1) with a spleen area in the upper limit of the normal range the clearance was slower than for normals (T 1/2 50.1 min).

Normal controls had pit counts of 0.0 – 3.2% (median 1.0%) and splenectomized patients had counts of 28.8 – 63.0% (median 48.1%). In 16 haemophilia patients the percent of erythrocytes with pits varied from 0.0% to 1.7%, and was similar to the normal controls, while patient 1, who had a long T 1/2 clearance, had a pit count of 8.2%.

For the normal controls the Q_{15} values varied from 1.1 to 2.8 (median 1.9). Six patients with haemophilia (including the three patients with normal T 1/2) had Q_{15} values of 1.9 to 2.5, which were in the same range as the normals while the Q_{15} obtained for 6 other patients varied from 3.1 to 4.2, higher than the normal upper value. Finally, case 1 had the lowest Q_{15} value, 1.1, in agreement with a long T 1/2 and high pit count.

Thus, spleen enlargement by scintillographic scanning and spleen hyperfunction by rapid removal of heat-damaged erythrocytes were simultaneously demonstrated for 6 of 13 haemophiliacs. Of three patients with normal function tests, two had enlarged and one had normal spleen. Finally, an 11 year-old boy had signs of splenic hypofunction as shown both by a slow clearance of heat-damaged erythrocytes and high pit counts; the spleen area was at the upper limit of the normal range for adults.

Discussion

The data presented here show an enlargement of the spleen in 59% of the investigated haemophilia patients, although in most cases the enlargement is not detectable by physical examination. Splenomegaly has been reported in 2% to 40% of patients with haemophilia [1 – 5], usually on the basis of physical findings, and on the basis of liver-spleen imaging with ^{99m}Tc sulfur colloid in a single study [5]. Some studies have shown a tendency of the more heavily transfused haemophiliacs to have a higher frequency of splenomegaly [5] while in others there is no correlation between the presence of splenomegaly and the amount of factor VIII transfused [2]. An explanation for the higher proportion of our patients with splenomegaly than those reported in the literature would be the sensitivity of the scanning method employed as compared to palpation. These patients were selected consecutively as they attended the clinic (no patient refused to participate in the study). The study could not be performed in children under 8 years of age, because cooperation during the examination is essential. Thus it may be possible that there may be a different proportion of patients with spleen enlargement among younger haemophiliacs.

The majority of the patients (9/13) had a hyperfunctional spleen as shown by a short clearance half-time. Normal clearance was observed in 3 cases, including 2 patients with splenomegaly and the single patient with haemophilia B, who had been minimally transfused only with plasma and had a normal spleen. Finally, splenic hypofunction was detected in one patient, (case 1) who also showed slow clearance and high pit count. This patient was the most heavily transfused of this group on the basis of his body weight (2287 units/yr/kg), except for patient 14 whose transfusion requirements were evaluated on the basis of only 7 months of follow up.

Therefore the spleen of haemophiliacs is usually enlarged and functionally abnormal although it is rarely palpable. Most frequently the enlargement is accompanied by hyperactivity, but hypofunction may also be seen occasionally. The abnormalities were not related to the predominant usage of any blood product in the present study. It cannot be concluded from the data whether the abnormalities are related either with the cumulative procoagulant dosage, its yearly dosage or the total exposure time, since no infants were studied and only two patients with haemophilia A had normal spleens. Nevertheless, the patient with haemophilia B, who had been minimally transfused only with plasma, had the smallest spleen and functional tests within the normal range, while patient 1, who was the second most heavily transfused, had the most striking functional abnormalities, and patient 14, who had received the largest yearly dosage, had the largest spleen.

The splenic enlargement and hyperfunction seen in the present study could be the result of the treatment with repeated transfusions of plasma products. Evidence of liver abnormalities and exposure to hepatitis B and non-A, non-B viruses [1–3, 5, 11], alterations of T-lymphocyte subpopulations and other immunologic dysfunction [4, 12–16], and the development of the acquired immunodeficiency syndrome [17] are also considered to be the consequences of frequent transfusions. However, there are no sufficient data to speculate on the significance of the splenic abnormalities reported here.

Acknowledgements

This work was partly supported by Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil.

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Abstracts

Structural basis of human erythrocyte glucose transporter function in reconstituted system. Hydrogen exchange. E. K. Y. Jung, J. J. Chin and C. Y. Jung (Biophysics Laboratory, Veterans Administration Medical Center and Department of Biophysical Sciences, State University of New York at Buffalo, Buffalo, N. Y.). *J. Biol. Chem. 261*, 9155 (1986).

Hydrogen exchange kinetic behavior of human erythrocyte glucose transporter protein in vesicles was studied in the absence and in the presence of D-glucose or a well known inhibitor, cytochalasin B. This is to detect a proposed channel of water penetrating into the protein through which the sugar molecule passes and to monitor any conformational changes induced by the substrate or inhibitor. Analyses of the kinetic data revealed several classes of hydrogens which exchange with readily distinguishable rates. Of 660 hydrogens detected per transporter, approximately 30% exchanged with rates generally characterized as those of free amide hydrogens indicating they are interfaced to solvent water. Since the tranporter is known to be embedded deep in the hydrophobic area of the membrane with minimum exposure to the outside of the membrane lipid bilayer, a significant portion of these free amide hydrogens must be at the purported channel rather than outside of the membrane. D-Glucose and cytochalasin B affected the exchange kinetics of these presumably channel-associated free amide hydrogens rather differently. D-Glucose reduced the apparent rate constants, but not the total number. Cytochalasin B on the other hand reduced the total number to one-half without significantly changing the apparent rate constants. The remaining 70% of the labeled hydrogens exchanged with much slower rates which vary 10—10,000-fold, indicating that they are internally structured peptide amide and side chain hydrogens. Both D-glucose and cytochalasin B further reduced the rates of these hydrogens, indicating a global stabilization of the protein structure.

G. Gárdos

Permeability alterations and antihaemolysis induced by amphiphiles in human erythrocytes. B. Isomaa, H. Hägerstrand, G. Paatero and A. Ch. Engblom (Department of Biology, Åbo Akademi, Turku, Finland). *Biochim. Biophys. Acta 860*, 510 (1986).

In an attempt to define the parameters in amphiphilic molecules important for their interaction with the erythrocyte membrane, the effects of cationic, anionic, zwitterionic and non-ionic amphiphilic agents ($C_{10}-C_{16}$) on osmotic fragility and transport of potassium and phosphate in human erythrocytes were studied. All the amphiphiles protected the erythrocytes against hypotonic haemolysis. Half-maximum protection occurred at a concentration which was about 15% of that inducing 50% haemolysis. The concentrations of amphiphiles required to induce protection or haemolysis were related to the length of the alkyl chain in a way indicating that a membrane/aqueous phase partition is the mechanism whereby the amphiphile monomers intercalate into the membrane. At

antihaemolytic concentrations all the amphiphiles increased potassium efflux and passive potassium influx. The increase in the fluxes was about the same in both directions through the membrane and there were no clear differences in the effects of the different amphiphilic derivatives at equi-protecting concentrations. Active potassium influx was decreased by cationic, zwitterionic and nonionic amphiphiles. The ability of the amphiphiles to inhibit the influx was not related to the length of the alkyl chain. Anionic amphiphiles had no or only a weak stimulatory effect on the influx. Phosphate efflux was reduced by all the amphiphiles. The inhibitory potency of the different amphiphiles decreased in the following order: anionic > zwitterionic, non-ionic > cationic. Short-chained amphiphiles were more potent inhibitors than long-chained. The possible participation on non-bilayer phases (mixed inverted micelles) in the intercalation of amphiphiles into the membrane is discussed.

Ilma Szász

Does diamide treatment of intact human erythrocytes cause a loss of phospholipid asymmetry? P. F. H. Franck, J. A. F. Op den Kamp, B. Roelofsen and L. L. M. Van Deenen (Department of Biochemistry, State University of Utrecht, Utrecht, The Netherlands). *Biochim. Biophys. Acta 857*, 127 (1986).

Diamide-treated human erythrocytes have been compared with native red cells as to the accessibility of their amino phospholipids to both phospholipase A_2 hydrolysis and fluorescamine labeling. Treatment of intact human erythrocytes with diamide resulted in considerably enhanced degradation of amino phospholipids upon subsequent incubation of the cells with bee venom phospholipase A_2. The hydrolysis of phosphatidylethanolamine (PE) in control cells reached a plateau value at 5% after 10 min. In diamide-treated cells, on the other hand, PE hydrolysis did not level off. Contrastingly, dose-response curves recorded for the labeling of PE with the very fast reacting NH_2-group-specific reagent, fluorescamine, showed identical results for both native and diamide-treated erythrocytes. In each of these two cases, a plateau was reached after approx. 15% of the PE had been labeled. These results strongly suggest that the enhanced phospholipase-A_2-induced hydrolysis of amino phospholipids in diamide-treated erythrocytes may reflect a destabilization of the lipid bilayer, rather than an *in situ* loss of phospholipid asymmetry.

G. Gárdos

The activation of phosphatase activity of the Ca^{2+}-ATPase from human red cell membranes by calmodulin, ATP and partial proteolysis. J. P. F. C. Rossi, P. J. Garrahan and A. F. Rega (Instituto de Quimica y Fisicoquimica Biologicas, Facultad de Farmacia y Bioquimica, Buenos Aires, Argentina). *Biochim. Biophys. Acta 858*, 21 (1986).

Depending on the assay conditions, the ability of the Ca^{2+}-ATPase from intact human red cell membranes to catalyze the hydrolysis of p-nitrophenylphosphate is elicited by either calmodulin or ATP. The response of the phosphatase activity to p-nitrophenylphosphate, ATP, Mg^{2+} and K^{+} is the same for the activities elicited by ATP or by calmodulin, suggesting that a single process is responsible for both activities. In media with calmodulin, high-affinity activation is followed by high-affinity inhibition of the phosphatase by Ca^{2+} so that the activity becomes negligible above 30 μM Ca^{2+}. Under these conditions, addition of ATP leads to a large decrease in the apparent affinity for inhibition by Ca^{2+}. In membranes submitted to partial proteolysis with trypsin, neither calmodulin nor Ca^{2+} are needed and phosphatase activity is maximal in media without Ca^{2+}. This is the first report of an activity sustained by the Ca^{2+}-ATPase of red cell membranes in the absence of Ca^{2+}. Under these conditions, however, ATP still protects against high-affinity inhibition by Ca^{2+}. These results strongly suggest that during activation by calmodulin, Ca^{2+} is needed only to form the calmodulin-Ca^{2+} complex which is the effective cofactor. Protection by ATP of the inhibitory effects of Ca^{2+} and the induction of phosphatase activity by ATP + Ca^{2+} suggests that activation of the phosphatase by Ca^{2+} in media with ATP requires the

combination of the cation at sites in the ATPase. Results can be rationalized assuming that E_2, the conformer of the Ca^{2+}-ATPase, is endowed with phosphatase activity. Under this assumption, either the calmodulin-Ca^{2+} complex or partial proteolysis would elicit phosphatase activity by displacing the equilibrium between E_1 and E_2 towards E_2. On the other hand, ATP + Ca^{2+} would elicit the activity by establishing through a phosphorylation-dephosphorylation cycle a steady-state in which E_2 predominates over other conformers of the ATPase.

G. Gárdos

ATPase activity and Ca^{2+} transport by reconstituted tryptic fragments of the Ca^{2+} pump of the erythrocyte plasma membrane. G. Benaim, A. Clark, and E. Carafoli (Laboratory of Biochemistry, Swiss Federal Institute of Technology, Zürich, Switzerland). *Cell Calcium 7*, 175 (1986).

The purified Ca^{2+}-ATPase of the erythrocyte plasma membrane has been submitted to controlled trypsin proteolysis under conditions that favor either its (putative) E_1 or E_2 configurations. The former configuration has been forced by treating the enzyme with Ca^{2+}-saturated calmodulin, the latter with vanadate and Mg^{2+}. The E_1 conformation leads to the accumulation of a polypeptide of M_r 85 KDa which still binds calmodulin, the E_2 conformation to the accumulation of one of M_r 81 KDa which does not. Both fragments arise from the hydrolysis of a transient 90 KDa product which has Ca^{2+}-calmodulin dependent ATPase activity, and which retains the ability to pump Ca^{2+} in reconstituted liposomes. Highly enriched preparations of the 85 and 81 KDa fragments have been obtained and reconstituted into liposomes. The former has limited ATPase and Ca^{2+} transport ability and is not stimulated by calmodulin. The latter has much higher ATPase and Ca^{2+} transport activity. It is proposed that the Ca^{2+} pumping ATPase of erythrocytes plasma membrane contains a 9 KDa domain which is essential for the interaction of the enzyme with calmodulin and for the full expression of the hydrolytic and transport activity. This putative 9 KDa sequence contains a 4 KDa "inhibitory" domain which limits the activity of the ATPase. In the presence of this 4 KDa sequence, i.e., when the enzyme is degraded to the 85 KDa product, calmodulin can still be bound, but no longer stimulates ATPase and Ca^{2+} transport.

Ágnes Enyedi

Passive permeability of human red blood cells to calcium. M. K. McNamara and J. S. Wiley (Department of Haematology, Austin Hospital, Melbourne, Australia). *Am. J. Physiol. 250*, C26 (1986).

Ca^{2+}-influx was measured into human erythrocytes in which efflux was blocked by either 1) introduction of an intracellular Ca^{2+} chelator, 2) introduction of the Ca^{2+} chelator followed by ATP depletion, or 3) depletion of the Ca^{2+} pump cofactors ATP and Mg^{2+}. The Ca^{2} influx under all three conditions was 14—20 μmol · l cells^{-1} · h^{-1}, which is an order of magnitude higher than the influx previously reported for cells depleted of either ATP or Mg^{2+} separately. The difference between the two values was explained by the finding of substantial Ca^{2+} efflux from the Ca^{2+}-loaded ATP-depleted cells, whereas this efflux was insignificant from cells loaded with quin 2 and then ATP depleted. Under these latter conditions Ca^{2+} influx estimates the unidirectional permeability to this cation. Studies using this technique showed that Ca^{2+} influx was the same in media of isotonic sodium, potassium, lithium, choline, or magnesium chlorides. Moreover the dependence of Ca^{2+} influx on external Ca^{2+} concentration was well described by the sum of saturable and non-saturable (linear) components.

G. Gárdos

Investigation of (Ca^{2+} + Mg^{2+})-ATPase phosphoprotein formation in erythrocyte membranes of patients with cystic fibrosis. B. G. Allen, M. Bridges, B. D. Roufogalis and S. Katz (Division of Pharmacology, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada). *Cell Calcium 7*, 161 (1986).

The $(Ca^{2+} + Mg^{2+})$-ATPase present per mg of protein in erythrocyte membranes of controls and patients with cystic fibrosis (CF) was determined by estimation of the levels of its phosphoprotein. In the presence of 10 mM free Ca^{2+}, which inhibits phosphoprotein decomposition, significantly less phosphoprotein intermediate, $E_{Ca}P$, was found in erythrocyte membranes from CF patients than in age- and sex-matched controls; this correlated with a significant decrease in $(Ca^{2+} + Mg^{2+})$-ATPase activity. These observations indicate a decrease in the number of functional $(Ca^{2+} + Mg^{2+})$-ATPase molecules in erythrocyte membranes from CF patients or an alteration in either the structure of the pump protein or the composition of its environment.

Ilma Szász

Characterization of multiple forms of phosphoinositide-specific phospholipase C purified from human platelets. M. G. Low, R. C. Carroll and A. C. Cox (Cardiovascular Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA). *Biochem. J. 237*, 139 (1986).

The origin and physiological significance of the multiple M_r forms of phosphoinositide-specific phospholipase C in human platelets were investigated. The higher-M_r (400000 and 270000) forms of the phospholipase C were converted into the 100000-M_r form without substantial loss of activity by incubation with a Ca^{2+}-dependent proteinase partially purified from human platelets. These three forms of the phospholipase C were purified approx. 200—500-fold outdated human platelet supernatants. SDS/polyarylamide-gel electrophoresis and gel-filtration analysis suggested that the higher-M_r forms of phospholipase C were complexes of 140000-M_r subunits, whereas the lower-M_r form consisted of a single 95000-M_r subunit. The substrate specificity of the purified phospholipase C was investigated by using ^{32}P-labelled polyphosphoinositide substrates purified from human platelets by a new method utilizing h.p.l.c. on an amino column. Activity against all three phosphoinositides was detected at micromolar concentrations of Ca^{2+}; this hydrolysis was markedly stimulated by phosphatidylethanolamine and inhibited by phosphatidylcholine. Comparison of the different forms of purified phospholipase C revealed no major differences in Ca^{2+}-sensitivity or substrate specificity. Thus, although the suggestion that the high-M_r forms of human platelet phosphoinositide-specific phospholipase C were converted into a lower-M_r form by a Ca^{2+}-dependent proteinase has been substantiated, the physiological significance of this process remains to be determined.

Ágnes Enyedi

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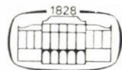

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HU ISSN 0017-6559

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VOLUME 20 · NUMBER 2

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

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AKADÉMIAI KIADÓ, BUDAPEST

1987

Printed in Hungary

Akadémiai Kiadó és Nyomda, Budapest

MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

Haematologia 20 (2), pp. 67—72 (1987)

Marked Changes in Red Cell Volume Response to Isotonic Glucose Solution During Storage

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(Received 27 February, 1986; accepted 21 May, 1986)

The changes of erythrocyte-volume caused by isotonic hexose solutions, glucose and galactose, during storage were studied. Blood was stored for 13 weeks at 4 °C. The determinations were carried out using a newly developed blood cell volume analyzer which can measure the continuous changes in cell volume. The erythrocyte volume gradually increased and reached a plateau after 13 weeks of storage. The percent volume increase in hexose solutions markedly decreased during the first 4 weeks of storage. These changes may have been due to denaturation of the membrane protein responsible for hexose permeation during storage.

Keywords: blood cell counter, erythrocyte volume, glucose, galactose, storage

Introduction

Red blood cells are usually preserved in glucose-containing solutions such as acid-citrate-dextrose (ACD) and citrate-phosphate-dextrose (CPD) to preserve the viability of the cells [1, 2]. The biconcave disk shape of the cells becomes echinocytic or stomatocytic during storage [3, 4]. For a while this transformation of shape is reversible, however, after prolonged storage microspheres cannot be regenerated into biconcave disc shape any more.

In an isotonic hexose (e.g. glucose) solution, red cell volume increases due to the permeation of the solute [5]. This permeability across the red cell membrane has been observed by radioisotopic [6], microhematocrit [7] or light scattering [8] methods. We have developed a new system to observe the volume response of erythrocytes to isotonic hexose solution using a continuous electric mean cell volume (MCV) analyzer [9]. The method is easy, rapid, and highly reproducible. Using this method, the changes in volume response of red cells during storage were studied.

Materials and Methods

CPD blood was kindly provided by the Osaka Blood Center of the Japan Red Cross and stored at 4 °C for 13 weeks. The blood was first examined biochemically and hematologically using routine procedures, and 6 samples exhibiting no abnormalities were chosen; their mean cell volume (MCV) was 90 fl with a mean hemoglobin concentration of 30 pg/cell.

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Volume changes in the isotonic glucose or galactose solution were examined using a continuous MCV analyzer (CMA, Sysmex-TOA Electronics Co., Kobe, Japan). Blood (2 μl) was suspended in a mixture (50 ml) of isotonic hexose, either glucose or galactose solution, and saline (volume ratio; 2 : 3) containing 1 mM sodium phosphate buffer (pH 7.4). Immediately after placing the suspension in the CMA, MCV was continuously measured for 10 min, and the data were recorded every 5 sec. After 100 determinations, the changes in MCV were plotted as a function of time. The apparatus was calibrated using standard resin particles (Dow Chemical Co., City Ind., USA) before every determination. All measurements were carried out at 30 °C.

Results

Figure 1 shows representative volume change curves obtained before and after 3 and 10 weeks of storage. Before storage, the cells showed good expansibility in glucose solution, and the volume reached the equilibrated level within 10

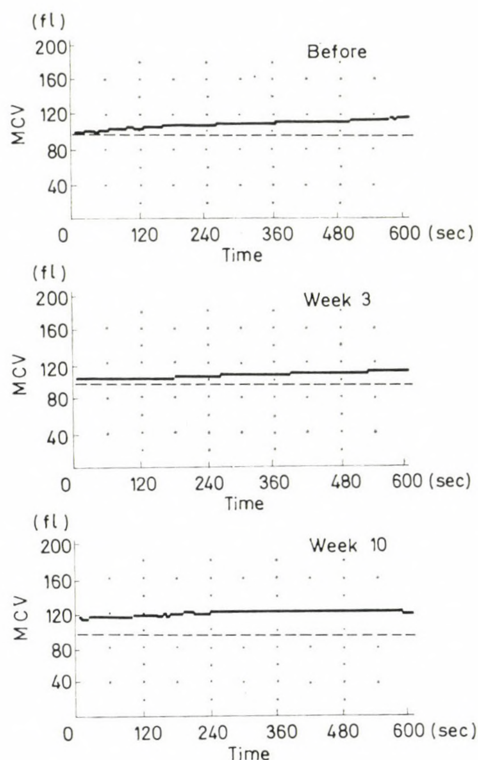

Fig. 1. Volume change curves in isotonic glucose solution drawn by continuous MCV analyzer before storage, and after 3 and 10 weeks of storage. Dashed line in each panel denotes the initial volume determined at week 0. MCV: mean cell volume

min. After 3 weeks of storage, the cells showed slight swelling, marked changes occurred in the presence of glucose. After 10 weeks of storage, the initial volume was increased and the volume response in glucose solution was low.

Figure 2 shows the time-course changes of the initial volume and the volume at 90% equilibration in a glucose solution. The initial volume remained constant during 4 weeks of storage, then gradually increased, reaching a plateau at 10

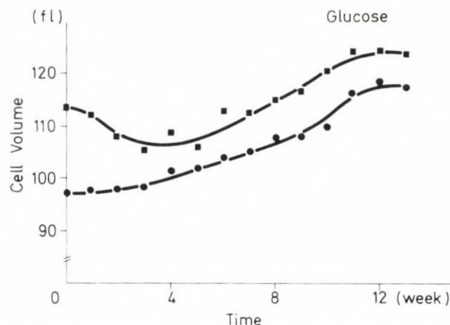

Fig. 2. The effect of storage on the initial (●) and 90% equilibrated (■) volumes in isotonic glucose solution. 90% equilibrated volume was the volume observed at 480 sec

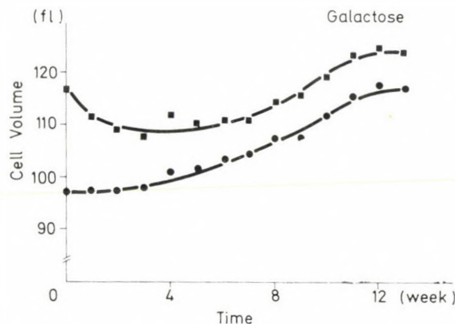

Fig. 3. The effect of storage on the initial (●) and 90% equilibrated (■) volumes in isotonic galactose solution. 90% equilibrated volume was the volume observed at 160 sec

weeks. On the other hand, the volume at the equilibrated level decreased gradually during the first 4 weeks, and then increased in parallel with the changes of the initial volume. The difference between the initial volume and the equilibrated volume, representing the expansible space, was large during the initial 4 weeks but decreased gradually. A similar tendency was observed when the cells were placed in galactose solution (Fig. 3).

The percent volume increase, 90% equilibrated volume/initial volume, of red cells in an isotonic glucose solution, is shown in Fig. 4. The value decreased during

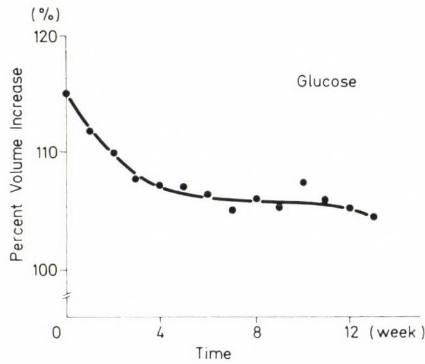

Fig. 4. The effect of storage on percent volume increase in isotonic glucose solution. The value was calculated as: 90% equilibrated volume/initial volume x 100 percent

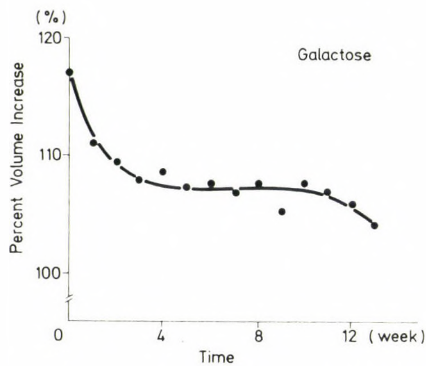

Fig. 5. The effect of storage on volume increasing ratio in isotonic galactose solution. Calculations were made as in Fig. 4

the first 4 weeks of storage, and was unchanged between 4 to 10 weeks, then decreased again after 10 weeks. A similar tendency was seen for cells in galactose (Fig. 5).

Discussion

Glucose, a major source of ATP in red cells [10], is transported by a carrier protein. The type of this transport is not considered to be simple diffusion, but a facilitated diffusion [7, 11, 12]. The glucose permeation of red cells increases the cell volume, since an isotonic glucose solution is not an isoosmolar solution.

The long-term storage of red cells induces an increase in volume [13], and this phenomenon was confirmed in the present experiments by the increase in the initial volume. Insufficiency of the membrane-sodium pump causes the leakage of potassium ion to increase the volume [13]. By contrast, a volume increase

observed in an isotonic hexose solution can be interpreted as a biophysical phenomenon based on hexose permeation, and the expansibility is regulated by several factors such as membrane component, the concentration gradient of permeable solutes, internal viscosity or temperature [12]. In our experimental system, the former two may be important for expansibility, since MCV and mean hemoglobin content regulating internal viscosity, and determination temperature were constant.

Expansibility of red cells in hexose solution was very high during the early storage period and the volume increase curve showed a parabolic pattern. Storage over 4 weeks resulted in decreased expansibility of the cells in the hexose solution. The volume increase curve changed from a parabolic to a linear form, thus, the increase must have been due to simple diffusion [7, 11, 12]. Long-term storage gradually decreased the level of intracellular ATP even in the presence of glucose [14]. The decrease caused an irreversible shape change, namely, spherocyte formation [15]. These cells demonstrated potassium leakage, membrane fragmentation and changes in membrane constituents [15–16]. Therefore, alteration in membrane proteins responsible for hexose transport may be assumed as a possible cause for the reduced red cell expansibility in isotonic hexose solutions.

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Lymphokine Effect upon the Erythrocyte Antibody Uptake by Human Monocytes

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(Received 17 February, 1986; accepted 6 June, 1986)

Preincubation of human monocytes with different amounts of human lymphokine at 37 °C dose dependently increased the uptake of EA cells at both 37 °C and 4 °C. Phenylmethanesulphonyl fluoride (PMSF), an inhibitor of serine esterases, inhibited the process. It seemed that the serine esterase and not the gamma interferon component of the lymphokine played the main role in the phenomenon.

Keywords: EA, lymphokine, monocyte esterase, gamma interferon

Introduction

The uptake of EA cells (rosette-formation, adherence and phagocytosis of sensitized sheep red blood cells (SRBC)) by monocytes or macrophages is a form of the Fc receptor mediated and dependent cell activation [1, 2]. Lymphokines promote the activation of macrophages via both Fc and C3b receptors [3, 4, 5]. The lymphokines are thought to represent effector molecules responsible for the expression of both humoral and cellular immune phenomena. The mechanism of several lymphokine actions is poorly understood, but observations concerning the enzymatic character of some lymphokine factors, for example of the human leukocyte migration inhibitory factor (LIF) and lymphotoxin (LT), might mean some new biochemical approach to the explanation [6, 7, 8, 9, 10].

In this study we examined the effect of human lymphokine (LK) upon the uptake of EA cells by human monocytes. Lymphokine stimulated the adherence and phagocytosis of sensitized SRBC to monocytes. This process seemed to be related to the esterase and not to the gamma interferon component of lymphokine.

Materials and Methods

Human monocytes were separated on Ficoll-Uromiro gradient according to Pawlowski [11].

Reaction of monocytes with SRBC: 0.1 ml mononuclear cell suspension containing $1-5\times10^5$ monocytes was placed into a plastic ring fixed to a glass slide with wax. After 30 min, the non-adhering cells were removed by washing; the

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monolayers contained 95–98% monocytes. Monocyte FcR activity was assayed by the uptake of sheep red blood cells sensitized with a subagglutinating dilution (1 : 128) of an IgG fraction isolated on Sephadex G-200 from rabbit anti SRBC serum (EA). The adherent cells were incubated with 0.2 ml of 2% EA in 0.3 ml of medium 199 at 37 °C for 30 min, in 5% CO_2 and 100% humidity. After washing, the monolayers were stained with 0.01% crystal violet solution or Wright stain, and the percentage of monocytes attaching to or ingesting three or more sSRBC (percentage of M-EA rosettes) was determined [12].

Lymphokine-containing supernatants were produced by stimulation of human peripheral mononuclear cells (lymphocytes and monocytes) with Concanavalin A (Con A, Sigma) at 37 °C for 48 hours. The cell and Con A-free protein containing supernatants, chromatographed on Sephadex G-75, concentrated by Amicon B-15, representing leukocyte migration inhibitory (LIF) activity were regarded as "lymphokine (LK)" [10]. The protein content of LK was found to be on the average, 0.25 mg/ml, the esterase activity 18.14 μmol/min/mg, tested by Z-Ala-2-naphtylester substrate. The gamma interferon (IFN-γ) activity of LK was in general 5IU/mg. The purified human gamma interferon was the product of EGIS Blood Products Laboratory, Budapest, Hungary).

Preincubation of monocytes with LK or phenylmetanesulphonylfluoride (PMSF, Merck) took place at 37 °C. The uptake of sSRBC was tested at 37 °C and 4 °C by monocytes treated and non-treated with LK.

Statistical analysis: the standard deviation (SD) of the averages from four separate experiments was determined; then Student's t test (after Fischer) was applied for estimating the differences [13].

Results

Preincubation of monocytes with LK increased their sSRBC uptake dose and time dependently. As can be seen in Table 1, 60 μg/ml of LK already increased the uptake of EA cells remarkably.

Table 1

Uptake of sensitized SRBC by human monocytes preincubated with different amounts of human lymphokine (LK) at 37 °C

Preincubation of monocytes with	M-EA (%) mean ± SD
PBS	52±8
30 μg/ml LK	55±6
60 μg/ml LK	*76±8
120 μg/ml LK	*84±9

* = $p < 0.01$

Figure 1 shows that the optimum preincubation time for the lymphokine effect was 60 minutes.

To determine whether or not the esterase component of LK plays some role in the augmentation of uptake of EA cells, the monocytes were preincubated with LK and with the esterase inhibitor PMSF at 37 °C. Washing the cells twice, the uptake of sRBC by monocytes was tested at both 37 °C and 4 °C. Table 2 demonstrates that the preincubation of monocytes with LK in the presence of PMSF at 37 °C prevented the formation of M-EA rosettes at both 37 °C and 4 °C.

Since the LK preparations usually contain some gamma interferon, and interferon is known to increase the expression of Fc receptors on the surface of monocytes [14, 15], it was important to clarify whether the gamma interferon or the esterase were dominant factors in the experiments. Therefore, we tested the

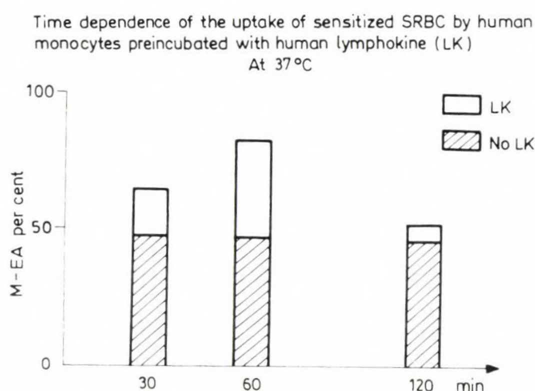

Fig. 1. Time dependence of the uptake of sensitized SRBC by human monocytes preincubated with human lymphokine (LK) at 37 °C (120 μg/ml LK)

Table 2

Uptake of sensitized SRBC by human monocytes preincubated with human lymphokine (LK) and PMSF (10^{-4} M)

Temperature of sSRBC uptake by monocytes	Preincubation of monocytes at 37 °C	M-EA (%) mean ± SD
37 °C	PBS/PMSF	48±4
	60 μg/ml LK	*70±6
	60 μg/ml LK + PMFS	45±5
	PBS/PMFS	46±6
4 °C	60 μg/ml LK	*71±4
	60 μg/ml LK + PMSF	42±5

* = $p < 0.01$

Table 3

Uptake of sensitized SRBC by human monocytes preincubated with human gamma interferon (IF) and PMSF (10^{-4} M)

Preincubation of monocytes with	M-EA (%) mean ± SD
PBS/PMSF	48±4
5 IU/ml IF	52±3
20 IU/ml IF	*74±6
20 IU/ml IF+ PMSF	*76±5

* = $p < 0.01$

influence of purified gamma interferon on the uptake of EA cells by monocytes in the presence of PMSF. As can be seen in Table 3, purified INF-γ in the concentration equal to the INF-γ content of LK (5IU/ml), did not cause any elevation in the formation and uptake of EA rosettes. On the other hand, 20 IU/ml of purified INF-γ resulted in a similar increase in the adherence of sSRBCs to monocytes like 60μg/ml of LK, but this effect could not be neutralized by PMSF.

Discussion

Although interferon was described as a specific antiviral substance [16], it is now clear that it does affect cell division and function [17]. For example, INF-α increases the expression of histocompatibility antigens and cell surface receptors [18], affects the transport of nucleic acid [19] and induces a rapid increase in cyclic GMP [20]. In addition, IN-α enhances the activity of sensitized cytotoxic T cells [21], of natural killer (NK) cells [22], of K cells in the antibody dependent cellular cytotoxicity (ADCC) assay [23] of macrophage functions [24].

Gamma interferon, one of the best characterized lymphokines, has been tested especially from the aspect of its participation in the activation processes of macrophages. Evidence was obtained that the macrophages have a gamma interferon receptor [26] and IFN-γ is one form of the lymphokine macrophage activating factors [26, 27, 28].

Our results suggested that beside gamma interferon, other lymphokine factor(s) with esterase activity may cause the increase of M-EA rosette formation, but by a different way. The serine esterase inhibitor substance clearly distinguished these two routes from each other because the effect of INF-γ was not inhibited by PMSF, while the influence of esterase was inhibited. LIF is an esterase, our LK showed both LIF and esterase activities, therefore LIF might be the cause of the PMSF sensitive activation of human monocytes. For direct proof we would need the purified form of LIF which is still far under the level of laboratory possibilities [29]. That was the reason why we used a "mixture" of lymphokine factors, con-

taining both INF-γ and LIF, to obtain indirect evidence of the role of LIF in monocyte activation.

The increase in the adherence and uptake of EA-cells by both LK and γ interferon seemed to be due to the Fc receptors, and it did not depend on the temperature, because there was no difference in the uptake of sSRBC at 37 °C and 4 °C. Gamma interferon enhances the number of Fc receptors on the cell surface [14, 15]. It is therefore suggested that esterase treatment of monocytes may probably stimulate the affinity of Fc receptors to bind sensitized sheep red blood cells in greater numbers.

It might also be taken into consideration that LIF (esterase) activity of lymphokines may modulate not only the migration of phagocytic cells, but have an influence on the cell surface receptors, too.

Acknowledgements

The authors wish to thank Dr. Klára Ónody for the gift of purified human gamma interferon (EGIS Blood Products Laboratory, Budapest) and Mrs. Júlia Esztergály for technical assistance.

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Alpha, Beta Thalassemia Produces High Levels of Hb Bart's in Newborns and High HbA_2 in Adults*

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(Received 6 June, 1986; accepted 25 June, 1986).

Keywords: α/β thalassaemia, Hb Bart's, HbA_2

Introduction

Several cases of interaction between α and β thalassemia have been reported in different populations. In all of them this combination was similar to the heterozygous state for high HbA_2 β-thalassemia [1]. The slight clinical variability observed in some instances was explained by the presence of different α-thalassemia determinants, though in most families the precise genotype has not been determined. A few well-documented examples indicate that, in combination with β-thalassemia, both α_1 and α_2 thalassemia mutations produce identical phenotypic expression [2, 3], although in the first case MCV and MCH tend to normalize [4]. Thus since both combinations are almost indistinguishable from the heterozygous state for β-thalassemia in adults [1], a differential diagnosis based on routine haematological and clinical data is not feasible. On the other hand, α/γ chain synthesis imbalance produces high levels of Hb Bart's in newborns. This indicates the presence of a thalassemia determinant, whereas the β-thalassemic gene would pass undetected.

Here we report the study of a case of α/β thalassemia which provides further information on this problem.

Materials and Methods

During a screening for abnormal haemoglobins in newborns we found a subject with 5% of HB Bart's. Since his mother was a heterozygote for high HbA_2 β-thalassemia, he could be the carrier of both thalassemic genes. The subject was reanalyzed together with his family 4 years later.

Haematological investigations were carried out by standard methods [5]. Serum iron was quantitated by the bathophenanthroline method [6]. Starch gel haemoglobin electrophoresis was carried out at pH 8.6 [7]. HbF was estimated by the alkali denaturation method [8], HbA_2 by DEAE cellulose column chromatography [9] and Hb Bart's after elution from cellulose acetate strips, according to Marengo-Rowe [10]. The relative rate of globin chain synthesis was measured

* A case report

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as described by Huisman and Jonxis [11], by incubating reticulocyte-enriched peripheral blood with 14 °C Leucine and globin chain separation by CM-cellulose column chromatography.

Restriction endonuclease analysis was carried out with Bam HI. 15 μg of DNA prepared from leukocytes were digested as recommended by the manufacturer (Boehringer, Mannheim), were electrophoresed in 0.9% agarose and then transferred to a nitrocellulose filter according to Southern [12]. Filter hybridization was performed as described by Martinez et al. [13] with JW 101 plasmid (kindly provided by Dr. A. Bank) nick translated in the presence of ^{32}P-dATP. Following hybridization filters were washed and autoradiographed at −70 °C for 3−5 days.

Results and Discussion

Table 1 shows the haematological findings of the family and Figure 1. the restriction mapping with Bam HI.

Table 1

Haematological data of the family

	Age years	Hb g/dl	PVC %	RBC $x10^6/\mu l$	MCV fl	MCH pg	MCHC %	RETICS %	HbF %	HbA_2 %	α/β globin chain ratio	α genotype
Father	35	14.0	48	6.47	74	22	29	1.3	0.38	2.0	0.75	$\alpha\alpha/-\alpha$
Mother	32	12.3	44	5.28	83	23	28	1.8	1.50	5.1	1.62	$\alpha\alpha/-\alpha$
Propositus	4	10.0	35	5.16	70	19	28	1.2	0.54	4.3	1.09	$-\alpha/-\alpha$
Brother	6	9.5	34	3.90	87	24	28	1.0	0.54	2.0	—	$\alpha\alpha/-\alpha$

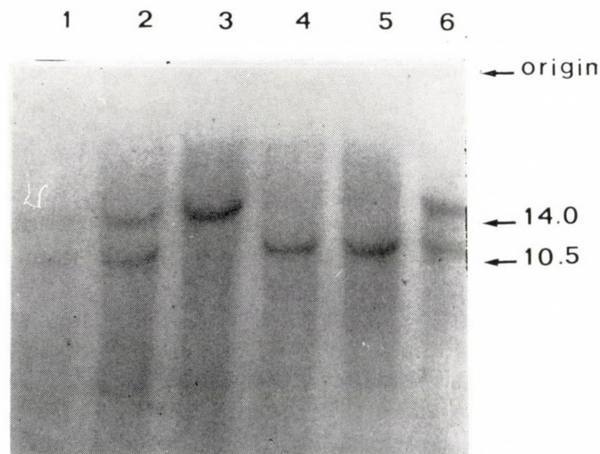

Fig. 1. Bam HI restriction mapping of the family. Lane 1: father; lane 2: mother; lanes 3 and 4: controls; lane 5: propositus; lane 6: brother

As is observable, the mother has high levels of HbA_2, a slight microcytosis and hypochromia, and an unbalanced synthetic ratio. DNA restriction analysis with Bam HI shows the presence of both the normal 14.0 kb fragment and of the abnormal 10.5 kb fragment produced by the $- \alpha$ 3.7 haplotype. She is, thus, a compound heterozygote for α-thalassemia-2 and high HbA_2 β-thalassemia.

The propositus has a somewhat more pronounced microcytosis and hypochromia, high levels of HbA_2 and a normal synthetic ratio. DNA restriction mapping with Bam HI shows the presence of only the 10.5 kb fragment. He is, therefore, homozygote for the α-thalassemia-2 determinant (as the DNA analysis of the father also indicated) and heterozygote for a high HbA_2 β-thalassemic gene.

It is apparent then that, as previously observed, the phenotypic expression of the two types of interaction is similar, and that the exact diagnosis can be made only by synthetic studies and gene mapping.

This case clearly demonstrates that family studies (including biosynthetic and gene mapping analysis) are necessary for the exact diagnosis of thalassemias: at birth this child would have been diagnosed as an α-thalassemia 1 heterozygote (or α-thalassemia-2 monozygote) (4.8% Hb Bart's) and at 4 years as a β-thalassemia heterozygote (4.3% HbA_2).

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Haematologia 20 (2), pp. 83–87 (1987)

Association of Dengue Hemorrhagic Fever with the HLA System

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(Received January 14, 1986; accepted March 18, 1986)

The frequency of HLA antigens was determined in 82 unrelated patients who had been hospitalized with the most severe form of dengue hemorrhagic fever: shock, dehydration and severe hemorrhages (DHF/DSS). The HLA-A1, HLA-B plank, HLA Cw1 and HLA-A29 antigens showed a significant difference when their values were compared with the normal control group.

Keywords: dengue hemorrhagic fever, shock syndrome, disease association, HLA system

Introduction

Endemic dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) have occurred in the Western Pacific and Southeast Asia and recently in our country (Bres 1979, Halstead 1982). In endemic areas this disease is among the principal causes of child-morbidity and mortality (Halstead 1982, Epidemiological Surveillance Report 1978).

The clinical pict ure of the disease varies from mild symptoms to shock and death. Sequential infections with different serotypes of dengue virus increase the risk of DHF/DSS (Fischer & Halstead, 1970). Nevertheless, primary infection with dengue virus may also result in DHF/DSS (WHO 1973, Scott et al. 1976).

When DHF is endemic, only few individuals exhibit severe disease while in the rest of the population the infection is relatively mild (Halstead et al. 1969). DHF/DSS has been observed in several members of the same family (Halstead 1970). All this suggests that genetically mediated factors are involved in the susceptibility to, and severity of the disease. If the differences are due to genetically mediated factors, it might be possible to identify genetic markers for the susceptibility to DHF/DSS. Different studies of blood groups and G6P dehydrogenase deficiency have been performed, but no relation with the susceptibility to the disease was found (Halstead 1966, Basanta et al. 1984). In 1981 Chiewsilp et al. performed a pilot study typing to find an association between DHF/DSS and the histocompatibility (HLA) system. They suggested other studies to confirm their results.

In the present report we determined the frequencies of HLA-A, B and C histocompatibility antigens in patients with DHF/DSS, to study their relation with the susceptibility to this disease.

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Materials and Methods

Patients

A total of 82 unrelated patients hospitalized in the William Soler and Enrique Cabrera hospitals, Havana City, were investigated. The clinical evidence of dengue infection was confirmed by positive serological test with serotype II dengue virus carried out at the Instituto de Medicina Tropical Pedro Kouri, Havana City (Kouri et al. 1982). These 82 patients had severe clinical manifestations: shock, dehydration and severe hemorrhages.

Normal controls

HLA-A, B and C frequencies were obtained from 276 unrelated healthy donors studied before the epidemia had occurred (Arce et al. 1976).

HLA typing

Lymphocytes were isolated from defibrinated blood by Ficoll-Telebrix centrifugation (Boyüm 1958). HLA typing was performed with the microlimphocytotoxicity technique recommended by NIH (Ray et al. 1974). The following specificities were included in the study: HLA-A1, 2, 3, 9, 10, 11, 28, 29, HLA-B5, 7, 8, 12, 13, 14, 15, 16, 17, 21, 27, 35, 40; HLA-Cw1, w2, w3, w4, w5.

Statistical methods

The HLA frequencies of the patients were compared with those of the controls by X^2 test with Yates's correlation and corrected p (α/no. of comparison).

Results

The phenotype and gene frequencies of the HLA-A, B and C antigens are shown in Table 1.

A significant increase of HLA-A1 ($p < 0.001$). HLA-B blank ($p < 0.01$) and HLA-Cw1 ($p < 0.002$) was found in the patients. The HLA-A29 antigen had a negative correlation with the disease ($p < 0.001$).

The ethnic composition of both groups was not matched because the control group showed no significant differences in HLA antigens between different ethnic groups (Arce et al. 1976).

Discussion

Several studies have shown an association between the presence of HLA antigens and some diseases. The first findings came from studies in mice (Lilly et al. 1964) which showed that susceptibility to virus-induced leukemogenesis

Table 1

HLA-A, B and C phenotype (a) and gene (b) frequencies in patients with severe form of DHF (compared with controls by X^2 test)

Normal controls = 276 Patients = 82

Antigens	Positive cases	(a)	(b)	Positive cases	(a)	(b)	X^2 YATES CO
A1	27	0.09	0.05	22	0.26	0.14	14.1405
A2	110	0.40	0.22	37	0.45	0.25	0.5233
A3	45	0.16	0.08	10	0.12	0.05	0.5353
A9	72	0.26	0.14	27	0.32	0.18	1.1562
A10	39	0.14	0.07	9	0.10	0.05	0.3043
A11	43	0.15	0.08	10	0.12	0.05	0.3372
A28	30	0.10	0.05	12	0.14	0.07	0.5298
A29	54	0.19	0.10	2	0.02	0.01	12.7839
blank	86	0.21	0.17	35	0.42	0.24	3.2545
B5	70	0.25	0.13	13	0.15	0.08	2.6978
B7	45	0.16	0.08	9	0.10	0.05	1.0163
B8	27	0.09	0.05	14	0.17	0.08	2.6336
B12	77	0.27	0.15	28	0.34	0.18	0.9082
B13	10	0.03	0.01	5	0.06	0.03	0.4463
B14	29	0.10	0.05	12	0.14	0.07	0.6938
B15	25	0.09	0.04	8	0.09	0.05	0.0007
B16	17	0.06	0.03	2	0.02	0.01	1.0795
B17	29	0.10	0.05	6	0.07	0.03	0.4126
B21	18	0.06	0.03	8	0.09	0.05	0.5604
B27	27	0.09	0.05	6	0.07	0.03	0.2119
B35	51	0.18	0.09	16	0.19	0.10	0.0025
B40	29	0.10	0.05	7	0.08	0.04	9.0973
blank	31	0.11	0.05	30	0.36	0.20	26.9816
Cw1	6	0.11	0.05	9	0.10	0.05	10.1058
Cw2	17	0.06	0.03	8	0.09	0.05	0.7662
Cw3	43	0.15	0.08	5	0.06	0.03	4.1131
Cw4	50	0.18	0.09	18	0.21	0.11	0.3808
Cw5	11	0.03	0.02	6	0.07	0.03	0.9022

was strongly influenced by a recessive gene in the mouse major histocompatibility system. At the same time, a specific immune response (Ir) closely linked to the histocompatibility system was demonstrated in several animal species. It has been suggested that histocompatibility antigens influence immune responsiveness and the susceptibility to virus diseases (Monro & Bright 1976, Braun 1979).

Identifiable HLA antigens may act as viral receptor sites (Braun 1979, Helenius et al. 1978) or, by mimicking receptors, they may competitively inhibit legitimate sites (Svejgeard & Ryder 1976).

The increased prevalence of HLA-A1 reported in patients with congenital rubella (Honeyman et al. 1975) and an increased frequency of HLA-A1, and A9 found in patients with primary dengue infection (Chiewsilp et al. 1981) correlate

well with our finding of a significant increase of HLA-A1 in patients with DHF/DSS. The significance of HLA-A1 in these diseases should, however, be further studied.

The deviations from the control group of HLA-A1, B blank, Cw1 and A29 suggest an association between the development of shock and the presence or absence of these HLA antigens. The presence of HLA-A29 appears to protect against shock.

Our HLA-B blank results were similar to those reported by Chiewsilp et al. in 1981, which suggested an association with the development of shock and this antigen. The antigen frequency for HLA-B locus in our work indicates the possibility of the association of this disease with only one antigen of this series. For this reason we might define our B blank as "only one antigen". These findings should be followed by investigations into other serologically defined HLA-DR antigens and B antigens, as well as cellulary defined HLA-D antigens. Studies on patients who did not develop severe forms of DHF should also be performed.

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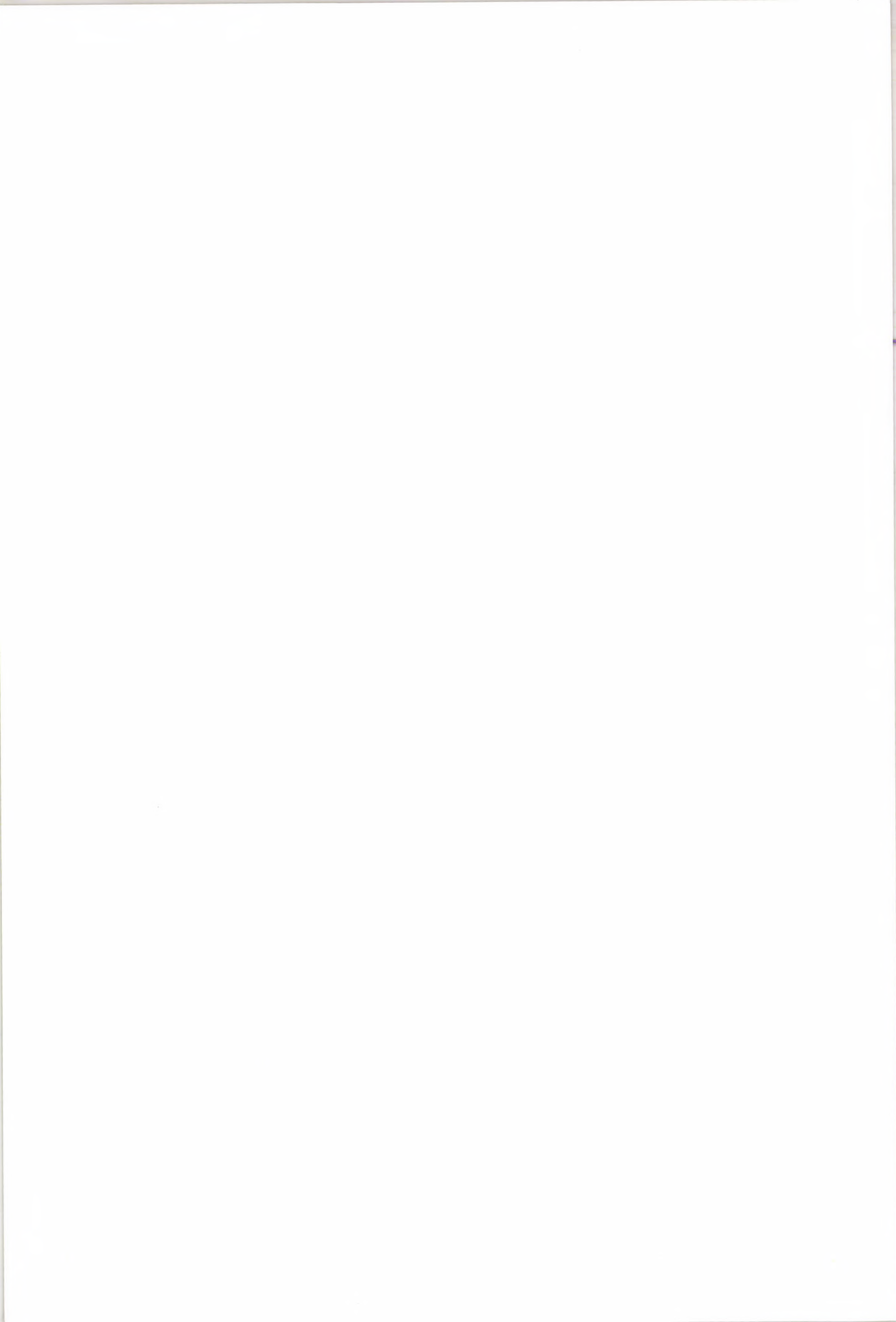

Haematologia 20 (2), pp. 89—99 (1987)

Proteinuria and Excretion of Ribonuclease in Patients with Chronic Granulocytic Leukaemia

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(Received 17 March, 1986; accepted 22 April, 1986)

Elevated RNase activity which occurs in serum and urine of CGL patients parallels the urinary protein excretion. Acid RNase and alkaline RNase activities in urine of CGL patients, as well as acid and alkaline RNase clearance values correlated with the urinary protein concentration. Mean urinary protein level in CGL patients was approximately twice as high as that in controls. The molecular mass of CGL urinary proteins ranged from 12 000 to 80 000 proving the LMWP type of proteinuria. No particular protein contributed to the elevation of LMWPs in CGL urine. Among numerous protein fractions, albumin, acid $alpha_1$ glycoprotein, prealbumin RNase and in a few cases LZM were observed. The results of this study suggest that the increase of RNase activity in serum and urine reflects a more general phenomenon of increase in excretion of the entire set of LMWPs.

Keywords: LMWP-proteinuria, plasma LMWP-pool, RNase activity, RNase apparent clearance, RNase fractional clearance, urinary proteins

Introduction

There exists some controversy over the mechanism of increase in plasma RNase activity in CGL patients. Some authors suggest that elevated RNase activity in CGL plasma and urine represents the effect of accumulation of leukaemic granulocytes [2, 17, 25]. Other authors [13, 15] are of the opinion that the increase in RNase activity in the plasma of CGL patients depends on the decrease of renal function. A manifold increase in the concentration of urinary RNase [2, 17] and of other low molecular weight proteins produces an overflow proteinuria in the majority of CGL patients. The aim of this study was to repeat the former investigations of RNase activity in the plasma and urine of CGL patients and to correlate the estimated RNase activity with creatinine clearance values.

Abbreviations: CGL — chronic granulocytic leukaemia; LMWP — low molecular weight proteins; LZM — lysozyme; RNase — ribonuclease; SDS-PAGE — sodium dodecylsulphate polyacrylamide gel electrophoresis

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Materials and Methods

The studied group consisted of 17 persons, 8 males and 9 females, 24 to 49 years of age, with previously untreated CGL. The white blood cell count of the patients ranged from 40 000 to 90 000 per μl with mature forms of granulocytes comprising more than 50%. Blasts comprised 0–10%, myelocytes 12–22%, and metamyelocytes 6–28%. Diagnosis of CGL was based on haematological tests and clinical observation of the patients in the Outpatient Clinic, Department of Haematology of the Medical Academy Kraków. Patients with myeloblastic exacerbation, acute and chronic infections, coexisting diseases of the renal and urinary tract and other diseases with possible effects on serum and urine protein levels were excluded from the studied group.

The control group consisted of 20 healthy persons who were blood donors and members of the medical staff of the Department of Biochemical Diagnostics, matched in sex and age to the studied patients.

Materials for the study were collected without any preservative. Urine volume was measured and 10 ml aliquots were centrifuged at 2000 *g* for 10 minutes and used for further study. The serum and urine samples were neither stored nor frozen. Blood drawn routinely into glass tubes was allowed to clot at room temperature for 1–2 hours. Serum was obtained by centrifugation at 800 *g* for 10 minutes. Materials for the study were collected from the patients before any cytostatic treatment had started.

Renal function was evaluated on the basis of routine urine examinations including microscopy of sediment, urinary protein tests and assays of 24-hour endogenous plasma creatinine clearance. Serum creatinine assays were performed with the Jaffé colour reaction with picrate, adapted to the Technicon SMA 12/60 Autoanalyser [27]. Urinary creatinine concentration was assayed manually with picrate using the procedure described by King [13].

Electrophoresis of serum and urine proteins was based on the procedure of Pesce et al. [21]. Urine and serum samples were studied by electrophoresis on 5% polyacrylamide gel at pH 4.1 and 7.8, respectively. Protein staining in the gel rods was performed using Coomassie Briliant Blue. The stained gel rods were stored in 1% acetic acid solution for more than one year for direct comparison. Proteins separated on SDS PAG electrophoresis were stained with amido black and stored in 1% acetic acid solution in water. Evaluation of the stained protein patterns was performed using the Autoradiogram-Gel Scanner and Beckman DU 8B spectrophotometer. Calculation of urine proteins' molecular weights was performed on the basis of SDS PAGE electrophoresis gel scan patterns using Beckman Gelscan Compuset program. To obtain legible protein patterns of urine electrophoresis at pH 4.1 and SDS PAG electrophoresis, it was necessary to use an approximately tenfold concentration of urine. This was obtained by dehydration of the urine samples placed in a dialysis tube (Diala-Por Membrane Dialysis Tube, cut off M. Wt. 6000–8000) using Aquacide II (Calbiochem) as a dehydrating agent.

Assays of urine LMWP concentration were performed using urine samples dialysed 20 hours against running tap water. LMWP concentrations were arbitrarily expressed in miligrams per litre of standard albumin solution which produced the same light absorption at 540 nm as the urine samples studied, according to the method of Lowry et al. [18].

RNase activity was assayed as described elsewhere [17, 26] using yeast RNA-sodium salt as substrate. Detection of RNase in the polyacrylamide gel rods was performed by incubation of the gel rods with RNA-substrate solution. Further staining of non-depolymerized RNA-substrate with 0.2% toluidine blue (Lachema) in 0.1% acetic acid solution of pH 3.0 displayed gel zones free of RNase [31]. Detection of LZM was performed by a similar procedure except that lyophilized Micrococcus lysodeicticus (Koch-Light) dried cells fixed in agarose gel slabs were used as substrate; clearing of the turbid gel slabs indicates the presence of lysozyme.

24-hour creatinine clearance values were calculated using the equation

$$Cl = \frac{C_u \times V_{min}}{C_p} \quad (1)$$

where C_u and C_p are the creatinine urine and plasma concentrations, respectively; V_{min} is the urinary excretion per minute. Acid and alkaline RNase clearance values were calculated using the same procedure, but urine and plasma RNase activities were used instead of C_u and C_p values, respectively. As the urine RNase activity (U-RNase) depends on the tubular reabsorption rather than on the glomerular filtration rate [8, 13, 19], the obtained values represented the "RNase apparent clearance" values. Ratios of RNase clearance to creatinine clearance values were calculated from the equation:

$$FR\text{-}Cl_{RNase} = \frac{C_u \times P\text{-}RNase}{C_p \times U\text{-}RNase} \quad (2),$$

where P-RNase is plasma RNase activity.

Results

The LMWP concentration in the urine of CGL patients was about twice as high as in healthy subjects (Table 1). None of the studied urine specimens gave protein-positive routine precipitation tests. Creatinine levels in the serum of CGL patients, as well as creatinine clearance values, did not essentially differ from those in normal subjects. Thus, no decrease in renal filtration rate was found in the studied patients.

Proteins contributing to the urine LMWP pool, studied by SDS-PAG electrophoresis showed molecular weights ranging from 12 000 to about 80 000 daltons (Fig. 1). Relative contents of the individual protein fractions differed in the

Table 1

Creatinine clearance and urine LMWP excretion in normal subjects and patients with chronic granulocytic leukaemia

	Serum creatinine $\bar{x}$ μmol per l. ± SD (from — to)	Urine creatinine $\bar{x}$ mmol per l. ± SD (from — to)	Creatinine clearance $\bar{x}$ ml per min ± SD (from — to)	Urine LMWP level $\bar{x}$ mg per l ± SD (from — to)
Normal (N = 20)	83.87±5.85 (75.0—95.6)	10.63±2.49 (6.49—15.7)	104.83±22.10 (75.0—128.0)	259±87.03 (181—420)
CGL patients (N = 17)	87.48±21.89 (60.0—120.)	10.84±2.96 (4.4—14.9)	124.0±32.26 (72.5—153.)	541.4±256.8* (262—1180)

* Difference is significant at $p < 0.01$

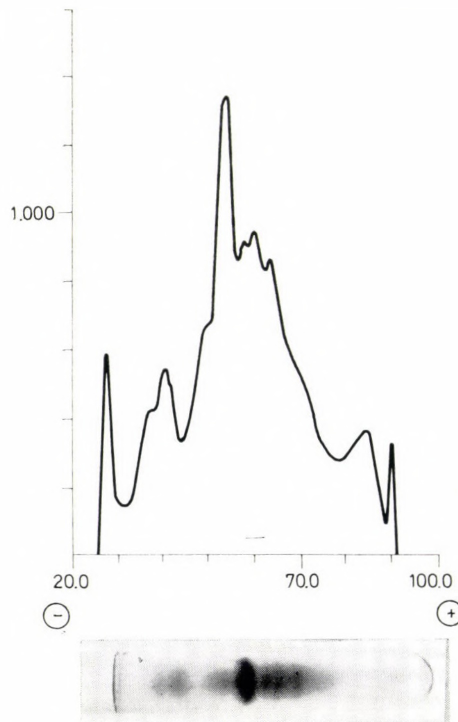

Fig. 1. SDS electrophoresis of proteins in concentrated urine of CGL patients. Heavy protein band in the middle of gel rod corresponds to molecular weight of 64 000 (—albumin). Further bands correspond to the molecular weights: 46 700; 36 300; 26 900; 13 000 (a shoulder); and 10 000. Bands preceding the albumin band correspond to molecular weights of 77 000, 90 000 and 103 000

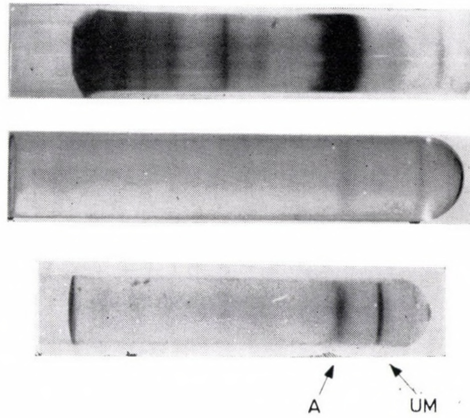

Fig. 2. Comparison of protein electrophoretic patterns of CGL serum (upper picture), normal human unconcentrated urine (middle picture), and unconcentrated urine of CGL patient (bottom picture). Electrophoresis was performed at pH 7.8. In the urine of a healthy subject a trace of albumin band (A) is only visible. In the urine of a patient with CGL the albumin band is more pronounced and a sharp band of acid glycoprotein (UM) is additionally present. The other serum proteins remain undetectable

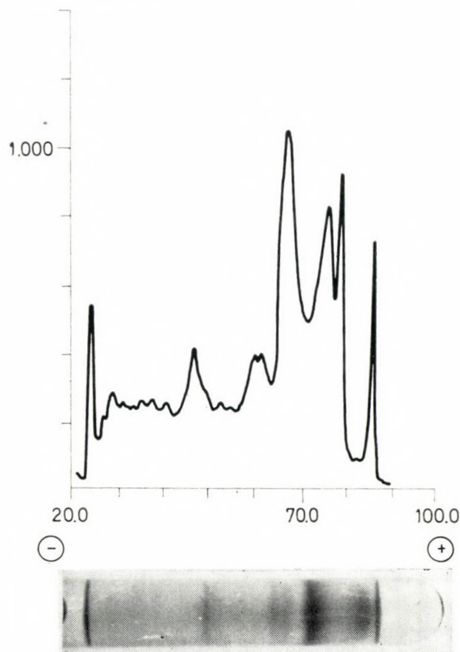

Fig. 3. Electrophoretic pattern of proteins from the concentrated urine of a patient with CGL. The fastest band was identified as acid $alpha_1$, glycoprotein followed by a diffuse band of prealbumin and a heavy band of albumin. The other bands were not identified. Electrophoresis was performed at pH 7.8

individual CGL subjects, but the albumin consisted of 36 to 52% of stained proteins. PAG electrophoresis at pH 7.8 showed the presence of albumin, acid $alpha_1$ glycoprotein, and in one third of the patients prealbumin (Figs 2 and 3). PAG electrophoresis at pH 4.1 showed the presence of a set of basic proteins visible as multiple protein bands (Fig. 4) including a broad zone of protein bands exhibiting RNase activity. LZM band was observed in the urine of three out of 17 CGL patients (Fig. 5).

Mean acid RNase activity in serum and urine of CGL patients was about 2.4 times higher than in the reference group (Table 2). Likewise, mean alkaline

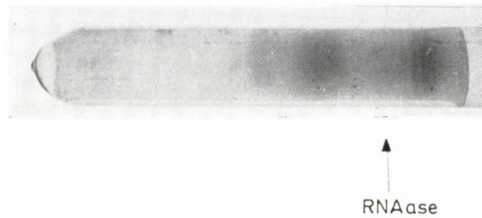

Fig. 4. Electrophoretic pattern of proteins from the concentrated urine of a patient with CGL. Electrophoresis was performed at pH 4.1, thus exhibiting urinary proteins of basic character. RNase activity corresponded to a broad zone between two diffuse protein bands

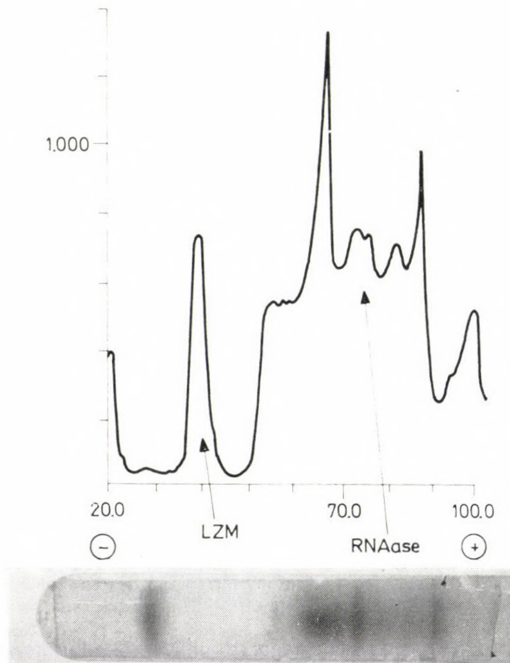

Fig. 5. Protein pattern obtained from concentrated urine of CGL patient with high LZM and RNase concentration. Electrophoresis was performed at pH 4.1

Table 2

Serum and urinary acid RNase activity, and urinary acid RNase excretion in normal subjects and patients with chronic granulocytic leukaemia

	Serum acid RNase activity $\bar{x}$ μ/ml ± SD (from — to)	Urine RNase activity $\bar{x}$ μ/ml ± SD (from — to)	Acid RNase apparent clearance value $\bar{X}$ ml min ± SD (from — to)	Acid RNase clearance to creatinine clearance ratio $\bar{x}$ ± SD (from — to)
Normal (N = 20)	0.45±0.13 (0.24—0.65)	1.84±0.94 (0.31—3.02)	2.43±0.36 (2.11—2.88)	0.0323±0.0161 (0.0023—0.0559)
CGL patients (N = 17)	1.13±0.50* (0.37—1.99)	4.86±2.33* (1.55—8.55)	4.00±1.11** (1.94—6.77)	0.0395±0.017 (0.012—0.065)

* Significant at $p \leq 0.01$
** Significant at $p \leq 0.05$

RNase activity was about 1.4 times higher than normal (Table 3). The level of RNase activity was somewhat less than that observed for a more numerous group of CGL patients previously studied [2, 26]. The acid RNase apparent clearance values in CGL patients were 1.5 times higher, and alkaline RNase clearance about 1.7 times higher than in normal subjects (Tables 2 and 3). The ratios of acid RNase clearance values to creatinine clearance values were in CGL patients about 1.2 of those in the controls.

Urinary acid and alkaline RNase activity in CGL patients (but not in controls) showed a correlation with the urinary protein level. A more pronounced correlation was found between the urinary protein level and the RNase apparent

Table 3

Serum and urinary alkaline RNase activity, and urinary alkaline RNase excretion in normal subjects and patients with chronic granulocytic leukaemia

	Serum alkaline RNase activity $\bar{x}$ μ/ml ± SD (from — to)	Urine alkaline RNase activity $\bar{x}$ μ/ml ± SD (from — to)	Alkaline RNase apparent clearance value $\bar{x}$ ml/min ± SD (from — to)	Alkaline RNase clearance to creatinine clearance ratio $\bar{x}$ ± SD (from — to)
Normal (N = 20)	1.44±0.47 (0.96—2.42)	2.13±0.88 (0.24—4.07)	0.97±0.31 (0.36—1.40)	0.0132±0.0084 (0.0013—0.0391)
CGL patients (N = 17)	1.93±0.61** (0.71—3.02)	3.38±1.67** (0.68—7.11)	1.71±0.77* (1.11—2.54)	0.0148±0.0076 (0.0070—0.0295)

* Significant at $p \leq 0.01$
** Significant at $p \leq 0.05$

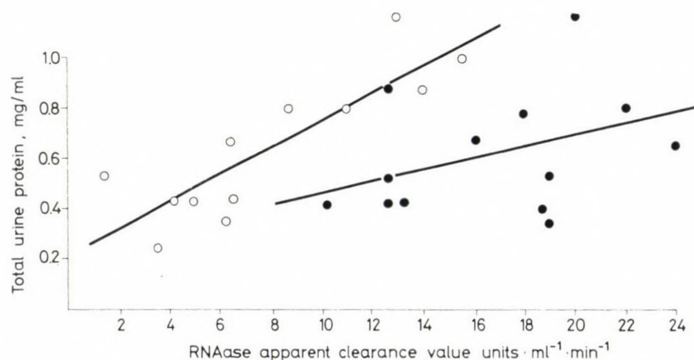

Fig. 6. Correlation between acid RNase clearance (white dots); alkaline RNase clearance (black dots); and urinary LMWP concentration in patients with CGL. Correlation coefficients were 0.547 and 0.392 for acid and alkaline RNase, respectively

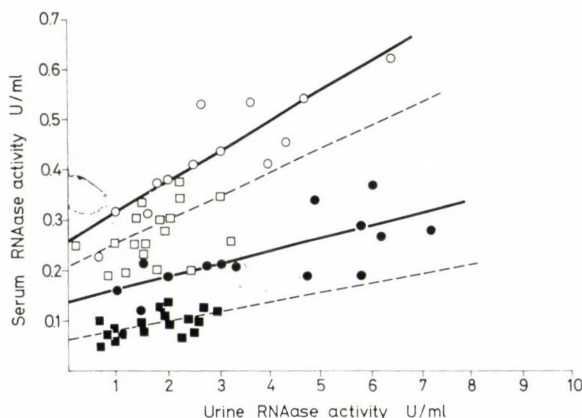

Fig. 7. Correlation between activities of acid and alkaline RNase in serum and urine of healthy subjects (black dots and black squares, respectively), and CGL patients (light dots and squares, respectively)

clearance values (Fig. 6). A correlation was observed between acid and alkaline RNase activity in serum and acid and alkaline RNase activity in urine. There were, however, two different relationships between the activities of serum acid and alkaline RNase and the activities of these enzymes in the urine of controls and CGL patients (Fig. 7).

The obtained results showed some parallelism between the increase in urinary LMWP level and urinary RNase activity, suggesting that both phenomena represent the same process of release to plasma and further renal filtration of LMWP deriving from CGL granulocytes.

Discussion

The present results have confirmed former findings [2, 17, 26] and extended our data on the mechanism of increase of acid RNase in urine and serum of CGL patients. The obtained result suggested that the augmentation of RNase activity in urine, and conceivably in plasma of CGL patients represented a more general phenomenon of increase in concentration of the entire set of LMWPs filtered into the urine from the plasma. In other studies on CGL, an increase was observed in the plasma concentration of LZM [4, 11, 15, 20], RNase [1, 2, 26], vitaminB_{12} binding protein [4, 6]. All these proteins belong to LMWP fraction removed from plasma by renal filtration [8, 12, 14, 19, 22, 25], thus the proteinuria described in this study fulfils the criteria of overflow proteinuria [19, 23, 26]. The primary cause of this type of proteinuria lies beyond the kidneys. As RNase is one of the excreted proteins, an increase in urinary RNase activity seems to presage the occurrence of this type of proteinuria in CGL. The proteinuria observed should not be confused with kidney malfunction manifested as nonselective proteinuria and a decrease in glomerular filtration rate, which may occur in the late phase of CGL [10, 11, 24]. This type of proteinuria would be detectable by protein precipitation tests, and much higher urinary protein concentrations [14, 24].

At normal glomerular structure, urinary protein concentration depends primarily on their reabsorption rate in the tubules [8, 19, 23]. Tubular reabsorption and transport may occur with LMWP loads exceeding many times their normal level [5]. A pronounced overflow proteinuria in certain haematological malignancies as in myeloma with Bence-Jones proteinuria [7, 9], Fe proteinuria and lysozymuria in myelomonocytic leukaemia [16] are well known for their damaging effect in the kidneys. It is conceivable that increased levels of LMWP in the glomerular filtrate of CGL patients lie below the level of nephrotoxicity. Heavy, and possibly harmful renal overloading with LMWP, as observed after X-ray therapy of CGL [28, 29, 30] is temporary and does not necessarily cause permanent impairment of the kidneys.

The clinical importance of LMWP proteinuria in CML may relate to the conditions causing an increase in the plasma LMWP level. Correlations between the leukaemic cell count and the plasma levels of LZM, RNase, vitamin B_{12} binding protein and basic granular proteins [1 – 4, 6, 17, 20, 26] suggest a leukocytic origin of many LMWPs in the plasma of CGL patients. Accumulation of granulocytes in CGL may cause a release of leukocytic proteases producing degradation of tissue proteins which then contribute to the plasma LMW pool. Both considered mechanisms concern a not yet well understood problem of interactions between CGL granulocytes and the leukaemic host, which deserves further investigation.

Acknowledgements

The author is greatly indebted to Dr. A. Skotnicki and Dr. Teresa Wolska from the Clinic of Haematology, Institute of Internal Medicine, Medical Academy Kraków, for the blood and urine samples of CGL patients, and to Mrs. Grazyna Jachym for her excellent technical assistance.

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Haematologia 20 (2), pp. 101–108 (1987)

Platelet Reactions in Acute *Plasmodium Berghei* Infection in Swiss Albino Mice

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(Received 6 December, 1985; accepted 7 May, 1986)

Swiss albino mice were infected by the intraperitoneal route with *P. berghei berghei* malaria parasite, and platelets, white cell counts and some coagulation parameters were monitored in order to find out whether changes reported in man also occurred in the mice. Parasitaemia developed form the 2nd post-infection day and reached significant levels by the 4th–6th day. Reduced circulating platelets which reached severe thrombocytopenic levels were observed. parallel with the increasing degree of parasitaemia. Anaemia which progressed to severe degree was also observed as was a slight leucocytosis attributed to the presence of normal mouse erythrocytes in the peritoneal space.

All untreated animals died by the 6th day of infection. Intramuscular chloroquine sulphate (20 μg/g body wt.) given for 7 days completely cured the malaria, and white cell and platelet counts were restored to preinfection levels in each animal about 2 weeks after treatment had ceased. Platelet hypersensitivity to exogenous ADP was observed within 48 hours of infection and persisted with the parasitaemia. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were prolonged while clottable fibrinogen concentration was reduced.

Keywords: malaria parasite, mouse platelet, parasitaemia, acute malaria

Introduction

The causes of thrombocytopenia or reduced platelet counts frequently encountered in acute *Plasmodium falciparum* or *P. vivax* malaria infections in man are still unsettled. Disseminated intravascular coagulation (DIC) (Devakul et al., 1966; Dennis et al., 1967) and immunological changes (Shulman et al., 1970; Beale et al., 1972; Srichaikul et al., 1975; Kelton et al., 1983) have been suggested to account for these observations, but they have been disputed by others (Essien et al., 1979; Horstman et al., 1981). Since it could still be validly argued that the platelet changes described could not in all instances be attributed to malaria infection as the studies were not always properly controlled, it was deemed necessary to conduct a properly controlled study that would answer these criticisms. A search for a readily available and cheap animal model which shows the platelet changes during acute malaria infection was therefore undertaken. This communication reports our findings.

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VNU Science Press, Utrecht
Akadémiai Kiadó, Budapest

Materials and Methods

Animals: Male and female inbred Swiss albino mice aged 7 – 10 weeks, each weighing 22 – 30 g and maintained at the Central Animal House, University College Hospital, Ibadan were used in the experiments. All animals were fed ad libidum with standard pellet diet and unrestricted water. A total of 202 animals were used in the study. Of this number, 172 were used in the platelet aggregation and blood coagulation studies of which only 7 infected mice died since they were sacrificed by the 5th day. It had earlier been observed that the animals usually died by the 6th post-infection day.

Infection. Each mouse was infected intraperitoneally with 0.15 ml of *P. berghei berghei* from an infected mouse blood diluted 1 in 3 (v/v) with sterile saline (Essien et al., 1984). It was calculated that an inoculum size of about 1.5×10^8 parasitized red cells per ml of blood was administered to each mouse.

Parasitaemia. Parasite levels in each animal were determined by examination of Leishman-stained blood smears and were expressed as percentage of the number of parasitized red blood cells, including multiple parasitized cells, seen per 1000 red cells.

Platelet and white cell (WBC) counts. A total of 30 animals were studied for these parameters. They were divided into 3 groups for this purpose. Group A comprised 16 animals infected with *P. berghei berghei* as described above. In group B, 7 animals received blood similarly treated as for the infected mice but the blood injected was from uninfected mice. These animals were treated as those in group A and served as placebo control group. Group C animals (7) were plain control mice fed normally.

Platelet and WBC counts were performed by standard methods (Dacie & Lewis, 1975) and counts were by phase contrast microscopy.

Malaria treatment. After parasitaemia had been established at about 14 – 18 %, usually from the 4th post-infection day, chloroquine sulphate (20 μg/gm body wt.) was administered intramuscularly daily for 7 days to each animal in group A. Preliminary studies had shown that 10 – 20 % parasitaemia and related thrombocytopenia existed by this time and indicated that chloroquine treatment for 4 days (Inyang & Essien, unpublished observations) did not give complete clearance of parasites in blood films. After treatment, thrombocyte counts were monitored until they returned to normal (pre-infection) levels. All the animals in this group survived.

Coagulation studies. Infected animal samples were normally collected and tested 5 – 6 days after infection when parasitaemia had usually attained the 24 – 36 % levels. After lightly anaesthesizing each animal with diethyl ether, blood for coagulation studies was collected by cardiac puncture and was mixed with trisodium citrate (9 : 1 v/v, blood : citrate). Platelet-poor plasma (PPP) was prepared as described below. Coagulation tests carried out included prothrombin time (PT), activated partial thromboplastin test (APTT) and clottable fibrinogen by the clot weight method (Hardisty et al, 1982). For these tests, blood from 4 – 7 animals were pooled. A total of 24 experiments was carried out.

Preparation of platelet-rich and platelet-poor plasma (PRP and PPP)

Blood samples for platelet aggregation studies were collected into heparinized polypropylene tubes at 15 – 20 μ/ml of blood because in a previous study (Rosenblum et al, 1983) it was reported that platelet aggregation was more regular and platelet counts were higher in heparinized than in citrated PRP. The pooled blood was centrifuged at 100 *g* at 23 – 25 °C for 30 min. the PRP was separated using none-contact process and kept at room temperature (18 – 25 °C) before use, usually within 1 hour of blood collection. The remaining blood was recentrifuged at 2600 *g* for another 20 minutes to obtain PPP, which was used to adjust the PRP to a platelet count in the $400-600\times10^3/\mu l$ range.

Platelet aggregation studies and release of labelled serotonin

Platelet aggregation was tested in the PRP sample at 37 °C using a Payton Aggregometer module (Model 800B) (Payton Associates, Scarborough, Canada). Each sample was stirred at 1000 rpm and stimulated with ADP (Sigma, St. Louis, U.S.A.). The points of minimum and maximum light transmission for each sample were preset using PRP and the corresponding PPP, respectively.

Uptake and release of ^{14}C-serotonin (^{14}C-5HT, Radiochemicals, Amersham U. K.) were studied in mice PRP. The pooled platelets were labelled by incubating PRP for 20 – 40 minutes with 0.06 uCi 1^{4}C-5HT/ml (Nunn, 1981). Samples were processed for measurement of uptake and release as described previously (Greenberg et al, 1975).

Results

Platelet and white cell counts

There was no significant difference in the platelet and WBC counts between the two control groups (the placebo and ordinary control animal groups) Fig. 1.

There was a rapid drop in platelet count with increasing parasitaemia which led to death in about 6 – 7 days if the animal was untreated. In animals on 7 days treatment course, the drop continued even after 4 days of chloroquine therapy, although the rate of fall was less steep. Three days after treatment had been completed, the platelet count started to rise. The difference between normal platelet count (day 0) and the lowest value (day 11) was significant statistically ($844.2\pm46.8\times10^9/L$ *vs* $317.5\pm62.8\times10^9/L$; $P<0.005$; $t=26.99$). The platelet counts returned to baseline values 16 days after chloroquine treatment had stopped i.e. on day 27 of observation. The difference between the counts on days 0 and 27 (844.2 ± 46.8 & $10^9/L$ on day 0 and 812.0 ± 82.8 & $10^9/L$ on day 27) was not significant.

With respect to WBC levels, the mean count in the infected animals on post-infection day 4 ($14.38\pm104\times10^9/L$) was significantly higher than the baseline

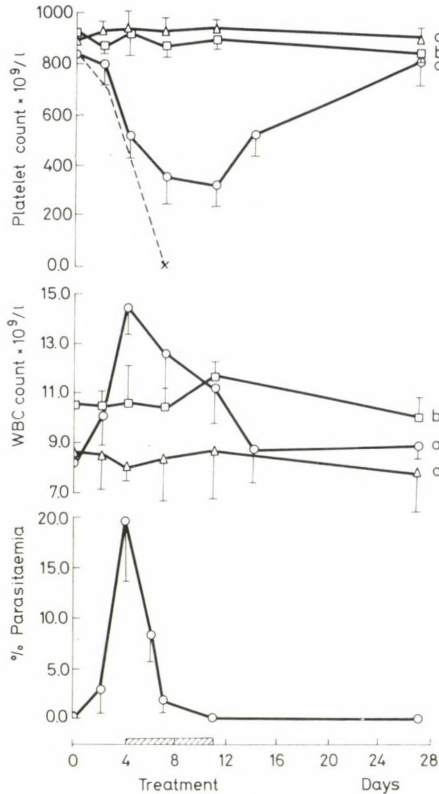

Fig. 1. Summary of changes in platelet and white blood cell counts with *p. berghei* parasitaemia. *Upper diagram:* a) platelet count in infected mice before, during and after treatment. b) platelet count in sham-infected (placebo) mice. c) platelet count in plain control mice. platelet count in untreated mice ...... denotes point of death of animals. *Middle diagram:* describes changes in white cell counts in the same groups of animals as described above. *Lower diagram:* changes in parasitaemia with treatment

value of $8.20 \pm 1.28 \times 10^9/L$) ($P < 0.005$: $t = 14.99$). The WBC count dropped during chloroquine treatment and reached pre-infection levels 3 days after treatment had stopped. There was no significant difference in the WBC counts between the 2 control groups.

Blood coagulation

The mean PT in the infected animal group was significantly longer than that in the control animal group. The mean APTT value of the infected mice was similarly longer than that of the control group. A decrease in the clottable fibrinogen weight in the infected mice was observed (Table 1).

Table 1

Summary of blood coagulation test results on *P. Berghei* infected mice and normal mice

Prothrombin time (seconds)		APTT (seconds)		Clottable fibrinogen weight (mg/dl)	
Control	Infected	Control	Infected	Control	Infected
8.13±1.94 (n = 8)	11.70±2.55 (n = 10)	29.0±7.2 (n = 11)	45.5±12.5 (n = 13)	253.3±41.5 (n = 10)	153.9±50.0 (n = 13)
P < 0.005; t = 3.27		P < 0.005; t = 5.08		P < 0.005; t = 5.03	

Values given for PT and APTT tests are mean duplicate determinations.

Mean % Parasitaemia in infected animals used was 39.3±20.1 (101 animals were used in this investigation).

n = no. of experiments. The limiting factor in the no. of experiments performed was the volume of plasma available

Platelet aggregation and serotonin release

With respect to platelet aggregation stimulated by low ADP concentration (0.5μM final concentration), infected PRP showed significantly increased aggregation response in infected compared to that of control animals (Table 2). With 1.0 μM ADP, however, the difference in the aggregation response of the two groups was not significant (Table 2).

Table 2

Results of platelet aggregation and serotonin release studies on *P. Berghei* infected and control mice (PRP)

	ADP			Collagen	
	% Aggregation		% Release	% Aggregation	% Release
	0.5 μM	1.0 μM			
Control	20.7±6.96 (n = 5)	37.2±15.98 (n = 4)	1.20±0.81 (n = 5)	43.5±6.2 (n = 10)	49.20±1.8 (n = 10)
Infected	38.45±12.86 (n = 6)	47.14±12.10 (n = 5)	1.41±1.03 (n = 6)	45.3±4.7 (n = 12)	48.8±4.3 (n = 12)

For 0.5 μM ADP-induced aggregation, there is a significant difference in % aggregation between the control and infected groups (P < 0.01; t = 2.83).

Mean % Parasitaemia in animals used for these experiments was 19.2±6.81.

n = no. of experiments.

% Aggregation values given are (Mean ± 1SD.)

Discussion

It has been shown in these studies that thrombocytopenia set in rapidly in Swiss albino mice experimentally infected with the malaria parasite, *P. berghei berghei*. This effect was reversed, although slowly, following treatment with chloroquine sulphate. As earlier demonstrated with *P. falciparum* infection in man, (Essien et al., 1981), the mice platelets (as PRP) also manifested a hypersensitivity response to exogenous ADP when compared to the response of the control group of the same mice colony. Thus the Swiss albino mouse is another suitable animal model which can be used in the study of platelet/malaria parasite interactions in addition to the golden hamster earlier described (Essien et al., 1984) but which proved more expensive and difficult to obtain and breed successfully in required numbers in certain circumstances.

Several problems were, however, encountered with the use of the mouse model. These include limited blood volume in each animal, a tendency of the infected mouse erythrocytes to haemolyse during centrifugation, and the severe anaemia which affected the animals when parasitaemia exceeded 30%. The latter factor would affect the blood/citrate ratio which had earlier been shown to account for increased platelet sensitivity to ADP in anaemic conditions (Packham et al., 1978).

This problem was overcome by choosing a level of parasitaemia which caused relatively mild anaemia but with adequate platelet changes in mice. The problem of haemolysis of infected erythrocytes during PRP and PPP preparations was overcome by careful handling of the blood. Pooling of blood from about 5 animals on each occasion ensured adequate PRP volume for the platelet function experiments. Platelet counts in PRP from infected and control groups of mice were equilibrated with corresponding PPP.

Blood coagulation parameters determined in the infected and control animals gave results which suggested co-existing consumptive coagulopathy in the infected animals. DIC mechanisms had earlier been suggested as being responsible for the thrombocytopenia associated with *P. falciparum*, *P. malariae* and *P. vivax* infections in man (Devakul et al., 1966; Dennis et al., 1967; Borochovitz et al., 1970), although more recent studies (Essien et al, 1976; 1979; Horstman et al, 1981) did not support such a mechanism in man. By contrast, increased platelet lysis was observed in association with hypersensitivity to exogenous ADP during acute *P. falciparum* infection (Essien and Ebhota, 1983). Although the mechanism(s) involved remained to be clarified, it was suggested that those findings could adequately account for the reduction in human circulating platelets observed in the disease in a minority (5%) in whom it reached severe thrombocytopenic levels (Essien et al., 1979).

Factors responsible for increased leucocyte counts observed in this and in an earlier report (Essien et al, 1984) remain unclear. Leucocytosis had been found in rats and mice with *P. berghei* infection mainly because of an increase in peripheral blood monocytes (Singer, 1954). Wellde (1971a, b; 1972) suggested that the leuco-

cytosis, which normally manifested as monocytosis in malaria infections, correlated with stimulation of the reticuloendothelial system to handle malaria pigments and the products of accelerated destruction of infected and non-infected erythrocytes.

Acknowledgements

We are grateful to the Staff of the Animal House, Biode Building, College of Medicine for the supplies of mice. Generous funds were provided to E. M. E. by the Medical Research Council U. K. (G975/195) and in part by the Federal Ministry of Science, Education and Technology, Lagos.

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Haematologia 20 (2), pp. 109—115 (1987)

Altered Monocyte Functions in Patients with Angioimmunoblastic Lymphadenopathy

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(Received 22 April, 1986; accepted 7 July, 1986)

Monocyte function was investigated in ten patients with angioimmunoblastic lymphadenopathy (AILD). Although spontaneous migration, phagocytosis and opsonisation of monocytes were unimpaired, the chemotactic response and erythrocyte-antibody-rosette (EA-rosette) formation were decreased significantly. The migratory response of normal monocytes was inhibited on preincubation with serum from AILD patients. It is suggested that the immune abnormality in AILD, previously thought to involve T—B and natural killer lymphocytes, extends to the monocyte-macrophage system.

Keywords: monocyte chemotaxis, Fc receptor function, angioimmunoblastic lymphadenopathy

Introduction

Angioimmunoblastic lymphadenopathy (AILD) is a recently described uncommon lymphoproliferative disorder that is characterized clinically by fever, sweating, weight loss, generalised lymphadenopathy, hepatosplenomegaly, and skin rash [1, 2, 3]. Coombs' positive autoimmune haemolytic anaemia, polyclonal gammopathy, eosinophilia, lymphopenia, cryoglobulinaemia and hypocomplementaemia have been described as laboratory features [4, 5].

The lymph nodes show a characteristic histology [1, 2, 3, 5]. The condition runs a variable clinical course and may respond to steroids, but often proceeds to lymphoma [1, 3, 5, 6, 7].

Immunologically, a decrease in the number of T-lymphocytes, negative skin tests and a polyclonal proliferation of B-lymphocytes can be observed [3, 5, 8, 9]. Garam et al. [10] found a decreased natural cytotoxicity, while Coupland et al. [11] established an elevated circulating immune complex in their patients.

In the present report, the monocyte function was studied in ten patients with AILD. Monocytes from AILD patients exhibited a marked defect in chemotactic function and in EA-rosette formation. These defects may contribute to the depressed immune response in patients with AILD. Thus, the immune defect in AILD, previously thought to involve mostly the T and B lymphocytes [4, 9, 10, 12], extends to the monocyte-macrophage system too.

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Materials and Methods

Patients

Ten patients with AILD (5 women and 5 men) were studied. Their age ranged from 31 to 62 (mean 54.5) years. Lymph node biopsy was performed in all cases. In the active state the patients were treated with 100 – 500 mg prednisone daily or with polychemotherapy (vincristine, cyclophosphamide, prednisone). Laboratory investigations were made before starting the treatment. Ten healthy volunteers were used as controls.

Preparation of monocytes

Monocytes were separated from 10 ml heparin anticoagulated venous blood sample on Ficoll-Uromiro gradient [13]. After washing, the cells were suspended in 1 ml of medium TC-199 and counted with a standard haemocytometer. Trypan blue viability and neutral red pinocytosis were performed on the cells to determine the percentage of phagocytic cells and their viability.

Phagocytosis test

A monolayer technique was used with the cells adherent to a glass slide in Boyden chamber. After washing, the monocytes ($0.5 - 1.0 \times 10^5$ cells) were incubated with 25 μl of a 0.2% suspension of baker's yeast containing 5×10^6 particles, together with 0.5 ml TC-199. Incubation was done at 37 °C for 60 min in 5% CO_2 and 100% humidity with constant rocking. After washing, the monolayers were stained with Türk's stain, and cells containing ingested particles were counted. The percentage of cells ingesting or binding particles, and also the number of yeast particles ingested per cell (PI = phagocytic index) were determined [14].

Monocyte opsonisation

Monolayers of adherent monocytes were incubated as above at 37 °C for 60 min with 25 μl of 2×10^8 particles/ml yeast which had been treated with 0.1 ml of AB human serum as opsonin and washed. After washing and staining, the percentage of cells ingesting or binding yeast particles and the number of yeast particles ingested per cell were determined (PI = phagocytic index) [14].

EA-binding of monocytes

Monocyte Fc receptor activity was assayed by adherence to it of sheep red blood cells (SRBC) coated with a subagglutinating dilution (1 : 128) of an IgG fraction isolated on Sephadex G-200 from a rabbit anti-SRBC serum. The adherent monocytes were incubated with 0.2 ml of 2% sensitized SRBC in 0.3 ml of medium TC-199 at 37 °C for 30 min. After washing, the percentage of cells binding or ingesting 3 or more SRBC was determined [14].

Chemotactic assay and evaluation

Chemotactic factor. The complement derived chemotactic factor (C_{5a}) was produced by incubation of 10 mg E. coli endotoxin (O26 : B_6 Difco) with 10 ml normal AB serum and 90 ml Parker's medium at 37 °C for 30 min then at 56 °C for 30 min. The chemotactic solution was kept at −20 °C for 8 weeks.

Simple disposable Boyden-chambers modified in our laboratory [15, 16] were used. The cell suspension was placed in the upper compartment of the chamber, separated by a 5 μl millipore filter (Sartorius Membranfilter GmbH, FRG) from the 1 ml chemotactic solution in the lower compartment.

Parker's 199 medium was substituted for the chemotactic solution to measure spontaneous migration. After filling and closing the gaps with warm paraffin, incubation followed at 37 °C for 90 min. The membranes were then stained with haematoxylin and evaluated by measuring in μm-s the average distance travelled by the leading front cells in ten microscopic fields (63 × 8 × 0.8) in each filter [15]. In order to investigate the role of serum factors, mononuclear leukocytes were suspended in 1 ml heated test serum and then incubated at 37 °C for 30 min.

Results

Phagocytosis and EA-rosette formation of monocytes

In Table 1 it is shown that the phagocytosis of monocytes is the same in patients with AILD as in controls. No significant differences were found between patients and controls in their ability to bind or phagocytose opsonised yeast. Monocytes from AILD patients showed, however, a significantly lower capacity of EA-rosette formation than did the monocytes of healthy subjects.

Table 1

Functions of monocytes in AILD patients and controls

Functions	Healthy controls (n = 10)	Patients (n = 10)	p
Chemotaxis (μm)	63.2±4.1	34.7±6.84	< 0.001
EA-rosette (%)	57.6±7.3	41.4±7.5	< 0.001
Opsonisation (PI)	3.6±0.52	3.6±0.27	N. S.
Phagocytosis (PI)	1.0±0.17	1.07±0.35	N. S.

Defective monocyte migration in AILD

Using the complement derived chemotactic stimuli, the chemotactic function of monocytes from AILD patients was compared with that of healthy subjects. As shown in Fig. 1, peripheral monocyte chemotaxis was markedly reduced in

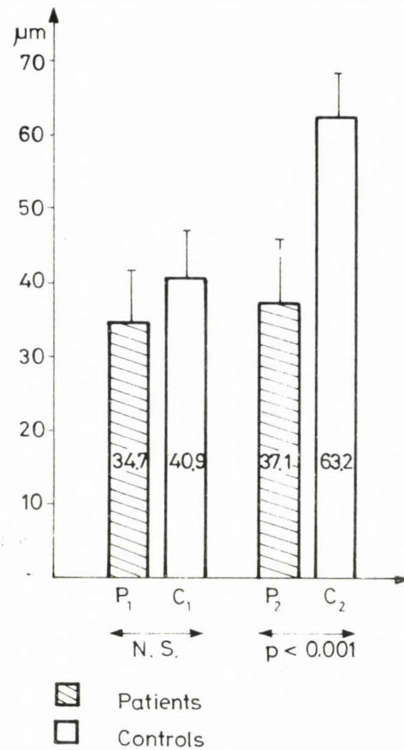

Fig. 1. Spontaneous migration and chemotaxis of monocytes P_1: spontaneous migration of monocytes in patients with AILD; P_2: chemotaxis of monocytes from AILD patients; C_1: spontaneous migration of control monocytes; C_2: chemotaxis of control monocytes

the AILD patients as compared to the controls ($p < 0.001$). In contrast, there were no significant differences in spontaneous migration between patients and healthy controls.

Effect on chemotaxis of serum from AILD patients

To determine whether a serum factor was responsible for the inhibition of monocyte migratory function in the AILD patients, monocytes from healthy control subjects were preincubated with serum from the AILD patients and the healthy subjects, and then tested for chemotactic function. As shown in Fig. 2, the migratory response of normal monocytes to C_{5a} was significantly inhibited by preincubation with serum from AILD patients. The chemotaxis of monocytes of AILD patients remained, however, unchanged after preincubation with control sera.

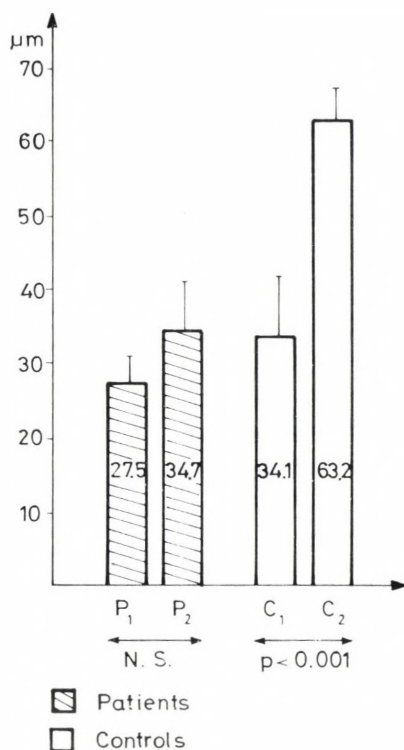

Fig. 2. Effect of sera on chemotaxis of monocytes P_1: chemotaxis of monocytes with AILD patients; P_2: chemotaxis of monocytes with AILD patients after preincubation with control sera; C_1: monocyte chemotaxis in healthy subjects after preincubation with AILD serum; C_2: monocyte chemotaxis in healthy controls

Discussion

We investigated the chemotactic capability, phagocytosis, and EA-rosette formation of peripheral blood monocytes in patients with AILD. In contrast to the chemotactic responses of monocytes from healthy subjects, monocytes from patients with AILD exhibited reduced "directed migration" to the complement derived chemotactic factor (C_{5a}), while the random migration of patients' monocytes exhibited no significant decrease in activity in the absence of chemoattractant when compared to the control subjects (Fig. 1, Table 1).

The defect in migratory capacity could contribute to the reduced immune-mediated inflammatory reaction to some infective organisms that were observed in patients with AILD. For example, M. tuberculosis, which occurs in AILD patients, elicits minimal or no granulomatous reaction [17], (unpublished observation). In addition, defective T cells may not generate sufficient quantities of

lymphokines, such as the macrophage activating factor, to recruit adequate monocytes/macrophages to the inflammatory foci [18, 19, 20].

Although, the mechanisms responsible for the suppressed monocyte migratory function are unclear, a number of factors warrant further investigations: reduced numbers of membrane chemoattractant receptors, prior in vivo downregulation, reduced transmission of the chemotactic signal, and/or serum factors. In this relation, Snyderman et al. [21, 22] have shown that a low molecular weight material from tumour cells and certain oncogenic murine leukaemia viruses inhibit macrophage migration. While trying to find the reasons for the diminished chemotaxis, the effect on chemotaxis of AILD serum are still being studied. In our investigations, sera from AILD patients inhibited the chemotaxis of monocytes in healthy control subjects, and we suppose that the abnormally elevated circulating immune complex level plays a role in the impairment of monocyte chemotaxis in AILD [11] (unpublished observation). Besides, the effect of the immune complex may have other causes such as unidentified inhibitory factors, which also impair the function of monocytes [23].

The present study demonstrated the intact phagocytosis and opsonisation of yeast particles by monocytes in patients with AILD. Still, monocytes from patients with AILD reacted with antibody-coated SRBC in a significantly lower degree than did normal monocytes, indicating a decreased monocyte IgG Fc receptor activity. The Fc receptor has an important role in the ingestion of IgG antibody-antigen complexes [14]. Thus the impaired Fc receptor function and the decreased monocyte chemotaxis may decrease the elimination of immunogenic molecules and immune complexes in AILD. The permanent and increased production of antibodies and pathologic immune complexes add to the depression of monocyte function in AILD.

Regardless of whether or not suppressed monocyte chemotaxis is a primary or secondary event in patients with AILD, the inability of peripheral blood monocytes to migrate to inflammatory stimuli and to chemotactic products of infectious organisms could contribute to the inadequate host response to certain infectious organisms and neoplasms characteristic of this disease.

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Haematologia 20 (2), pp. 117—119 (1987)

Short Communication

Lupus Anticoagulant and False Positive Serological Tests For Syphilis

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(Received 30 April, 1986, accepted 7 July, 1986)

A 41-year old male who presented for blood donation for the first time was rejected on the grounds of a positive reaginic serological test (VDRL and RPR) and a specific antitrepomena test (FTA) for syphilis. Later studies revealed the existence of a lupus anticoagulant and this was made responsible for the positive serology. Our observation suggests the need to search for the existence of a lupus anticoagulant in blood donors with non-specific or specific positive antitreponema test.

Keywords: lupus anticoagulant, syphilis

Introduction

In spite of excellent methods for the diagnosis and testing of syphilis, the disease is still widespread. Accordingly, to perform serological tests for syphilis in blood donors is a regular practice of the health services of different countries; positive findings constitute justification for rejecting the possible donor. Invasion of a human host by Treponema pallidum leads to the production of non-specific antibodies (reaginic or anticariolipin antibodies) directed against lipoidal antigens of the treponema or against a lipoidal antigen resulting from host-parasite interaction [1]. Unfortunately, the cardiolipin antigens used in the detection of reaginic antibodies yield a large number of false-positive cases in many conditions other than syphilis [2]. Sometimes, the presence of cardiolipin antibodies manifest as an inhibitor of blood coagulation, named lupus anticoagulant [3], an immunoglobulin which reacts with platelet wall phospholipids and interferes with the phospholipid portion of the prothrombin activator complex [4]. The presence of a lupus anticoagulant and other anticariolipin antibodies are interpreted as markers of hypercoagulability [5, 6].

In the present report we underline the need for investigating the presence of a lupus anticoagulant as a factor for positivity in serological syphilis test, even in a specific treponemal test such as the fluorescent treponemal antibody (FTA), in donor patients before starting specific treatment.

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Report of a Case

A 41 year old male patient with no history of haemorrhagic episodes or thromboembolic disease presented voluntarily for the first time to donate blood. The man was rejected on the basis of positive findings in the reaginic serological test according to the Veneral Disease Reference Laboratory (VDRL) and rapid plasma reagin (RPR). Additionally, a specific antitreponema test (the fluorescent treponemal antibody (FTA)) proved to be positive. Rheumatoid factor, antinuclear and antimitochondrial antibodies were negative. The serological study of his wife was negative. The patient received treatment with penicillin. One year later, in view of the persistent positivity of the syphilis serological test, complementary studies were performed with the following results: Platelet count: 321 000; prothrombin time: 15 sec (control 12); thromboplastin time: 56 sec (control 28); thrombin time and fibrinogen were normal; the tissue thromboplastin inhibition test [7], which explores the existence of a lupus anticoagulant, was positive (dilution 1 : 500: 195 sec, patient plasma vs 90 sec of normal plasma). At this time a specific antitreponemal test was performed (Treponema pallidum immobilization, (TPI)) which proved to be negative.

Discussion

The existence of false-positive results in the reaginic syphilis tests due to the presence of anticardiolipin antibodies is an established fact in very diverse clinical situations, such as autoimmune diseases, pulmonary hypertension, thromboembolic diseases, women with repeated miscarriages and even in asymptomatic subjects [2–4]. On the other hand, the existence of false-positive results in a specific antitreponemal antibody test such as FTA, is less well known. Also, the incidence of anticardiolipin antibodies is extremely high in subjects with lupus anticoagulant [8], and the incidence of false specific tests is unknown. Furthermore, patients with syphilis do not have lupus anticoagulant [9]

In view of the fact that our patient presented a specific and non-specific serology of positive syphilis, he was diagnosed and treated accordingly. The fact that we found lupus anticoagulant led us to reconsider the diagnosis and the negativity of the TPI test provided no conclusive proof of a past or present treponemal infection.

Our observations suggest the need to search for the existence of a lupus anticoagulant in blood donors with nonspecific (VDRL or RPR) or aspecific (FTA) positive antitreponemal test as the mechanism responsible for these reactions; in this way the subject may consider himself or herself free from personal and family problems and may also avoid additional and unnecessary therapy, as was the case with our patient.

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Haematologia 20 (2), pp. 121—124 (1987)

Abstracts

Control of heme synthesis during Friend cell differentiation: Role of iron and transferrin. J. D. Laskey, P. Ponka and H. M. Schulman (Lady Davis Institute for Medical Research of the Sir Mortimer B. Davis-Jewish General Hospital, Montreal, Quebec, Canada). *J. Cell. Physiol. 129*, 185 (1986).

In many types of cells the synthesis of δ-aminolevulinic acid (ALA) limits the rate of heme formation. However, results from our laboratory with reticulocytes suggest that the rate of iron uptake from transferrin (Tf), rather than ALA synthase activity, limits the rate of heme synthesis in erythroid cells. To determine whether changes occur in iron metabolism and the control of heme synthesis during erythroid cell development Friend erythroleukemia cells induced to erythroid differentiation by dimethylsulfoxide (DMSO) were studied. While added ALA stimulated heme synthesis in uninduced Friend cells (suggesting ALA synthase is limiting) it did not do so in induced cells. Therefore the possibility was investigated that, in induced cells, iron uptake from Tf limits and controls heme synthesis. Several aspects of iron metabolism were investigated using the synthetic iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH). Both induced and uninduced Friend cells take up and utilize Fe for heme synthesis directly from Fe-SIH without the involvement of transferrin and transferrin receptors and to a much greater extent than from saturating levels of Fe-Tf (20 μM). Furthermore, in induced Friend cells 100 μM Fe-SIH stimulated 2-^{14}C-glycine incorporation into heme up to 3.6-fold as compared to the incorporation observed with saturating concentrations of Fe-Tf. In contrast, Fe-SIH, even when added in high concentrations, did not stimulate heme synthesis in uninduced Friend cells but was able to do so as early as 24 to 48 h following induction. In addition, contrary to previous results with rabbit reticulocytes, Fe-SIH also stimulated globin synthesis in induced Friend cells above the level seen with saturating concentrations of transferrin. These results indicate that some step(s) in the pathway of iron from extracellular Tf to protoporphyrin, rather than the activity of ALA synthase, limits and controls the overall rate of heme and possibly hemoglobin synthesis in differentiating Friend erythroleukemia cells.

A. Egyed

Comparative study of iron mobilization from haemosiderin, ferritin and iron (III) precipitates by chelators. G. J. Kontoghiorghes, S. Chambers and A. V. Hoffbrand (Department of Haematology, Royal Free Hospital School of Medicine, Hampstead, London, UK). *Biochem. J. 241*, 87 (1987).

The heteroaromatic chelators, 1,2-dimethyl-3-hydroxypryrid-4-one, maltol, mimosine and 2,4-dyhydroxypyridine-N-oxide, have been shown to mobilize iron from human spleen haemosiderin, ferritin and also from iron (III) precipitates, all containing equal amounts of iron, at physiological pH. In the case of almost every chelator, the least-solubilized polynuclear iron form was ferritin, whereas haemosiderin was more soluble and the iron (III) precipitate the most soluble of all. Most of the chelators were more efficient than desferrioxamine at releasing

iron from ferritin, but less efficient in the removal of iron from the other two polynuclear iron forms. It is suggested that the chelator differences in iron mobilization may be related to variations in the chelator molecular structure, the protein structure, iron forms and in the mechanism of iron release.

A. Egyed

Substrate-induced conformational change of human erythrocyte glucose transporter: inactivation by alkylating reagents. A. L. Rampal and Ch. Y. Jung (Biophysics Laboratory, Veterans Administration Medical Center, Buffalo, N. Y, USA). *Biochim. Biophys. Acta 896*, 287 (1987).

The glucose transport carrier in human erythrocyte membranes, when transporting glucose, undergoes a conformation change. In an attempt to delineate the extent of this substrate-induced conformational change, transport incativation by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, N-ethylmaleimide, iodoacetamide, and 2,4,6-trinitrobenzenesulfonic acid was examined in the presence and in the absence of d-glucose. All these alkylating agents inactivated the carrier. With each of these reagents, with the exception of trinitrobenzenesulfonic acid, d-glucose modified the rate of inactivation as well as the activation enthalpy (ΔH) of the inactivation. The inactivation by trinitrobenzenesulfonic acid was not affected by the sugar. Based on these findings, it is suggested that the substrate-induced conformational change mostly occurs within the transmembrane hydrophobic domain while the hydrophilic extramembrane domains are largely outside of this change.

B. Sarkadi

Furosemide-sensitive Na^+ and K^+ transport and human erythrocyte volume. W. Ch. O'Neill and R. B. Mikkelsen (Department of Medicine and Therapeutic Radiology, Tufts-New England Medical Center, Boston, MA, USA). *Biochim. Biophys. Acta 896*, 196 (1987).

The relationship between cation transport and cell volume in human erythrocytes was investigated by measuring ouabain-sensitive K^+ influx, ouabain-resistant, furosemide-sensitive K^+ influx, and ouabain + furosemide-resistant K^+ influx, and maximal ouabain binding in microcytic, normocytic and macrocytic red cells. A significant correlation was found between the mean corpuscular volume and furosemide-sensitive K^+ influx normalized either to cell number ($r = 0.636$, $P < 0.001$) or to cell volume ($r = 0.488$, $P < 0.001$). No relationship was seen between mean corpuscular volume and ouabain-sensitive K^+ influx, and the number of ouabain-binding sites per cell was only weakly correlated with mean corpuscular volume ($r = 0.337$, $P < 0.05$). A slight, negative relationship existed between mean corpuscular volume and ouabain + furosemide-resistant K^+ influx expressed per volume of cells ($r = -0.359$, $P < 0.01$), and an apparent relationship between furosemide-sensitive K^+ influx and mean corpuscular hemoglobin concentration ($r = 0.446$, $P < < 0.01$) disappeared when microcytic samples were excluded from analysis. Furosemide-sensitive transport, including Na^+ influx and K^+ and Na^+ efflux, was completely absent in microcytic cells from one patient with α-thalassemia minor. In addition, these cells exhibited a furosemide-resistant, Cl^--dependent K^+ influx. Exposure of normal erythrocytes to hypotonic conditions (196 mosM) increased furosemide-sensitive K^+ influx by a mean of 45% ($P < 0.05$), while exposure to hypertonic conditions (386 mosM) had no significant effect. The results indicate that furosemide-sensitive transport and cell volume are interrelated in human erythrocytes. However, the inability to fully recreate this relationship with in vitro manipulation of cell volume suggests that this relationship is established prior to red cell maturation.

B. Sarkadi

Oxidative inactivation of the calcium-stimulated neutral proteinase from human red blood cells by divicine and intracellular protection by reduced glutathione. A. Morelli, M. Grasso and A. De Flora (Institute of Biochemistry, University of Genoa, Italy). *Arch. Biochem. Biophys. 251*, 1 (1986).

Calpain, the micromolar Ca^{2+}-requiring form of Ca^{2+}-stimulated neutral proteinase

purified from human red cells, is remarkably inactivated during autoxidation of divicine (2,6-diamino-4,5-dihydroxypyrimidine), an aglycone implicated in the pathogenesis of favism. Inactivation of purified calpain is produced, in decreasing order of efficiency, by transient, probably semiquinonic species arising from autoxidation of divicine, by the H_2O_2 that is formed upon autoxidation itself, and by quinonic divicine, respectively. Purified procalpain, the millimolar Ca^{2+}-requiring form that can be converted to the fully active calpain form by a variety of mechanisms, is less susceptible than calpain itself to inactivation by the same by-products of divicine autoxidation. When intact red cells are exposed to autoxidizing divicine, procalpain undergoes a significant loss of activity. At 1 mM divicine, intracellular inactivation is observed with procalpain only, while the activity of a number of red cell enzymes is unaffected. Inactivation of procalpain is consistently greater in red cells from glucose-6-phosphate dehydrogenase-deficient subjects than in normal cells. Restoration of normal levels of glucose-6-phosphate dehydrogenase activity by means of entrapment of homogeneous human glucose-6-phosphate dehydrogenase in the deficient red cells results in (a) normal stability of intracellular reduced glutathione; (b) decreased susceptibility of procalpain to inactivation by autoxidizing divicine. These fiindings suggest that in the glucose-6-phosphate dehydrogenase-deficient red cells the procalpain-calpain system is a major target of divicne cytotoxicity.

G. Gárdos

Proton countertransport by the reconstituted erythrocyte Ca^{2+}-translocating ATPase: Evidence using ionophoretic compounds. A. Villalobo and B. D. Roufogalis (Laboratory of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada). *J. Membrane Biol. 93*, 249 (1986).

Human erythrocyte Ca^{2+}-translocating ATPase was solubilized from calmodulin-depleted membranes using the detergent Triton X-100, and subsequently purified by calmodulin-affinity chromatography. The purified enzyme was reconstituted in artificial phospholipid vesicles using a cholate-dialysis method and various phospholipids. The reconstituted enzyme was able to translocate Ca^{2+} inside the vesicles, both in the absence and in the presence of the Ca^{2+}-chelating agent, oxalate, inside the vesicles. The tightness of coupling between ATP hydrolysis and cation translocation was investigated by the use of different ionophoretic compounds. The efficiency of Ca^{2+}-translocation was measured by the ability of the ionophores to stimulate ATP hydrolytic activity of the reconstituted enzyme. It was found that the maximum stimulation of the ATP hydrolytic activity was induced by the electroneutral $Ca^{2+}/2H^+$ ionophore A23187 (9 to 10-fold). A Ca^{2+}-ionophore unable to translocate H^+, CYCLEX-2E, was less efficient in stimulating the activity of the reconstituted enzyme (two-to threefold). However, the combined addition of CYCLEX-2E plus protonophores further increased the ATP hydrolytic activity (around fourfold), whereas, the protonophores did not further stimulate ATP hydrolysis in the presence of A23187. Furthermore, in the absence of Ca^{2+} ionophore, the electroneutral $K^+(Na^+)/H^+$ ionophoretic exchanger, nigericin, or the electroneutral $Na^+(K^+)/H^+$ ionophoretic exchanger, monensin, stimulated the rate of ATP hydrolysis in the reconstituted enzyme two- or threefold, respectively. These results suggest that the Ca^{2+}-ATPase not only translocates Ca^{2+} but also H^+ in the opposite direction.

G. Gárdos

A contribution of calmodulin to cellular deformability of calcium-loaded human erythrocytes. J. Murakami, N. Maeda, K. Kon and T. Shiga (Department of Physiology, School of Medicine, Ehime University, Shigenobu, Onsen-Gun, Ehime, Japan). *Biochim. Biophys. Acta 863*, 23 (1986).

The effect of intracellular calcium on the deformabilityl of human erythrocytes was studied with a rheoscope, especially in relation to the dynamic structure of membrane cytoskeleton. The appropriate calcium-load-

ing and calcium-depletion were performed to intact erythrocytes with A23187 in potassium buffer. The total calcium content was varied in the range of 0.25 to 3 times as much as normal content, without complete ATP depletion and shape change (the reduction of mean cell volume and the condensation of hemoglobin due to dehydration were avoided). Increasing the intracellular calcium content by about 1.5 times of normal, the deformability was distinctly decreased, while calcium depletion did not affect the deformability. Reduced deformability of the calcium-loaded erythrocytes was restored by the treatment with calmodulin inhibitors, W-7 or trifluoperazine. However, such an effect by calmodulin inhibitors was not detected on normal or calcium-depleted erythrocytes. In conclusion, the interaction between calcium-calmodulin complex and cytoskeletal proteins may affect the membrane stiffness which is regulated through the change of the cytoskeletal structure, and contributes to the deformability of erythrocytes.

G. Gárdos

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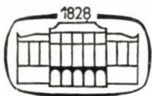

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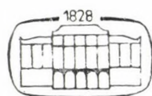

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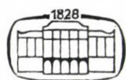

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VOLUME 20 • NUMBER 3 • 1987

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

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Haematologia 20 (3), pp. 125–130 (1987)

Hemoglobin Barts Hydrops Fetalis Syndrome

ELLEN BOWMAN, J. WATTS, R. BURROWS, D. H. K. CHUI*

Departments of Pediatrics, Obstetrics and Gynecology and Pathology, McMaster University School of Medicine, Hamilton, Ontario, Canada L8N 3Z5

(Received 19 June, 1986; accepted 29 August, 1986)

A 25 year old Vietnamese-Canadian pregnant woman was referred to our regional perinatal center at 31 weeks gestation after a routine ultrasound examination showing fetal ascites. A diagnosis of non-immune hydrops fetalis was made, and a Caesarean section was performed two days after hospital admission. An infant with Hb Barts hydrops fetalis was delivered who expired one hour after birth. α-Globin gene mapping of fetal DNA confirmed the diagnosis of homozygous α-thalassemia with deletion of all four α-globin genes. Both parents were shown to have α-thalassemia trait with deletion of both α-globin genes on one chromosome. This report further illustrates the need for a simple screening test for couples at risk of giving birth to infants with homozygous α-thalassemia. The availability of such a test would facilitate genetic counselling and prenatal diagnosis, thereby improving the quality of obstetrical care provided to these women at risk.

Keywords: α-thalassemia, carrier detection, embryonic ζ globin, hydrops fetalis

Introduction

Alpha thalassemia is a hereditary condition with diminished or absent α-globin chain synthesis, due to either deletional or non-deletional abnormalities involving the α-globin genes. In homozygous α-thalassemia due to the deletion of all four α-globin genes, i.e. Hb Barts hydrops fetalis, a syndrome not infrequently encountered in the Southeast Asian populations, the fetuses are usually stillborn during the third trimester or die shortly after birth [1, 2]. We have recently encountered an infant with homozygous α-thalassemia in our hospital. This report raises the number of pertinent issues regarding the diagnosis, prevention and management of Hb Barts hydrops fetalis syndrome. In view of the recent advances in obstetrical and neonatal care as well as in the understanding of the molecular genetics of α-thalassemia, these issues have assumed clinical importance.

Case History

Mrs. A. D. was a healthy 25 year old Vietnamese-Canadian in her second pregnancy. Her first pregnancy, two years previously, terminated spontaneously at term with the birth of a healthy, normal male. This second pregnancy was

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uneventful until 31 weeks' gestation when a routine obstetrical ultrasound revealed fetal ascites. Repeat scanning of the fetus at the Regional Perinatal Centre demonstrated massive cardiomegaly, pericardial effusion, marked hepatomegaly, ascites, distended umbilical vessels and enlarged placenta. The diagnosis of alpha thalassemia was suspected but not confirmed antenatally.

As amniocentesis showed an immature lecithin-sphingomyelin ratio and the fetal status was felt to be poor but stable, corticosteroids were administered to the mother. With close fetal monitoring, Caesarean section was performed forty-eight hours later, and a baby girl was delivered.

At birth, the condition of the baby girl was very poor with Apgar score of one at both 1 and 5 minutes. There was generalized oedema, cyanosis, and poor cardiac output. Despite active resuscitation including volume expansion and pharmacologic circulatory support, her condition continued to deteriorate rapidly with a persistent bradycardia, and the baby expired one hour after delivery. At autopsy, the baby girl weighed 2.2 kg. There were cardiomegaly (17.5 g) with serosanguinous pericardial effusion, serous ascites, and marked extramedullary haematopoiesis in the grossly enlarged liver (150 g), spleen, adrenals and kidneys. The placenta was enlarged, weighing 1.0 kg.

Shortly after the delivery, the mother developed post-partum pre-eclampsia manifested by hyperuricaemia and diastolic hypertension which required treatment with parenteral hydralazine. At discharge 5 days later, she had become normotensive without the need for any medication.

Results

Table 1 shows the haematological values of members of the family under study. At birth, the baby girl had severe anaemia with marked erythroblastosis (137×10^9 normoblasts per liter of blood). Hb Barts was detected in her haemoly-

Table 1

Haematological data

	Father	Mother	Son	Infant Girl
Age (years)	28	25	2	(Died one hour after birth)
Hb (g/L)	137	122	182*	62*
Mean corpuscular volume (fl)	70	72	104*	94*
Serum ferritin (μg/L)	98	62	Not done	Not done

Complete blood counts were done by standard laboratory procedures, using a Coulter S electronic counter. Serum ferritin was determined by radioimmunoassay (normal range 18–300 μg/L).

* These values were obtained at birth.

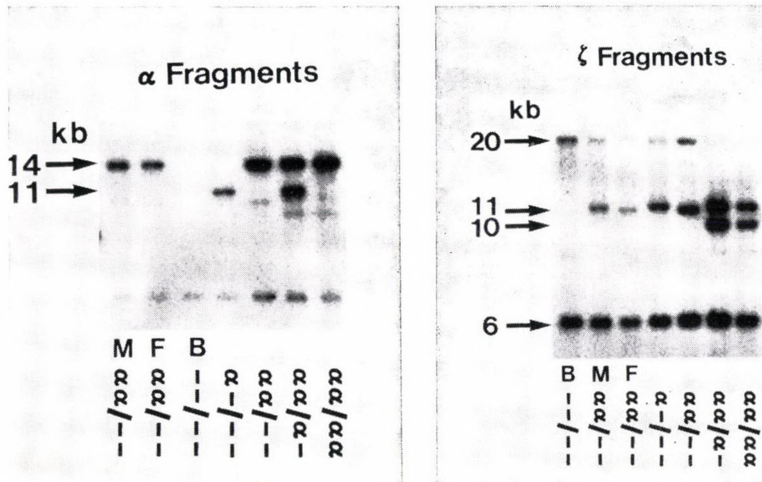

Fig. 1. Autoradiographs showing α-globin and ζ-globin gene-specific restriction fragments. Genomic DNAs were obtained from four unrelated Oriental individuals with known ζ-α globin genotypes, i.e. $\alpha\,\alpha/\alpha\,\alpha$, $\alpha\,\alpha/\alpha$-, $\alpha\alpha$/--, and α-/-- for comparison. M, DNA from the mother; F, father; B, baby girl

(α-/) represents genotype in which one α globin gene is deleted from one chromosome, the so-called rightward 3.7 kb deletion. (--/) represents the > 17.5 kb deletion involving all $\psi\alpha$- and α-globin genes on the same chromosome, a deletion which is not infrequently encountered in Southeast Asia

Left panel. Genomic DNAs digested with Bam HI and hybridized to ^{32}P labelled 1.6 kb α-globin gene-specific probe which was obtained by digestion of the JW101 plasmid with Mbo II. Chromosome containing both α-globin genes gives rise to the 14 kb fragment. Chromosome containing only one α-globin gene gives rise to 11 kb fragment. The other faint bands represent ζ-globin gene specific fragments which cross-hybridized with the α-globin gene probe. Note that the baby girl has no α-globin gene-specific fragment

Right panel. Genomic DNAs digested with Bam HI and hybridized to ^{32}P labelled 2.0 kb ζ-globin gene-specific probe which was obtained by digestion of the p^{ζ} plasmid with Hinf 1. Chromosome containing either both α- or one α-globin genes gives rise to 10/11 kb and 6 kb bands. Chromosome containing the > 17.5 kb deletion involving both $\psi\alpha$- and α-globin genes gives rise to 20 kb and 6 kb bands

sate by cellulose acetate electrophoresis. Both parents were Vietnamese-Canadian, and had very mild anaemia with microcytosis. HbH inclusion bodies were found in the peripheral blood erythrocytes of both parents after their blood was incubated with brilliant cresyl blue (British Drug House) at 37 °C. Their serum ferritin levels were normal.

ζ-Globin chains were detected by a specific ζ-globin chain radioimmunoassay [3] in the peripheral blood haemolysates from the baby girl, as well as from both parents (0.09% in the father's haemolysate and 0.07% in the mother's haemolysate). The presence of ζ-globin chains in both parents' haemolysates was confirmed by Triton X-100/urea/polycrylamide gel electrophoresis [3] (data not shown).

DNAs were extracted from the liver tissue of the baby obtained at autopsy, as well as from the leukocytes of the peripheral blood of both parents. The genomic DNAs were digested with the restriction endonuclease Bam HI, electrophoresed, transferred to nitrocellulose filter paper, and hybridized with α- and ζ-globin gene specific probes as described previously [4]. The results indicated that both parents had α-thalassemia trait due to a >17.5 kb (kilobases) deletion involving all $\psi\alpha$- and α-globin genes on one chromosome, not infrequently encountered in the Southeast Asian populations. This deletion is sometimes designated as $(-^{SEA}/)$. The baby had homozygous α-thalassemia due to the deletion of all four α-globin genes on both chromosomes (Fig. 1).

Discussion

The human α-globin gene cluster is normally made up of an embryonic ζ-globin gene, a non-functional pseudo-ζ-globin ($\psi\zeta$) gene, two non-functional pseudo-α-globin ($\psi\alpha$) genes, and two α-globin genes. These six gene loci are closely linked within about 30 kb on the short arm of chromosome 16. In Hong Kong, for example, the gene frequency for the >17.5 kb deletion involving all $\psi\alpha$- and α-globin genes on the same chromosome has been estimated to be 3% [5]. Homozygous α-thalassemia is by far the major cause for hydrops fetalis syndrome in Southeast Asian populations [5]. There are, for example, approximately 15,000 pregnancies with hydropic fetuses every year in Thailand alone [6]. With an increasing Asian population in North America, it is likely that increasing numbers of Hb Barts hydrops fetalis syndrome will be recognized in the U.S. and Canada as well [2, 7, 8, 9].

The recent advances in obstetrical care and molecular biology have made possible prenatal diagnosis of Hb Barts hydrops fetalis syndrome in specialized centres by gene mapping of cells of fetal origin, obtained either by chorionic villi biopsy during the first trimester of pregnancy or by amniocentesis during the second trimester [6, 10, 11]. Genetic counselling and prenatal diagnosis for this invariably fatal condition are important for a number of reasons. Firstly, it can relieve the physical burden for the mother and the emotional strain for the entire family created by a pregnancy which results in a perinatal death usually in the third trimester. Secondly, pregnancy involving a homozygous α-thalassemia fetus is associated with an increased risk of maternal complications such as hydramnios, pre-eclampsia, ante-partum and post-partum haemorrhage, and difficult vaginal or Caesarean delivery, all with considerable maternal morbidity and even mortality [5]. Thirdly, early definitive diagnosis and intervention of the pregnancy will prevent unnecessary Caesarean sections being performed, or aggressive neonatal resuscitation including exchange transfusions being undertaken which might lead to an infant whose viability is totally dependent on recurrent transfusions of blood [8, 9]. Lastly, the availability of prenatal diagnosis early in pregnancy will allay the anxiety of most of the couples at risk regarding the prospect of begetting a fetus with homozygous α-thalassemia.

The couples at risk for becoming parents of infants with homozygous α-thalassemia are presently identified mainly by a history of previous deliveries of hydropic infants. The ζ-α globin genotypes of these couples can be confirmed definitively by the rather laborious procedure of DNA analysis. Clinical suspicion of the diagnosis can be aroused by knowledge of the parents' racial origin and their routine haematological results, as well as the early onset of hypertension and/or hydramnios during pregnancy [5]. Ultrasonography can be a useful diagnostic tool, but is rarely helpful before twenty weeks' gestation [5].

More recently, it has been found that adult individuals heterozygous for the >17.5 kb deletion involving all $\psi\alpha$- and α-globin genes on one chromosome have a minute but clearly detectable amount of the embryonic ζ-globin chains in their haemolysates identifiable by immunological technique [4]. The parents of the hydropic infant girl in this study had 0.09% and 0.07% ζ-globin chains in their haemolysates. Therefore, the presence of embryonic ζ-globin chains can serve as a marker for the deletion, and can potentially be useful, in populations where the gene frequency of the >17.5 kb deletion is high, for screening couples who are at risk of becoming parents of homozygous α-thalassemia fetuses [4]. We are presently actively engaged in developing a simple, rapid, specific, and sensitive immunological assay for embryonic ζ-globin chains in adult haemolysates. Such a test, when widely available, should help to improve the quality of obstetrical care provided to women at risk of producing an infant affected by the Hb Barts hydrops fetalis syndrome.

Acknowledgement

We are grateful to Dr. H. A. Heggtveit for the autopsy results. We thank M. Patterson and M. K. Berdusco for expert assistance, Dr. J. Wilson for providing the JW101 plasmid, and Dr. T. Maniatis and colleagues for providing the pζ plasmid. This study was supported in part by a research grant from the Medical Research Council of Canada (MT-5004 to DHKC). Dr. R. Burrows was supported in part by the Sick Children's Hospital Foundation of Toronto.

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Haematologia 20 (3), pp. 131—153 (1987)

Effect of Acute Lead Intoxication on the Ultrastructure of Rat Erythroblasts and Reticulocytes. Morphometric Analysis and Röntgen Micro-analysis

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(Received 29 September, 1986; accepted 12 October, 1986)

Effect of 17 hr intoxication of lead on the different maturation stages of erythroid cells were studied in rat. Morphometric methods were used to analyse the lead-induced ultrastructural changes in the early, intermediate, late erythroblasts and reticulocytes. It was found that the toxic effect of lead increases with maturation. Energy-dispersive Röntgen micro-analysis showed that fibrillar structures within vesicles and endoplasmic reticulum contained lead.

Keywords: bone marrow, electron microscopy, erythropoiesis, lead, morphometry, Röntgen micro-analysis

Introduction

Lead intoxication causes multiorgan pathology [1]. The haematologic effect of lead toxicity results in anaemia. This is a consequence of two separate and independent events, namely, shortening of half-life of the mature erythrocytes [2] and inefficient erythropoiesis. The latter results from inhibition of haem biosynthesis [3], reduced globin synthesis [4, 5] and increased breakdown of erythroblasts in the bone marrow [6]. Morphological features of red cell precursors, after lead intoxication, are swollen iron-loaded mitochondria [7] and basophilic stippling [8]. Other morphological alterations induced by lead are not known. The reason for this may be that the changes are more quantitative rather than qualitative and hence not seen in a morphological investigation alone. We report here morphological and morphometric changes observed in the bone marrow red cell precursors, 17 hours after a single intravenous injection of lead.

Furthermore we describe fibrillar lead-inclusions in vesicles and endoplasmic reticulum of erythroblasts and reticulocytes from rats 6 hours and 17 hours after lead administration. The lead content of these fibrillar inclusions has been demonstrated by means of energy-dispersive Röntgen micro-analysis.

Material and Methods

Animals

Fifteen adult white male Wistar rats (200—250 gm body weight) were divided in two groups. Five rats served as control and the other rats (Pb-rats) were injected,

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Akadémiai Kiadó, Budapest

via the tail vein, with a lead acetate solution at a dose of 40 mg Pb/kg rat. Six (Pb-6h) and seventeen (Pb-17h) hours later the rats were ether-anaesthesised and peripheral blood and bone marrow were taken for investigation.

Haematological parameters of peripheral blood of control and Pb-rats with 17 hours lead intoxication were determined on Coulter Counter Model S IV. Lead concentration of the femoral bone marrow was determined by atomic absorption spectrometry (Perkin Elmer 372 + graphite furnace HGA 76b).

Electron microscopy

The femoral bone marrow was fixed by perfusion via the dorsal aorta with 2% glutaraldehyde in cacodylate buffer (0.07 M, 3.7% succrose, pH 7.3) for 5 min. Small pieces of the femoral bone marrow were removed and immersed at 4 °C in 3% glutaraldehyde in cacodylate buffer for 2h. The blocks were then rinsed in cacodylate buffer and postfixed for 1h at 4 °C, in 2% OsO4 in cacodylate buffer. After rinsing in distilled water, the blocks were stained in 1% uranyl acetate for 30 min., dehydrated in ethanol and propylene oxide and embedded in Spurr's low viscosity medium. Silver sections were cut with a diamond knife using an LKB Ultratome III; they were double stained with lead citrate and uranyl acetate, and examined with a Zeiss EM 10 microscope at 40 and 60 kV.

Identification of maturation stages

Classification of morphologically recognizable erythrocyte precursors in early, intermediate and late erythroblasts was based on nuclear morphology (Figs 1, 2, 3, 4) [9, 10]. Late erythroblasts were divided in two groups: (EN+)erythroblasts with an eccentric nucleus, surrounded at least partially by a narrow ruffled rim of cytoplasm devoid of cell organelles and (EN-)erythroblasts without these morphological signs of nuclear extrusion [11, 12, 13]. Rat reticulocytes have no nucleus.

Röntgen micro-analysis

Bone marrow samples obtained 6 and 17 hours after lead administration were processed without post-osmification and without "en bloc" uranyl acetate staining when used for Röntgen microanalysis. This was performed on uranyl acetate stained ultrathin sections in a Philips EM 400 analytical microscope with a Tracor/Northern 2000 analyser and computer attached to it. The ultrathin sections were loaded in a berylium low background holder tilted 30° towards the analyser, which is positioned perpendicular to the specimen rod. Multiple 100 s. static beam point analyses were performed, with a 40 nm spot size at 80 kV. The obtained spectra were recorded and printed. Peak identification was done by the computer.

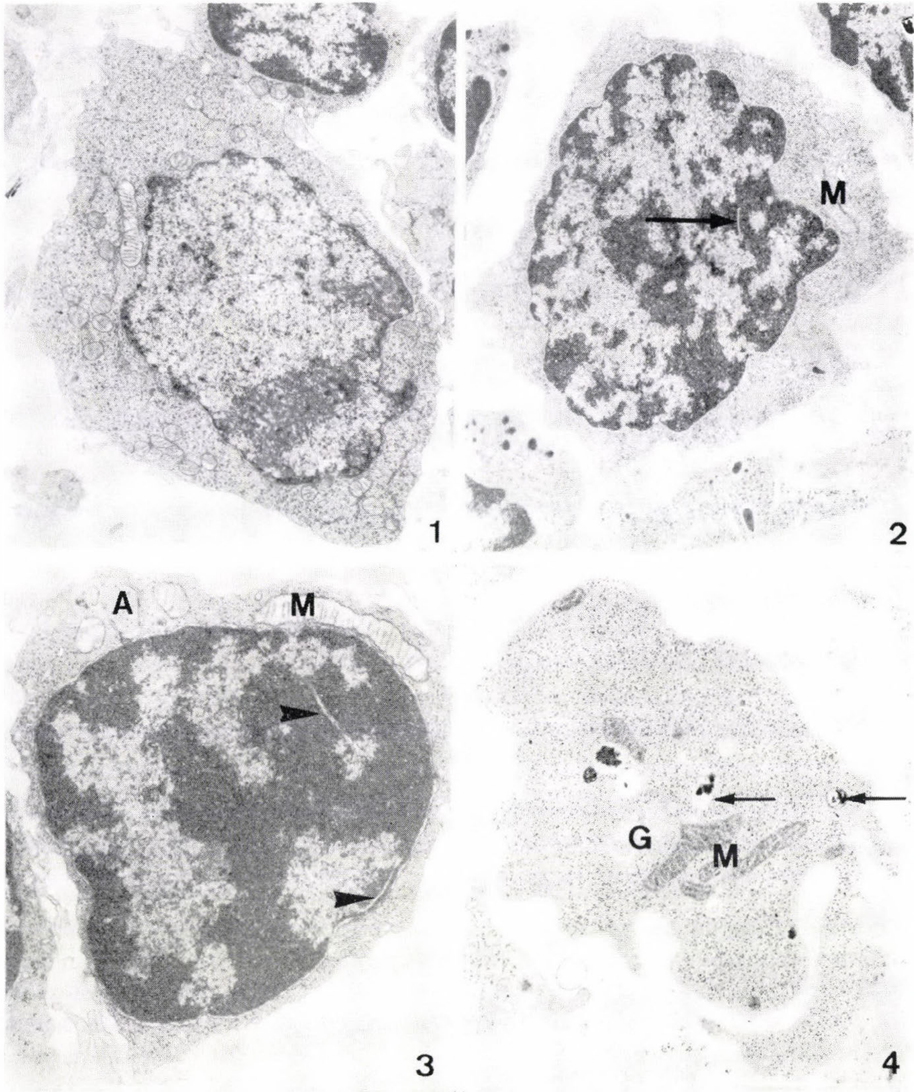


Plate 1

Four erythroblast maturation stages of rats, 17 hours after lead administration

Fig. 1. Early erythroblast. This cell has been classified as early erythroblast for its predominantly euchromatic nucleus with two large nucleolar complexes. The influence of lead is visible in this early stage of maturation by the irregular outline of the nuclear and cytoplasmic boundary. × 8,000

Fig. 2. Intermediate erythroblast. The heterochromatin content of the nucleus is increased. In addition to the irregular nuclear and cytoplasmic boundary, the influence of lead is visible in the presence of a nuclear cleft (arrow). Numerous large mitochondrial profiles (M) are visible. × 8,000

Fig. 3. Late erythroblast without morphological signs of nuclear extrusion; i.e. "(EN-)erythroblast". The nucleus contains predominantly clumped heterochromatin. In addition to the irregular nuclear and cytoplasmic outline and the nuclear clefts (arrowheads), the influence of lead is visible in the presence of swollen mitochondria (M) and autophagocytosed cytoplasm (A). × 12,500

Fig. 4. Reticulocyte. This reticulocyte section with a very irregular shape contains iron-loaded mitochondria (M), a Golgi area (G), and autophagosomes with degraded material (arrows). × 12,500

Morphometric procedure

At least three blocks were sectioned from each rat. From a single section of each block, micrographs were recorded on 70 mm film at two levels of magnification: level I (× 9,950) and level II (× 24,400). The magnifications were determined by including a grating replica of 2160 lines/mm (Balzers Union) on each film strip.

A double square test system (1 : 9, 121 : 1089) [14] was superimposed onto the negative and the two are then projected together. At magnification I (final magnification: × 43,000) the nucleus, cytoplasm, mitochondria, rough endoplasmic reticulum and autophagosomes were assessed. At magnification II (final magnification: × 100,000) the mitochondrial cristae and ribosomes were estimated. The reference area of nucleus and cytoplasm was estimated by counting coarse test points and the area of mitochondria, autophagosomes and autophagosomal components by counting fine test points lying over the structures. The length of the membranes of the nuclear clefts, rough endoplasmic reticulum and mitochondrial cristae were estimated by counting the number of intersections occurring between those membranes and the fine horizontal and vertical test lines. The number of ribosomes was counted in an at random cytoplasmic area (0.20 × 0.20 μm); the number of single and clustered (2 to 6) ribosomes were counted in a cytoplasmic area (0.28 × 0.28 μm), free of other organelles. The number of mitochondrial and autophagosomal profiles per cell section was also noted. The number of point and intersection counts and the number of ribosomes, mitochondria and autophagosomes were collected with a manual optical sample analyser (Kontron Messgeräte G.m.b.H., Munich, Germany).

Stereological and other calculations

Variables were computed per cell section. Volume and surface densities were calculated according to Weibel [15, 16]. The numerical density of ribosomes was computed using the formula of Abercrombie and Hennig as modified by Simar and Weibel [17]; the mean diameter of the ribosomes was 23 nm, the smallest profile still recognizable as a ribosome has a mean diameter of 16 nm and we assumed a 70 nm thickness of the "silver" sections [18]. The mean area of mitochondrial profiles (AM) is calculated as the number of test points overlying mitochondrial profiles divided by the number of such profiles. The per cent of polyribosomes is defined as the ratio of the number of ribosomes occurring in clusters and the total number of ribosomes. The minimal sample size for each variable was determined by the progressive mean technique [19, 20]. In this procedure repeated sampling of cell section is carried out and the mean value of the parameter from 5, 10, 15 and 20 micrographs is plotted graphically. The sample size for a parameter per rat is considered adequate when the inclusion of an additional 5 micrographs fails to alter the progressive mean by 10%.

The level of significance ($P < 0.05$) for the difference in the results between control and Pb-rats and between subsequent maturation stages was analysed with non-parametric test: the Kruskal-Wallis one way analysis of variance by ranks [21].

Canonical discriminant analysis

Each of the cell sections has been described by giving scores of n normalised variables. The whole set of the sections can now be seen as a swarm of points in a n-dimensional subspace. Our aim was to obtain 2-dimensional representation that allows the best possible separation between the sections of the four different maturation stages of the Control- and Pb-rats (8 groups) and at the same time to indicate which variable contributes most to this separation. This is realised by canonical discriminant analysis [22]. In this procedure two new variables (canonical variables) are created. The point of each section is obtained by plotting the canonical variables one against another. In the same plot the original variables can be presented as vectors. Their position and length is defined by their correlation with the canonical variables.

Projecting the section points on these variable vectors results in a satisfactory reconstruction of the scores of the original variables. The class means on canonical variables gives the position of the means of the 8 groups of sections on the 2 canonical variables.

Results

Characteristics of Pb-rats, 17-hours after lead administration.

Whereas control rats were found to contain 0–26 μg Pb/gm bone marrow the bone marrow of the Pb treated-rats (17h after lead acetate injection) contained 90–122 μg Pb/gm.

Table 1

Haematological parameters of peripheral blood (mean value ± standard deviation)

	Control rats	Pb-rats (17h)	P-value
No of rats	10	5	
Hg g/dl	15.2 (± 0.7)	15.6 (±0.4)	0.125
RBC 10^{12}/l	7.5 (± 0.8)	7.7 (±0.3)	0.425
PCV	0.53 (± 0.08)	0.48 (±0.04)	0.325
MCV fl	70.3 (±10.7)	61.8 (±5.5)	0.176
MCH pg	20.2 (± 1.4)	20.2 (±0.8)	0.898
MCHC g/dl	29.2 (± 4.3)	32.4 (±2.1)	0.175

The haematological parameters of the peripheral blood were not significantly affected during this period of acute lead intoxication (Table 1). However the number of late erythroblasts per 100 nucleated bone marrow cell was reduced by more than 50% in the Pb-rats. Electron microscopic observation of the bone marrow shows a prominent phagocytosis of late erythroblasts by macrophages (Figs 5, 6).

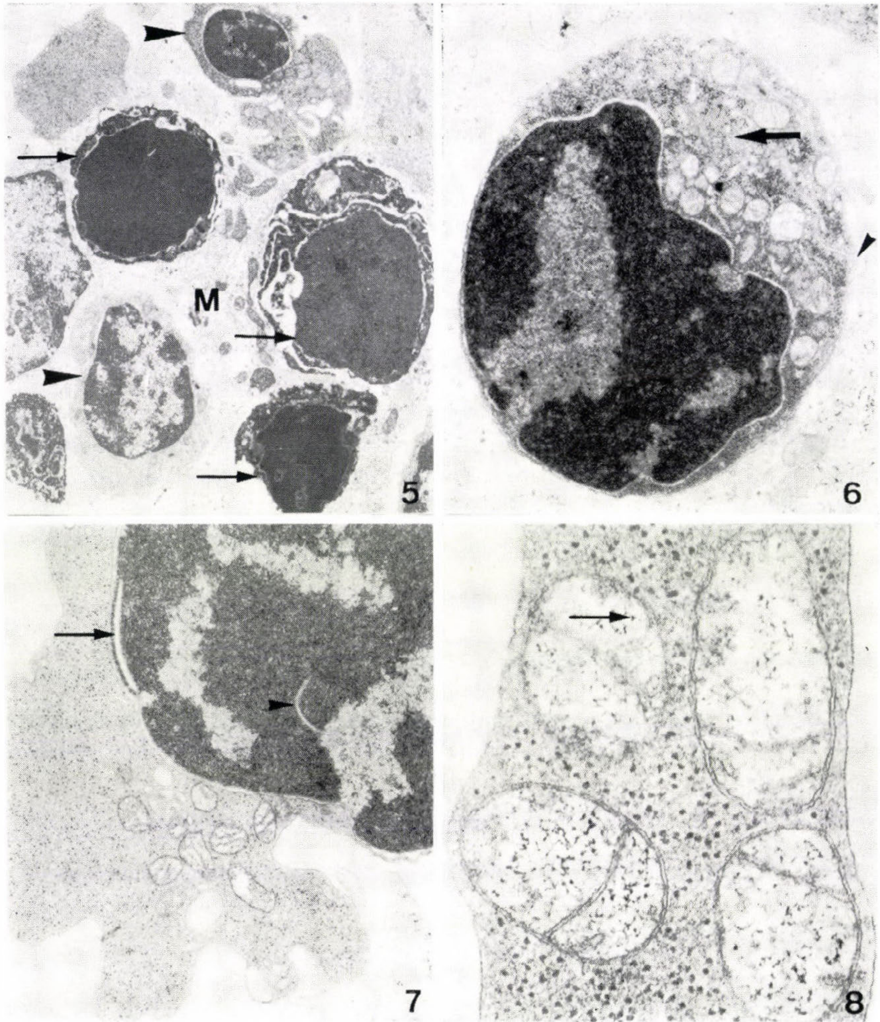


Plate 2

Fig. 5. Bone marrow macrophage (M) containing three partly degraded cells (arrow), probably late erythroblasts, and two almost intact late erythroblasts (arrowhead). × 5,000

Fig. 6. Late erythroblast within the cytoplasm of a bone marrow macrophage. The nucleus of the late erythroblast is pyknotic and the cytoplasm is crowded with ribosomes, mitochondria and tubular profiles (arrow), which are in open communication with the macrophage cytoplasm (arrowhead). × 12,500

Fig. 7. Two nuclear clefts inside the nucleus of a late erythroblast from a lead-intoxicated rat. One nuclear cleft is positioned in parallel to the nuclear envelop (arrow) and another is randomly placed (arrowhead). Except for their ends, both lie completely within the heterochromatin. × 17,000

Fig. 8. Four swollen iron-loaded mitochondria in a bone marrow reticulocyte from a lead-intoxicated rat. The swelling affects only the inner compartment, which contains a flocculent matrix, electron dense iron granules (arrow) and few cristae. × 45,000

Shape of the erythroid cells

Even from a simple inspection of the electron micrographs it is evident that erythroid precursor of the Pb-rats have a more irregular form and that the nuclear and plasma membranes show deeper invaginations (Figs 1, 2, 3, 4).

Nuclear clefts

Nuclear clefts are membrane-limited flat cisternae lying in the nucleus (Figs 2, 3, 7). Some authors [23] make a distinction between "nuclear blebs" which are positioned in parallel to the nuclear envelop and nuclear clefts which are randomly placed in the nucleus. In our stereological analysis we have not followed this distinction. Instead, each flat cisternae without nuclear pores was called "nuclear cleft". Except for the extreme ends, the nuclear clefts lie completely within the heterochromatin. Sometimes both ends are in contact with the euchromatin. No ultrastructural difference was observed between "nuclear clefts" in cells from the control and the Pb-rats. However erythroid cells of the Pb-rats contained more "nuclear clefts" than those from the control rats. Furthermore, the surface area of the "nuclear clefts" membrane per nuclear volume from the late erythroblasts of Pb-rats was significantly greater than from the control rats (Table 2, item 1). Significant increase in the value of this parameter occurs during maturation of early to intermediate and to late erythroblasts from Pb-rats.

Ribosomes

Lead intoxication did not cause a statistically significant change in the number of ribosomes per unit volume of erythroid cell cytoplasm. However in contrast to the normal situation, in Pb-rat erythroid cells, there was no significant drop in the number of ribosomes as the cells mature from the late erythroblast stage to reticulocytes (Table 2 item 2). In comparison with the cells at the same maturation stage in control rats, the intermediate erythroblasts and the subsequent maturation stages of the Pb-rats showed a significantly lower percentage of polyribosomes (Table 2 item 3). This decrease did not occur at the same rate in every erythroid cell, since one could find two neighbouring reticulocytes one with predominantly single ribosomes and the other containing mainly polyribosomes (Fig. 9). Occasionally a cluster of ribosome-like structures was observed in reticulocytes of Pb-rats (Fig. 10).

Mitochondria

Under our experimental conditions lead intoxication had no effect on the ultrastructure of mitochondria of the early and intermediate erythroblasts (Figs 1, 2). Nor could any statistically significant difference be found in the mean cross-section area (AM) of the mitochondria or the surface density (SVCRM) of the cristae in the mitochondria at these maturation stages (Table 2 item 5 and 6).

Table 2

Parameters (mean ± S.D.) of early-, intermediate-, late erythroblast and reticulocytes of lead-intoxicated (Pb-17h) and control (Ctl) rats

Component	Symbol of parameter	Dimension units	Rats	Early ebl.	Intermediate ebl.	Late ebl.	Reticulocytes
Nuclear clefts (CL)	$SV_{CL,N}$	$\mu m^2/100\ \mu m^3$	Pb	0.0 ± 0.0*	0.6 ± 3.0*	3.3 ± 10.4	—
			Ctl	0.0 ± 0.0	0.1 ± 0.8	0.1 ± 0.4^{+}	—
Ribosomes (R)	$NV_{R,C}$	$10^3\ \mu m^{-3}$	Pb	3.7 ± 2.0	3.9 ± 2.3*	2.8 ± 2.1	1.9 ± 1.4
			Ctl	3.7 ± 1.9	4.2 ± 2.3*	2.7 ± 2.2*	1.6 ± 1.1
Polyribosomes	%		Pb	67 ± 10*	60 ± 11*	49 ± 17*	35 ± 21
			Ctl	69 ± 13*	72 ± 12^{+}*	66 ± 16^{+}*	49 ± 22^{+}
Mitochondria (M)	$VV_{M,C}$	$\mu m^3/100\ \mu m^3$	Pb	10.8 ± 4.1	11.2 ± 3.9*	7.3 ± 5.1	4.3 ± 4.1
			Ctl	10.7 ± 5.0	9.4 ± 3.6^{+}*	7.0 ± 3.6*	2.1 ± 1.7^{+}
	AM	$10^{-3}\ \mu m^2$	Pb	145 ± 44*	102 ± 33	100 ± 53*	133 ± 103
			Ctl	163 ± 171*	106 ± 38*	81 ± 39^{+}*	66 ± 29^{+}
Mitochondrial cristae (CR)	$SV_{CR,M}$	$\mu m^2/\mu m^3$	Pb	12.4 ± 5.6	11.8 ± 5.2	11.1 ± 7.1*	8.5 ± 5.1
			Ctl	11.8 ± 5.4	12.7 ± 6.3	13.9 ± 6.1^{+}	13.4 ± 7.6^{+}
RER	$SV_{RER,C}$	$\mu m^2/100\ \mu m^3$	Pb	48.9 ± 39.2*	20.9 ± 24.5*	11.5 ± 27.8*	13.1 ± 57.7
			Ctl	44.8 ± 32.9*	23.4 ± 23.2*	6.7 ± 9.6	8.2 ± 9.6
Autophagosomes (AUT)	$VV_{AUT,C}$	$\mu m^3/100\ \mu m^3$	Pb	0.04 ± 0.23	0.12 ± 0.44*	1.88 ± 3.82	0.66 ± 1.25
			Ctl	0.00 ± 0.00	0.00 ± 0.00*	0.17 ± 0.64^{+}*	0.37 ± 0.65

ebl.: erythroblasts

VV, volume density; SV, surface density; NV, numerical density

The first subindex corresponds to cell component and the second subindex corresponds to reference volume (N = nucleus; C = cytoplasm)

AM: mean area of mitochondrial profiles

$^{+}$ significant differences between Control- and Pb-rats ($P \leq 0.05$)

* significant differences between subsequent maturation stages ($P \leq 0.05$)

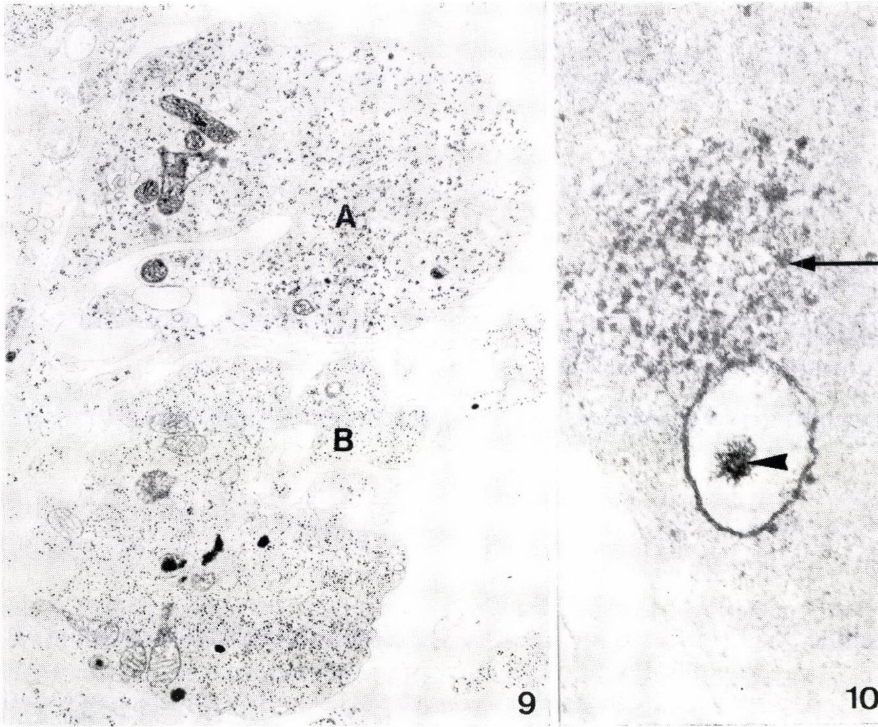


Plate 3

Reticulocytes in the bone marrow of lead-intoxicated rats

Fig. 9. Reticulocyte "A" contains predominantly polyribosomes and reticulocyte "B" contains predominantly single ribosomes. × 13,000

Fig. 10. Fragment of a reticulocyte with a RER cisterna containing a fibrillar lead inclusion (arrowhead) and a cluster of ribosome-like structure (arrow). This aggregation of "ribosomes" is also seen in the light microscope as "basophilic stippling". × 92,500

However the volume density of mitochondria in cytoplasm (VVMC) is significantly greater in the intermediate erythroblasts of the Pb-rats (Table 2 item 4). This suggests a net gain in mitochondrial material at this stage. In the more advanced maturation stages, one found both normal (Fig. 22) and swollen mitochondria (Figs 3, 7, 8). All the mitochondria within a cell were either normal or swollen. The swelling affected only the inner mitochondrial compartment (Fig. 8) and the swollen mitochondria had a flocculent matrix and fewer cristae. This was born out by the measurement of cristae surface density per unit volume of mitochondrion (SVCRM), which was significantly lower for the Pb-rats (Table 2 item 6).

One would expect the mean cross-section area of the swollen mitochondria (AM) to be greater than that of the apparently normal mitochondria. This is confirmed by the morphometric analysis (Table 2 item 5 and Table 3 item 2). The oc-

Table 3

Mitochondrial and autophagosomal parameters (mean ± SD) of reticulocytes with swollen and normal mitochondria of lead-intoxicated (Pb-17h) and control rats

	Control rats		Pb-rats
Ultrastructure of mitochondria	normal	normal	swollen
N of analysed cell sections	76	33	130
Mean area of mitochondrial profiles (10^{-3} μm^2)	66 ± 29*	79 ± 39*	127 ± 98
Per 100 μm^3 cytoplasm			
— mitochondria (μm^3)	2.08 ± 1.69*	4.44 ± 3.60	5.55 ± 3.91
— autophagosomes (μm^3)	0.37 ± 0.67*	1.75 ± 2.52*	0.36 ± 0.08
— autophagocytosed mitochondria (μm^3)	0.13 ± 0.36	0.32 ± 0.97*	0.0 ± 0.0
— autophagocytosed cytoplasm (μm^3)	0.03 ± 0.16*	0.21 ± 0.70*	0.03 ± 0.20
— degraded material in autophagosomes (μm^3)	0.22 ± 0.52*	1.20 ± 2.12*	0.32 ± 0.76

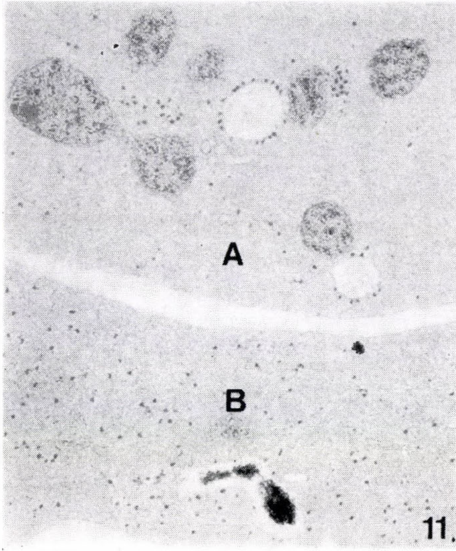
* significant differences ($P \leq 0.05$)

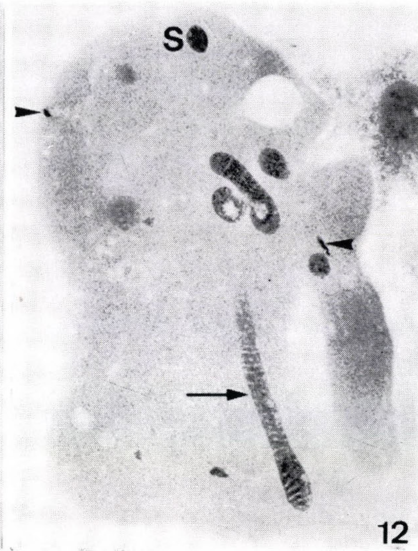
currence of swollen mitochondria in 36% of late erythroblasts from Pb-rats does not result in an increase of volume density of mitochondria in the cytoplasm (Table 2 item 4) of these cells. But in the reticulocytes of Pb-rats, with 65% of the sections containing swollen mitochondria, there is a 2.8 fold increase of the volume fraction of mitochondria in the cytoplasm, when compared with reticulocytes of control rats (Table 2 item 4 and Table 3 item 3). In 7% and 31% of the late erythroblasts and reticulocytes respectively mitochondria contained electron dense deposits in their inner compartments (Figs 4, 8, 11). Mitochondria with these granules were easily recognizable in thick sections by the changes in electron density in the deposits and in cristae (Fig. 12). Energy dispersive Röntgen micro-analysis revealed that the electron dense granules were composed of iron and phosphorus and contained no lead (Fig. 13).

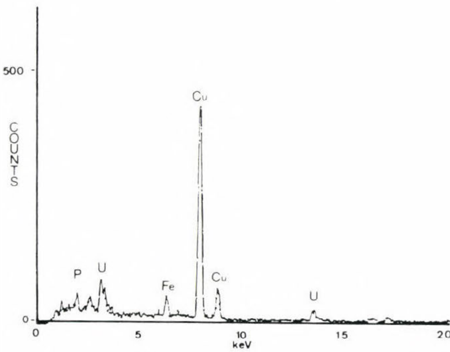
Rough Endoplasmic Reticulum (RER)

The surface density of membranes of RER in the cytoplasm (SVRERC) of late erythroblasts and reticulocytes from the Pb-rats was increased by 73% and 60% respectively (Table 2 item 7). However the Kruskal-Wallis statistical test failed to show a significant difference. The reason for this is the patchy distribution of RER in the cytoplasm: many cell sections contain no RER and a few have a large amount.

Cisternae of the endoplasmic reticulum of the late erythroblasts and the reticulocytes of the Pb-rats were found to be swollen and contained electron dense fibrillar inclusions (Figs 10, 11, 12, 18). These inclusions were so infrequent that they were not observed in the samples of stereological analysis. However explora-







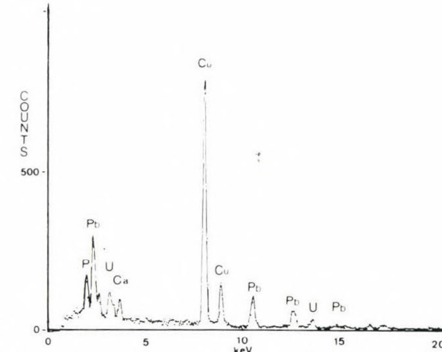


Plate 4

Iron-loaded mitochondria and fibrillar lead-inclusions in bone marrow reticulocytes of rats, 17 hours after lead-intoxication

Fig. 11. Fragments of two reticulocytes: (A) with iron-loaded mitochondria and swollen RER-cisternae and (B) with a RER-cisterna containing a large fibrillar lead inclusion. $\times$ 31,000
Fig. 12. Thick section of a reticulocyte, without Os04 fixation and contrasted with uranyl only. Iron-loaded mitochondria (arrows), two fibrillar lead-inclusions (arrowheads) and a siderosome (S) are visible. $\times$ 16,000
Fig. 13. Röntgen-spectrum of iron granules in a mitochondrion showing an energy peak for iron (Fe) and an energy peak for phosphorus (P). The copper (Cu) peaks are due to the grid and the uranyl (U) peaks are due to the contrasting with uranyl acetate
Fig. 14. Röntgen-spectrum of fibrillar lead inclusions showing 4 energy peaks for lead (Pb), one energy-peak for phosphorus (P) and one for calcium (Ca). The copper (Cu) peaks are due to the grid and the uranyl (U) peaks are due to the contrasting with uranyl acetate

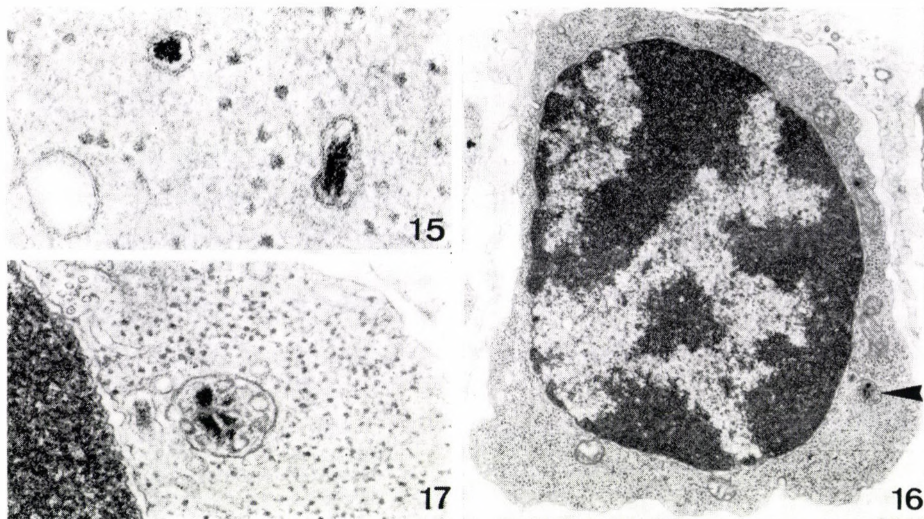


Plate 5

Fibrillar lead inclusions in late erythroblasts of rats, 6 hours after lead administration

Fig. 15. Two vesicles with electron dense fibrillar inclusions. × 105,000
Fig. 16. Late erythroblast with a multivesicular body (arrowhead) × 12,500
Fig. 17. Detail of the multivesicular body of Fig. 16 showing the vesicles with electron dense fibrillar inclusions. × 42,000

tion of a large number of electron micrographs for these electron dense bodies showed that they were present in the "endoplasmic reticulum" of late erythroblasts and the "rough endoplasmic reticulum" of reticulocytes from Pb-rats. Six hours after lead administration they were found in vesicles either free in the cytoplasm or inside multivesicular bodies (Figs 15, 16, 17). Energy dispersive Röntgen micro-analysis shows that these fibrillar deposits contain lead, phosphorus and calcium (Fig. 14).

Electron dense deposits were also found in vacuoles of bone marrow macrophages (Fig. 19) and endothelial cells (Fig. 20) of Pb-rats. X-ray micro-analysis of the inclusions in endothelial cells and macrophages revealed the presence of lead, phosphorus and calcium (Fig. 21). In contrast to the fibrillar lead deposits in erythroid cells, the inclusions in endothelial cells have a pleiomorphic structure. But lead deposits in bone marrow macrophages have either a pleiomorphic or a fibrillar structure.

Autophagosomes

Autophagosomes were identified as described previously [11]. In control rats, the autophagosomes were observed in late erythroblasts but in the Pb-rats they were already present in the young and the intermediate erythroblasts. The late

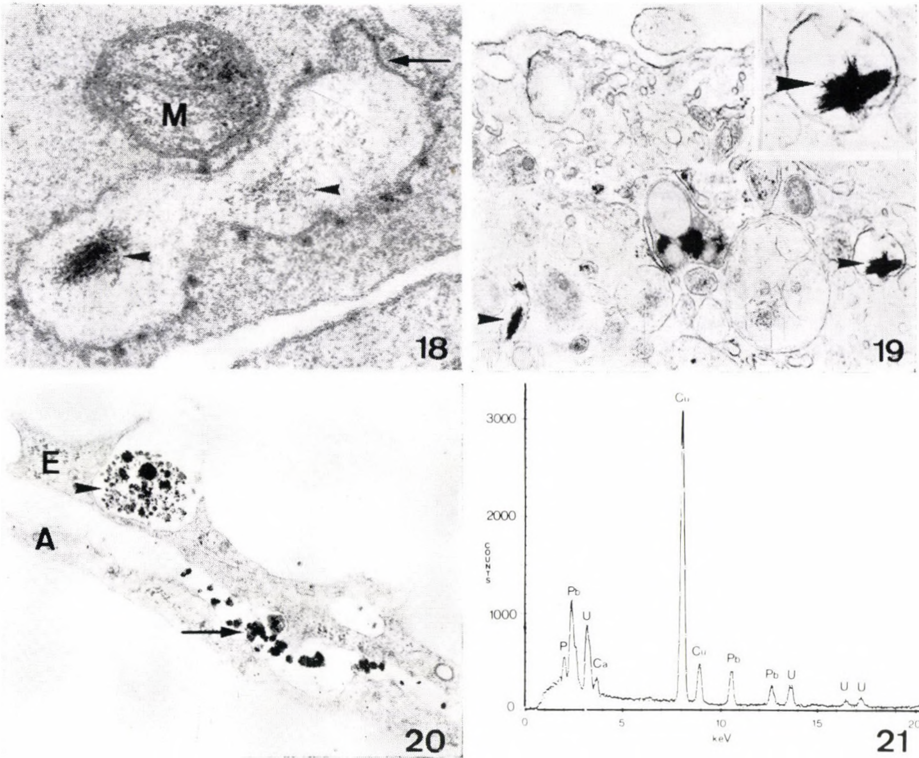


Plate 6

Bone marrow of rats, 17 hours after lead administration

Fig. 18. Fragment of reticulocyte showing an iron-loaded mitochondrion (M) and a cisterna of RER with 2 fibrillar lead-inclusions (arrowheads). The fusion of a vesicle with the RER cisterna is visible (arrow). × 80,000

Fig. 19. Two electron-dense fibrillar inclusions (arrowheads) inside vesicles of a macrophage. × 21,000 (insert × 48,000)

Fig. 20. Pleiomorphic electron dense inclusions (arrowhead) inside a vacuole of endothelial cells and in the intercellular space (arrow) between endothelial (E) and adventitial cells (A). × 18,000

Fig. 21. Röntgen-spectrum of pleiomorphic electron dense inclusions in endothelial cells showing 3 energy peaks for lead (Pb) and one energy peak for phosphorus (P) and one for calcium (Ca). The copper (Cu) peaks are due to the grid and the uranyl (U) peaks are due to the contrasting with uranyl acetate

erythroblasts of the Pb-rats had 16 times more autophagosomes per volume cytoplasm than the intermediate erythroblasts of the same rats and 11 times more than the late erythroblasts of the control animals (Table 2 item 8).

Table 4 shows that the increase in the volume density of autophagosomes in the cytoplasm of Pb-rats is already evident before nuclear extrusion i.e. in (EN−)

Table 4

Autophagosomal parameters (mean ± SD) of (EN−) erythroblasts, (EN+) erythroblasts and reticulocytes of lead- intoxicated (Pb-17h) and control (Ctl) rats

	Rats	(EN−) ebl.	(EN+) ebl.	Reticulocytes
N of analysed cell sections	Pb	87	84	202
	Ctl	47	88	76
% of sections with autophagosomes	Pb	50	54	31
	Ctl	4	25	34
Per 100 μm^3 cytoplasm				
— mitochondria	Pb	9.0 ± 4.5*	6.1 ± 4.8*	4.3 ± 4.1
	Ctl	7.9 ± 3.8*	5.2 ± 3.2*	$2.1 \pm 1.7^+$
— autophagosomes (μm^3)	Pb	1.1 ± 1.9	2.9 ± 6.3*	0.7 ± 1.6
	Ctl	$0.1 \pm 0.6^{+*}$	0.4 ± 1.0	0.4 ± 0.7
Per 100 μm^3 autophagosomes				
— mitochondria (μm^3)	Pb	2 ± 9	2 ± 7	9 ± 26
	Ctl	0 ± 0	23 ± 37	27 ± 39*
— cytoplasm (μm^3)	Pb	16 ± 31	21 ± 34*	11 ± 27
	Ctl	0 ± 0	18 ± 37	4 ± 14
— degraded material (μm^3)	Pb	78 ± 37	75 ± 37	78 ± 38
	Ctl	100 ± 0	59 ± 44	69 ± 44

(EN−) ebl.: (EN−) erythroblast
(EN+) ebl.: (EN+) erythroblast
$^+$: significant differences between Control- and Pb-rats ($P \leq 0.05$)
*: significant differences between subsequent maturation stages ($P \leq 0.05$)

erythroblasts. This elevated level persisted in the (EN+) erythroblasts but decreased in the reticulocytes. The latter had a four times lower volume fraction of autophagosomes in the cytoplasm than the (EN+) erythroblasts of the Pb-rats. (Table 4 item 4.) But in Table 3 it is shown that, when reticulocytes of Pb-rats are divided into two groups according to the ultrastructure of their mitochondria, reticulocytes with normal mitochondria have a higher volume density of autophagosomes in the cytoplasm than reticulocytes with swollen mitochondria (Table 3 item 4).

When analysing the content of autophagosomes it was found that in contrast with control rats, autophagosomes from (EN+) erythroblasts and from reticulocytes of Pb-rats contain a lower volume fraction of mitochondria, but a higher volume fraction of cytoplasm and degraded material (Table 4 items 5, 6, 7).

In reticulocytes with swollen mitochondria, the autophagosomes contained no mitochondria but did reveal 10% cytoplasm and 90% degraded material within it (Table 3 items 5, 6, 7).

Canonical discriminant analysis

The canonical discriminant analysis was carried out on 556 sections of control rats and lead intoxicated rats (Pb-17h). Each section was described by the scores of 6 variables which relate to the cytoplasmic components. The variables of the nucleus were dropped because reticulocytes have a "missing value" for these variables. From the graph (Fig. 22) it appears that consecutive maturation stages of the cell from both control- (figures) and the Pb-rats (letters) divide along the first canonical axis (Can 1). The sections through the early erythroblasts score the highest and those through reticulocytes the lowest values.

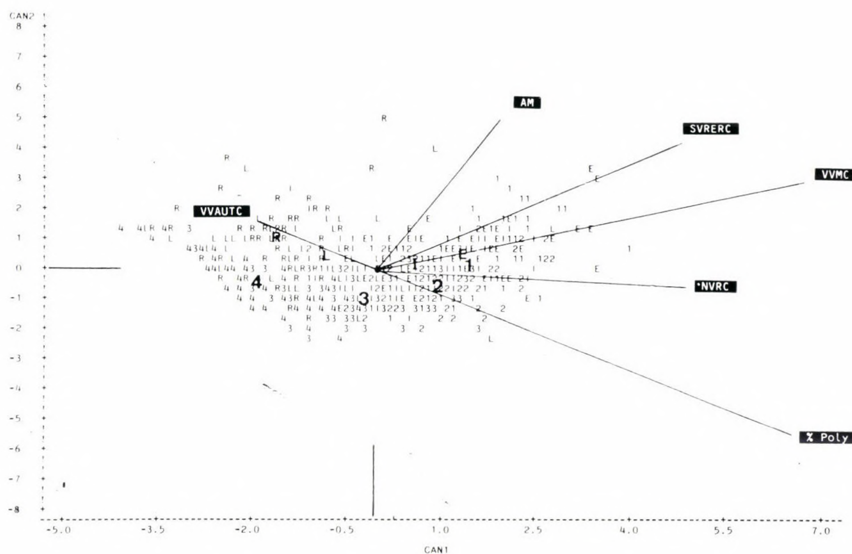


Fig. 22. Plot on the first and second canonical variable (Can 1, Can 2) of 556 sections described by the scores of 6 normalised original variables of early (E,1) intermediate (I,2), late (L,3) erythroblasts and reticulocytes (R,4) of lead-intoxicated (letters) and control (figures) rats. The class means are indicated by large letters and figures. The 6 original variables are represented as vectors. 200 sections are hidden and 52 sections had missing value. Description of the plot can be found in the results

This is illustrated by the position of the class means on the first canonical variable. Except for the volume density of autophagosomes in the cytoplasm, all the original variables have positive correlation with Can 1. When the points of cell sections are projected on the variable vectors one finds that the projection of the young erythroblast points is greater than those of the more mature forms. It can be deduced that as the erythroid cell maturation proceeds all the variables (taken into consideration) score lower, excepting the variables for autophagosomes which score higher in more mature cells.

The graph also separates the sections from Pb-rats (E, I, L, R) and those from the control rats (1, 2, 3, 4) along the second canonical axis (Can 2). The mid points of the young erythroblasts from both the Pb-rats and the control rats (E-1) lie close to each other, but the distances for the other corresponding pairs (I-2, L-3, R-4) are greater. This illustrates that the effect of lead intoxication becomes more pronounced with maturation of the erythroid cells. The size of the projection of the original variable on the second canonical axis indicates which variable is most affected by lead. This projection is a graphic representation of the correlation coefficient (CC) between the original variables and Can 2. Thus %polyribosomes (% poly, CC = −0.58), mean area of mitochondrial profile (AM, CC = 0.53), surface density of RER membranes (SVRERC, CC = 0.48), volume density of mitochondria in the cytoplasm (VVMC, CC = 0.30) and volume density of autophagosomes in the cytoplasm (VVAC, CC = 0.21) are seen to be influenced by lead. However the number of ribosomes per unit volume of cytoplasm (NVRC, CC = −0.05) seems to be unaltered by lead intoxication in this plot (Fig. 22). Although reticulocytes of Pb-rats score higher for NVRC than reticulocytes of control animals, when we drop the influence of the erythroblasts, performing a canonical discriminant analysis with sections of reticulocytes only, it is shown that NVRC contributes to the separation of reticulocytes from control and Pb-rats.

Discussion

Using morphological and morphometric analysis techniques we have studied the ultrastructure of the early, intermediate and late erythroblasts and reticulocytes, 6 and 17h, after an acute intravenous administration of lead (40 mg/kg). From the knowledge of previous cell kinetic data [24] and the inhibitory effect of lead on the growth and maturation of Friend leukemic cells [25], it was assumed that we should observe the effect of lead intoxication while the erythroid cells had passed through only one phase of their maturation cycle.

Haematological parameters

Control rats were found to contain 0−26 μg Pb/gm bone marrow, while the Pb-rats contained 90−122 μg Pb/gm bone marrow. Had the injected lead distributed uniformly throughout the rat body, the bone marrow would have shown 40 μg Pb/gm bone marrow. However the bone marrow contained 2 to 3 times more lead than expected if the injected lead had been distributed homogeneously in the rat. This suggests that there is preferential accumulation of lead in the bone marrow.

After 17hrs. of exposure to lead, haematological parameters of the peripheral blood showed no evidence of lead intoxication (Table 1) and the mean cell haemoglobin was normal. However morphological observations and stereological analysis of red cell precursors revealed that cell structures had undergone changes and

that these became more pronounced as the cells advanced through their maturation stages. The reduced number of late erythroblasts in the bone marrow in the Pb-rats could be explained partly by inefficient erythropoiesis and partly by increase in erythrophagocytosis of the late erythroblasts (Figs 5, 6).

This is indirectly supported by the observations in mice in which acute lead-induced transient hypoplasia has been explained by intramedullary destruction of the intermediate erythroblasts [6]. In addition, it has been shown that impaired protein synthesis in erythroblasts enhances their phagocytosis by macrophages [26].

Shape of erythroblast and reticulocytes

Examination of electron micrographs of the erythroblasts, their nuclei and reticulocytes of the Pb-rats showed deeper invagination than in the corresponding cells of control rats. The increase in the surface area relative to the volume of the cells may be the result of decrease in the volume of the cell. This is substantiated by the fact that mean cell volume of the Pb-rats erythrocytes was reduced without any decrease in mean cell haemoglobin (Table 1). The reduction in the cell volume in the Pb-rats may be due to the inhibition of the membrane-bound Na/K ATPase [27, 28, 29] with consequent loss of ions and water [30].

"Nuclear clefts"

More nuclear clefts were observed in the erythroblast nuclei of the Pb-rats. This is confirmed by the greater surface density of the nuclear cleft membrane per nuclear volume (Table 2 item 1). The nuclear clefts are characterized by their localisation in the heterochromatin of the nucleus (Fig. 7). Nuclear clefts and blebs are a frequent finding in the human dyserythropoietic conditions which arise as a result of defective DNA synthesis [23, 31, 32]. In this context it is significant that lead found in the nucleus can bind to the DNA and thus disturb its biosynthesis [33, 34].

Ribosomes

Although a decrease in percentage polyribosomes is already discernible in the early erythroblasts of the Pb-rats, it is much more significant in the more mature stages of the cells. Since protein synthesis occurs on the polyribosomes, these results suggest that the erythroblasts of the Pb-rats syntheses less protein. This correlates well with the previous in vitro experimental data showing that lead inhibits protein synthesis in reticulocytes by disaggregation of polyribosomes [35, 36]. Since in vitro addition of haem can abolish the lead effects [4, 36] and inhibition of haem synthesis or haem deficiency can cause inhibition of protein synthesis [37], we are not certain whether disaggregation of the polyribosomes and hence inhibition of protein synthesis is the primary effect of lead or is secondary to its inhibition of haem synthesis.

The number of ribosomes per unit volume of cytoplasm in early, intermediate and late erythroblasts of the Pb-rats is not different from those of control rats. Compared with the control rats the reticulocytes of the Pb-rats contain 25% more ribosomes. The explanation for this might lie in the fact that lead is a potent inhibitor of the enzyme pyrimidine 5-nucleotidase, even at 10 μM concentrations [38, 39]. This enzyme is necessary for the breakdown of RNA including the rRNA.

Mitochondria

Neither the examination of electron micrographs nor the morphometric analysis revealed any change in the mitochondria of the early and the intermediate erythroblasts. However 36% of the late erythroblasts and 65% of the reticulocytes of the Pb-rats contained mitochondria with swollen inner compartment. These mitochondria had also fewer cristae and their inner matrix contained flocculent material which probably represented irreversibly denatured proteins [40]. Previous studies with the renal tubular cells and erythroid cells have shown that lead causes uncoupling of the mitochondrial oxidative phosphorylation [41, 42]. It has been proposed [43, 44] that the energy released by the uncoupling of oxidative phosphorylation is used to transport ions, including Pb^{2+}, into the mitochondria. However, although we have found that mitochondria in 7% of late erythroblasts and 31% of the reticulocytes contained electron dense material, energy-dispersive Röntgen microanalysis revealed no lead [45]. In fact the material was found to be composed of iron and phosphorus (Fig. 13). This can presumably be due to the fact that the concentration of lead in the mitochondria was too low for the energy-dispersive Röntgen analysis technique. Inhibition by lead of haem synthesis prior to that of iron uptake and uncoupling of oxidative phosphorylation by lead would cause accumulation of both iron and phosphorus. Since iron is essential for haem synthesis it would follow that those cells (late erythroblasts and reticulocytes) which are synthesising haem more intensely, prior to their inhibition by lead, would accumulate more iron into their mitochondria than those which are not so active in haem synthesis. This is supported by our finding of iron-loaded mitochondria only in the late erythroblasts and reticulocytes and not in the earlier stages.

Uncoupling of oxidative phosphorylation prevents ATP synthesis. In the absence of ATP, the ionic concentration gradient across the mitochondrial membrane can no longer be maintained. This would result in passive osmotic swelling of mitochondria. We have observed swollen mitochondria particularly in late erythroblasts and reticulocytes (Table 2 item 5) and the swelling is limited to the inner compartment (Fig. 8).

Experiments with rat cardiac muscle indicate that mitochondrial swelling requires a threshold concentration of lead [46]. This would suggest that the absence of mitochondrial swelling in the young erythroblasts may be due to low concentrations of lead inside those cells.

Rough endoplasmic reticulum

Energy dispersive Röntgen micro-analysis revealed not only calcium and phosphorus, but also lead in the electron dense fibrillar inclusions in the late erythroblasts and reticulocytes of Pb-rats [45]. However the sulphur K_α peak also lies in the same energy field (2–2.3 keV) as the lead M peak (Fig. 14), and in the absence of sulphur, the peak height is smaller. The very tall peak at 2–2.3 keV suggested the presence of sulphur in the lead inclusions. This is not altogether unexpected. It is well known that lead binds quite strongly to the sulphhydryl groups which in the form of cysteine are abundant in metallothioneins. Lead does not induce its own metallothionein, but it can bind to that of other metals [34]. The presence of calcium together with lead in the fibrillar inclusions suggests that lead ions are pumped into vesicles by the same pump that is used for calcium ion translocation. This is supported by ultrastructural study of kidney cells incubated in vitro with free lead ions in various media [47].

When late erythroblasts and reticulocytes of rats with a 6 hour lead intoxication are screened for fibrillar lead inclusions, these inclusions are found predominantly in small vesicles of late erythroblasts and reticulocytes (Figs 15–17). These vesicles occur either free in the cytoplasm or inside multivesicular bodies. 17 hours after lead intoxication these vesicles are fused to larger cisternae which are found in the late erythroblasts and reticulocytes. In the reticulocytes the endoplasmic membrane is occupied by ribosomes (Fig. 18) on which, presumably, protein synthesis continues to occur. Moreover there is an increased surface of RER membranes per volume cytoplasm in Pb-rats (Table 2 item 7). We assume that formation of fibrillar Pb-inclusions in the endoplasmic reticulum probably serves to detoxify lead.

Lead, together with phosphorus and calcium, is found in pleiomorphic electron dense deposits in vacuoles of bone marrow endothelial cells and macrophages (Figs 20, 21). The same material is found in the intercellular space between endothelial and adventitial cells. The presence of lead in pleiomorphic inclusions of macrophages and endothelial cells may be explained by endocytosis of plasma protein-bound lead [48]. It can be assumed that lead bound to the fibrillar inclusions of bone marrow macrophages is not taken up by endocytosis, but by the same mechanism that is used for the uptake of lead into vesicles with fibrillar inclusions of erythroid cells.

Autophagosomes

The results of autophagosomal analysis can only be explained by two apparently opposing mechanisms. First, there is an increased sequestration of cellular material. This is supported by the volume density of autophagosomes which increases with maturation of erythroid cells and reaches a maximum in the late erythroblasts (Table 2, item 8 and Table 4). On the other hand, a decrease in volume density of autophagosomes in the reticulocyte cytoplasm suggests a second mechanism of inhibition of the autophagocytic process at this maturation stage.

The analysis of the autophagosomal content of (EN−)-, (EN+)-erythroblasts and reticulocytes showed that there was increased autophagocytosis of cytoplasm as well as mitochondria. In contrast to the normal animals [11] there is no selective sequestration of mitochondria. Whereas in the autophagosomes of (EN+)erythroblasts of the control animal the ratio of mitochondria to cytoplasm is one, in the lead-treated rats this ratio is 1 : 10. This ratio is of the same order of magnitude as the volume density of the mitochondria in the cytoplasm in the corresponding maturation stage (Table 4).

Samples used for X-ray microanalysis were not treated with osmium and the autophagosomes in these samples could not be visualised. This suggests that the electron dense material in the autophagosomes of osmium-preparations is composed of lipids rather than heavy metal.

Although lead induces greater changes in the reticulocytes than in the earlier stages (Fig. 22), the volume density of the autophagosomes in this maturation stage is much smaller (Table 2, item 8). When the reticulocytes are divided into two groups one with normal mitochondria and the other with swollen mitochondria one finds that autophagosomal volume density in cytoplasm of the cells with swollen mitochondria is extremely low (Table 3, item 4). In fact in the reticulocytes with swollen mitochondria one finds that 90% of the autophagosomal content consists of degraded material without any recognizable mitochondria or cytoplasm (Table 3, item 5, 6, 7).

Previous studies with other cell types have shown that autophagic vacuoles are isolated using preexisting cell membranes [49]. We have found that in erythroid cells, the isolating membranes originate in the endoplasmic reticulum. The wrapping mechanism of endoplasmic reticulum during autophagosome formation [50] and the elimination of residual bodies requires ATP. 30% of late erythroblasts and 60% of reticulocytes of Pb-rats contain swollen and presumably functionally abnormal mitochondria. Therefore, as a result of limited energy supply there is decreased sequestration of material and elimination of residual bodies.

Conclusion

Intoxication with high doses of lead over a short period was used to study its effect on the different maturation stages of the erythroid cells. We have shown that toxic effects of lead increase with the maturation of cells. The most profoundly affected systems were mitochondria and polyribosomes. This could be represented graphically by the canonical discriminant analysis (Table 22). Since the effect of lead on the various enzyme systems in the erythroid cells depends on a certain threshold value [46], it suggests that lead concentration in the more mature cells is greater than in the younger cells. This is indirectly supported by the presence of lead inclusion bodies in the late erythroblasts and reticulocytes. The greater concentration of lead in the mature cells may be due to an increased uptake.

Acknowledgement

We thank Lena Verhelst and Vic Van Duppen for technical assistance, Dr. W. Goossens for determination of lead concentration in bone marrow, Dr. Manuel Lesaffre, Dr. Jan Meijnaerts and Dr. Magda Vuylsteke for statistical analysis of the data, Dr. W. C. de Bruijn for the Röntgen micro-analysis and useful discussions and Ria Swinnen for secretarial assistance. The work was supported by the Nationaal Fonds voor Geneeskundig Wetenschappelijk Onderzoek (grant number 3.0051.80).

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Haematologia 20 (3), pp. 155–163 (1987)

T-Cell Depletion of Cercopithecus Aethiops Monkey Bone Marrow with Campath-1 Monoclonal Antibody and Complement

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(Received 11 July, 1986; accepted 5 September, 1986)

The effectiveness of anti human monoclonal antibody (Campath 1) plus complement (C') in removal of T-cells from Cercopithecus aethiops monkey bone marrow was studied. Recovery of haemopoietic progenitor cells (CFU-GM) was also investigated in vitro after treatment with Campath-1 plus C'. The results showed that the cell-yield was 37.2 ± 9.8 after Ficoll separation and $44.2 \pm 13.2\%$ after Campath-1 + + C' treatment. The CFU-GM yields referred to the original total CFU-GM were 88.9 ± 24.0 and $40.0 \pm 14.3\%$, respectively. After Campath-1 treatment, CFU-GM per 10^5 bone marrow cells was 269.3 as compared to the pretreatment value of 213.0. In the T-cell-depleted bone marrow suspensions mature T lymphocytes could not be detected. Following cryopreservation more than 70% of CFU-GM could be recovered in T-cell depleted bone marrow suspensions kept in frozen state for two months.

Cercopithecus aethiops monkeys can be used as a model to study T-cell depletion of bone marrow with Campath-1 plus C' for studying allogeneic bone marrow transplantation.

Keywords: monkey bone marrow, Campath-1, T-cell depletion, CFU-GM, cryopreservation of monkey bone marrow

Introduction

Transplantation of allogeneic bone marrow has been applied with increasing success in treatment of patients with certain haematological, immunological and immunogenetic diseases. Graft-versus-host disease (GVHD), however, is a cause of considerable morbidity and mortality in human allogeneic bone marrow transplantation (BMT). 50–70% of the patients receiving bone marrow from fully-matched HLA-A, B, C, DR compatible siblings will still develop some degree of acute GVHD [1, 2, 3, 4, 5]. Experimental and clinical evidence has shown that GVHD is mediated by immunocompetent T lymphocytes of donor origin, and it can be abolished by removing mature T-cells from the donor bone marrow [6, 7, 8, 9]. According to clinical observations the ratio of GVHD decreased markedly

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in the recipients grafted with T-cell depleted bone marrows [10, 11, 12, 13, 14, 15, 16].

Different methods have been tried to deplete T-cells of the bone marrow, including discontinuous albumin gradient centrifugation [17], continuous in vitro culture [18], complement-mediated cytolysis by heteroantisera [19], monoclonal antibody CT-2 and OKT-3 + 11 [12, 15, 20]. Other possibilities to remove T-cells of bone marrow are E rosette formation with sheep red blood cells (SRBC) [13, 20, 21], combined agglutination of T-cells with soybean agglutinin (SBA) and E rosettes [22]. None of these methods, however, could be employed with completely satisfactory results. Recently, T-cell elimination from allograft with Campath-1 monoclonal antibody (MoAb) and human C' has been described as an effective method of preventing GVHD [16, 23]. Campath-1 is a MoAb with pan-lymphocyte specificity reacting with human as well as Rhesus and Cynomolgus monkey lymphocytes, hence they have been used to study the elimination of T-cells for the prevention of GVHD [24, 25]. We noted that Campath-1 cross-reacted with Cercopithecus Aethiops (CA) lymphocytes in the complement-mediated cytotoxicity test and E rosette inhibition assay (data to be published).

In the present work we used Campath-1 and C' to eliminate T-cells from CA monkey bone marrow. The effectiveness of Campath-1 + C' in killing T lymphocytes in the marrow were examined, and the survival of granulocyte-macrophage progenitor cells (CFU-GM) of T-cell depleted bone marrow was studied. Finally, the recovery of progenitor cells after storing in liquid nitrogen and thawing is documented in this paper.

Materials and Methods

Monoclonal antibodies

Campath-1, monoclonal rat antihuman pan-lymphocyte antibody (IgM) was a generous gift of Prof. H. Waldman (Division of Immunology, Laboratory Block, Cambridge).

Animals

CA monkeys of both sexes (from Van-Der Bilj, The Netherlands) were provided by the National Institute of Hygiene, Budapest. The monkeys were 4 to 6 years old.

Mononuclear cell preparation

Both femora were removed aseptically and maintained in heparinized buffered salt solution. The samples were cut into small pieces: bone marrow cells were removed by aspiration, and washed with McCoy-5A medium. The cell suspension was centrifuged at 2000 rpm for 10 minutes. The fat layer was removed and the

pellet resuspended in McCoy-5A medium. Optimum concentration ($1-1.5\times10^7$ nuclear cells per ml of cell suspension) was layered over a Ficoll-Hypaque gradient (density = 1.077 g/ml) as described by Boyum [26]. After centrifugation at 400 *g* for 40 minutes at 18 °C, the mononuclear cells on the interface were collected and washed three times in McCoy medium. After this procedure the cell suspensions contained less than 10% granulocytes + monocytes as shown by Wright-Giemsa staining.

MoAb + complement treatment

Lymphocytes were removed from the bone marrow with Campath-1 and C' as described by Waldman and Hale et al. [16, 23]. Monkey bone marrow mononuclear cells ($50-100\times10^6$ cells per ml) were incubated with Campath-1 in final concentration of 100 μg/ml for 30 minutes at room temperature. Fresh human AB serum (as complement) was added up to 25% final concentration, and the mixture was incubated at 37 °C for 45 minutes. Aggregates of dead cells were discarded and living cells were recovered from the residual material.

Detection of residual immunocompetent cells

Immunocompetent cells in untreated and treated bone marrow suspensions were assayed by two methods: E rosette formation with AET-treated sheep red blood cells (SRBC) [27], and microcytotoxicity test with Campath-1 MoAb and rabbit complement [31]. Campath-1 concentration was 100 μg/ml of cell suspension. Preliminary tests showed that this was the optimum concentration.

Assay of haematopoietic progenitors

A modification of the semisolid agar assay described by Pike and Robinson [28] was applied to culture granulocyte-macrophage progenitor cells (CFU-GM). 1×10^5 normal or T-depleted bone marrow cells were cultured in 35 mm Petri dishes in a medium containing 0.3% agar, 35% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol and 10% human placental conditioned medium (HPCM) in McCoy-5A medium. HPCM was prepared according to the method of Burgess et al. [29]. The plates were incubated at 37 °C in an atmosphere of high humidity containing 5% CO_2. On the ninth day the colonies with more than 50 cells were counted by inverted microscope.

Cryopreservation

T-cell-depleted bone marrow suspension was stored in a frozen state for two months. A 1.5 ml volume of viable cells (10^7 cells/ml) in RPMI-1640 medium supplemented with 20% fetal calf serum and 10% dimethylsulphoxide (DMSO) was put in 2 ml tubes and frozen in a program freezer [30]. The frozen cells were stored in liquid nitrogen.

Results

The bone marrow cell yield was 37.2 ± 9.8% after Ficoll-Hypaque separation, and 44.2 ± 13.2% after treatment with Campath-1 + C' (Table 1).

The CFU-GM yield referred to the original CFU-GM content of the bone marrow were: 88.9 ± 24.0% after Ficoll-Hypaque separation, and 40.0 ± 14.3% after Campath-1 treatment (Table 2). CFU-GM per 10^5 cells from bone marrow

Table 1

Yield of C. aethiops bone marrow cells after Ficoll separation and subsequent Campath-1 + C' treatment

No. of monkey	Nucleated cells (× 10^6)	Ficoll-isolated cells (× 10^6)	Yield (%)	Campath-1 + C'-treated cells (× 10^6)		Yield (%)
				before	after	
1	132	26	19.2	15.0	6.5	43.3
2	308	90	29.8	22.5	8.5	36.0
3	335	114	43.2	28.6	12.9	45.1
4	70	21	30.0	10.0	6.8	68.0
5	212	62	29.4	55.7	22.3	40.0
6	203	85	41.4	60.1	27.0	45.0
7	216	81	37.5	63.0	23.2	36.8
8	327	169	50.5	50.8	25.5	50.3
9	367	164	44.8	55.0	31.5	57.2
10	450	210	46.8	51.6	27.2	52.7
Mean ± SD			37.2 ± 9.8			44.2 ± 13.2

Table 2

Yield of CFU-GM of C. aethiops monkey bone marrow cells after Ficoll Separation and Campath-1 + C' treatment

No. of monkey	Number of original CFU-GM	After Ficoll separation		After treatment with Campath-1 + C'	
		Abs. number CFU-GM	Yield* (%)	Abs. number CFU-GM	Yield* (%)
3	89.402	100.163	112.0	25.596	28.6
4	78.400	77.728	111.8	36.830	46.9
5	138.000	—	—	57.024	41.3
7	123.120	103.530	84.4	37.740	30.6
8	21.774	20.700	95.4	14.466	66.4
9	85.329	27.520	47.1	20.090	23.5
10	12.376	10.353	83.6	5.252	42.4
Mean ± SD			88.9 ± 24.0		40.0 ± 14.3

* Related to the original CFU-GM content of the bone marrow sample
abs: absolute number

Table 3

CFU-GM concentration in C. aethiops bone marrow after Ficoll Separation and Campath-1 + C' treatment

No. of monkey	CFU-GM per 10^5 bone marrow cells		
	Untreated cells	Ficoll isolated* cells	Campath-1 + C'* treated cells
1	136.0 ± 9.5	150.3 ± 10.2	ND
2	38.6 ± 3.5	212.7 ± 12.5	167.6 ± 7.9
3	41.5 ± 2.1	91.3 ± 3.7	131.7 ± 17.9
4	74.3 ± 2.5	380.9 ± 16.7	390.8 ± 29.0
5	46.1 ± 3.7	–	176.0 ± 14.4
6	–	127.7 ± 21.0	148.7 ± 16.3
7	112.0 ± 9.3	390.8 ± 24.1	538.0 ± 12.8
8	57.3 ± 6.3	203.6 ± 8.7	355.4 ± 29.1
9	37.0 ± 5.3	138.0 ± 29.6	282.6 ± 7.6
10	67.2 ± 2.6	222.3 ± 24.1	433.0 ± 24.0
Mean	67.7	213.0	269.3

* Mean ± SD of three samples

samples examined after each step of T-cell depletion is shown in Table 3. Mean of the CFU-GM counts per 10^5 cells was 67 in the unseparated bone marrow, 213 after Ficoll separation and 269 after treatment with Campath-1 + C'. These re-

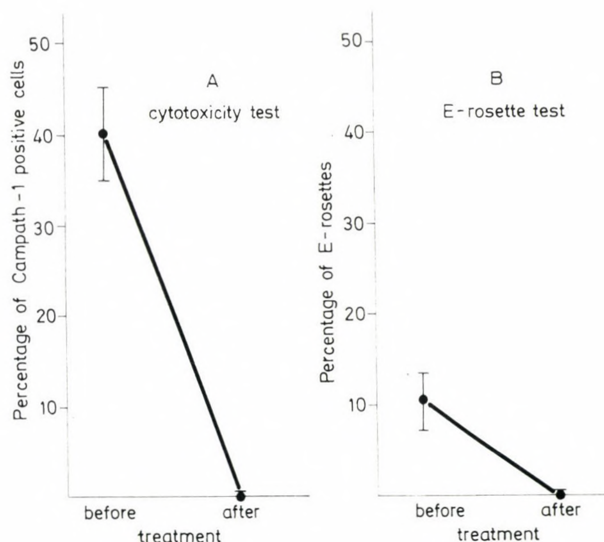

Fig. 1. Effectiveness of T-cell depletion with Campath-1 + C': T-cell-numbers of bone marrow suspensions before and after treatment was examined by microcytotoxicity test (A) and E rosette formation (B)

sults showed that treatment with Campath-1 + C' does not inhibit the colony formation of CFU-GM.

The bone marrow samples were tested for T-cell contamination before and after treatment; the E rosette formation test and cytotoxicity were used. The results were as follows. Before treatment, E-rosette-forming cells were 6 to 15% and Campath-1-positive cells were 32 to 49%. After treatment, the ratio of residual T-cells decreased significantly, mature T lymphocytes could not be detected in the T-cell-depleted bone marrow suspensions (Fig. 1).

We studied the recovery of nucleated cells and CFU-GM of T-depleted bone marrow suspensions which had been kept in frozen state for two months and then thawed. Table 4 shows the recovery of bone marrow cells (73.5%) and CFU-GM (70.9%) after thawing. The results indicated that T-cell depleted monkey bone marrow can be stored in liquid nitrogen for long periods without considerable loss of CFU-GM.

Discussion

Experimental and clinical evidence of allogeneic BMT shows that a nearly complete removal of T-cells from bone marrow graft (by both physical and specific methods) is absolutely necessary to prevent GVHD in HLA-matched, and particularly in mismatched BMT [1, 7, 12, 13, 21, 22, 24]. Recent clinical studies have demonstrated that GVHD could be prevented by T-cell elimination from the donor bone marrow graft with total lymphocyte-specific complement-binding monoclonal antibody, Campath-1 [16, 23, 25]. The advantage of MoAb Campath-1, IgM antibody is its ability to fix human complement effectively [16, 23]. Campath-1 reacts with both human T and B lymphocytes of peripheral blood, and crossreacts with lymphocytes of Rhesus monkeys [23, 24, 25]. Gerritsen et al. [24] succeeded in depleting T-cells for the prevention of GVHD from the bone marrow of Rhesus monkeys. They noted that the removal of all lymphocytes by Campath-1 looks promising, but experiments indicated that in a few cases GVHD could not be prevented with this procedure. We found, moreover, that lymphocytes from various sources (bone marrow, peripheral blood, lymph-node, spleen and thymus) of CA monkeys crossreacted with Campath-1 plus C' in the cytotoxicity test. On the other hand, CA lymphocytes possessing CD-2 antigen form E rosettes with either untreated SRBC or AET (2-aminoethylisothiorinium bromide) treated SRBC, and SRBC-E rosette formation was inhibited by preincubating the monkey lymphocytes with antihuman T lymphocyte MoAb (data to be published). Using the above observations as a starting point, we attempted to deplete T-cells from CA bone marrow by treating it with Campath-1 plus C'. T lymphocyte-depleted bone marrow cells did not react with Campath-1 in the cytotoxicity test and E rosette forming technique (Fig. 1). Previous studies have demonstrated that Campath-1 and human C' are not toxic to haemopoietic progenitor cells of human and Rhesus monkey origin [16, 23, 24, 25]. Similar results were obtained with other methods for human T-cell depletion [10, 11, 18, 20, 21, 22, 32, 33]. In this study,

we also observed that T-cell removal with Campath-1 and C' from CA bone marrow does not inhibit the growth of CFU-GM in vitro; due to the elimination of lymphocytes the concentration of CFU-GM in T lymphocyte-depleted bone marrow even exceeded the CFU-GM concentration before treatment (Table 3).

We studied the yield of haemopoietic progenitor cells after cryopreservation of T-cell-depleted bone marrow suspensions for two months. Over 70% of the cryopreserved CFU-GM-forming progenitor cells were recovered (Table 4). Similar results were described previously in studies of human bone marrow [27, 33].

Table 4

Survival of nucleated cells and CFU-GM of T-cell depleted bone marrow suspensions after 2 months storage in liquid nitrogen

No. of monkey	Cell number ($\times 10^6$)		Yield (%)	CFU-GM per 10^5 cells		Yield (%)
	before freezing	after thawing		before freezing	after thawing	
6	19	16.4	86.8	239.3 ± 10.5	203.3 ± 24.3	84.9
7	37	24.8	66.2	433.0 ± 24.0	299.5 ± 22.5	69.1
8	72	56.0	77.7	355.0 ± 29.2	184.6 ± 9.2	52.0
9	25	16.6	66.4	140.6 ± 13.6	122.6 ± 14.7	87.1
10	37	26.5	70.4	357.3 ± 17.1	220.7 ± 11.1	61.7
Mean $\pm$ SD			73.5 ± 8.8			70.9 ± 15.0

The present results indicate that Campath-1 + C' crossreacts with lymphocyte surface antigens of not only Rhesus and cynomolgus, but CA monkeys, too. CA was used by us as an experimental model for depleting T-cells from bone marrow graft in the study of allogeneic bone marrow transplantation.

Acknowledgements

We wish to express our special thanks to Prof. H. Waldman (Division of Immunology Laboratory Block, Cambridge, England) for gift of Campath-1 MoAb serum. We thank Dr. L. Nemes, Head of the Department of Experimental Animals of the National Institute of Hygiene, Budapest, Hungary, for access to monkey specimens.

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Haematologia 20 (3), pp. 165—170 (1987)

Dipeptidyl Peptidase IV Activity in Cells of T-lymphoid Origin is Decreased in Cultures with 12-0-Tetradecanoylphorbol-13-acetate (TPA)

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(Received 3 June, 1986; accepted 26 August, 1986)

Dipeptidyl peptidase IV (DPP IV) is a specific enzyme for cells of T-lymphocytic lineage. It has been attempted to induce DPP IV activity in DPP IV negative T-lymphoid leukaemias by 12-0-tetradecanoylphorbol-13-acetate (TPA). Isolated cells from peripheral blood of 6 healthy blood donors and 22 patients with various types of leukaemia were cultivated in RPMI-1640 medium with 20% fetal calf serum alone (control) or supplemented with 20% human placenta conditioned medium (HPCM) or with 16 nmol/l TPA for 3 days. The percentage of DPP IV positive lymphocytes from blood donors remained unchanged in control and HPCM cultures, but decreased significantly in TPA cultures. Leukaemic cells from three DPP IV positive cases of acute T-lymphoblastic leukaemia (T-ALL) reacted to the TPA treatment in a similar manner. Leukaemic cells of two DPP IV negative T-ALL cases remained negative in all three types of cultures, thus no induction of DPP IV activity was found. Other normal blood cells as well as leukaemic cells of 7 null-ALL, 1 preB-ALL, 3 B-CLL and 6 AML patients were DPP IV negative before and after cultivation in all types of culture. These findings showed that DPP IV is specifically expressed in cells of T-lymphocytic lineage even after short-term cultivation. HPCM was found to have no effect on DPP IV activity in T-lymphoid cells.

Keywords: DPP IV, haemopoietic cells, HPCM, short-term cultivation, T-ALL, TPA

Introduction

Dipeptidyl peptidase IV (DPP IV, E.C. 3.4.14.5) activity demonstrated with glycyl-L-proline-4-methoxy-2-naphthylamide as substrate was described in about 40% of peripheral blood lymphocytes [1]. Only a subset of T-lymphocytes reacted positively [1, 2]. The major part of DPP IV positive cells was OKT4+ but some OKT8+ lymphocytes were also positive [3—7]. All other blood and bone marrow cells were DPP IV negative [1—7]. The diagnostic value of DPP IV activity in cells of some T-malignancies is well established [2, 4, 6—15].

Short-term cultivation of leukaemic cells with 12-0-tetradecanoylphorbol-13-acetate (TPA) or with human placenta conditioned medium (HPCM) with subsequent cytochemical examination of cultivated cells is a useful tool for the

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classification of leukaemias [16, 17]. Furthermore, TPA may induce differentiation of leukaemic cells in some T-lymphoblastic leukaemia cell lines, e.g. MOLT-3 [18] or JM, HPB-ALL [19, 20], but DPP IV activity in leukaemic cells during short-term cultivation was not examined.

The aim of this study was therefore to examine the expression of DPP IV activity in blood cells of healthy blood donors as well as in leukaemic cells of patients with various types of leukaemia after 3 day cultivation in control, HPCM and TPA cultures, with special regard to the possibility of inducing DPP IV activity in originally DPP IV negative leukaemic T-cells.

Materials and Methods

Cells were isolated from heparinized blood (10 ml) of 6 healthy blood donors and 22 patients with various types of leukaemia as buffy coats or mononuclear cells from the interphase of Ficoll-Verografin gradient (1.077 g/ml). Blood samples were obtained usually from untreated patients with high leukaemic cell counts (often more than 50×10^9 leukocytes/l). Diagnosis of leukaemia was based on morphology, cytochemical and surface phenotype examination of leukaemic cells, and standard diagnostic criteria (for details see ref. 17). Surface phenotyping for T-markers was carried out with OKT monoclonal antibodies (Ortho Diagnostic Systems Inc., Raritan, NJ, USA) and with UCHT1 monoclonal antibody [21], a generous gift of Dr. P. C. L. Beverley. We studied leukaemic cells from 5 patients with acute T-lymphoblastic leukaemia (T-ALL), 7 patients with null-ALL, 1 patient with preB-ALL, 3 patients with chronic B-lymphatic leukaemia (B-CLL), and 6 patients with acute myeloid leukaemia (AML – according to the FAB classification: 1 M_1, 1 M_3, 2 M_4, 1 M_5, 1 M_6).

The isolated cells were cultivated in 5 ml RPMI-1640 medium (prepared in our Institute) with penicillin (100 U/ml) and streptomycin (100 μg/ml) and 20% pretested fetal calf serum alone (control cultures) or supplemented with 20% HPCM (HPCM cultures) or with 16 nM/l TPA (TPA cultures) respectively, in polystyrene Petri dishes (∅ 60 mm, Koh-i-noor, Dalečin, Czechoslovakia) at 37 °C in humidified air with 5% CO_2 for 3 days. The concentration of seeded nucleated cells was about 2×10^5/ml (for further details see ref. 17). Smears of cultivated cells were prepared and stained for DPP IV according to Lojda [1]. Usually 200 cells were found, the number of DPP IV positive cells was recorded and expressed in percentage. HPCM was prepared as described previously [22].

Results

The percentage of DPP IV positive lymphocytes in the blood of 6 healthy blood donors varied from 26 to 53.5%. Approximately the same number of DPP IV positive lymphocytes was found in control and HPCM cultures while only 0.5 – 15% cells of lymphocytic lineage were DPP IV positive in TPA cultures where

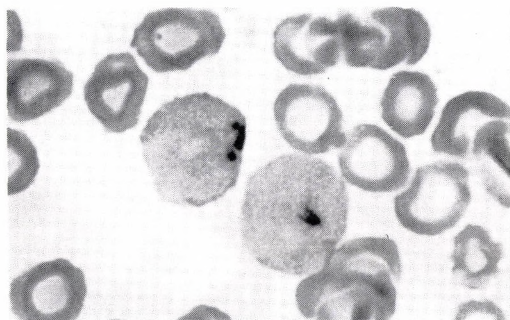

Fig. 1. Two DPP IV positive (dark dots) leukaemic cells in the blood smear of patient P. C. (magn.: × 1,300)

Table 1

Percentage of DPP IV positive lymphoid cells in blood and in control, HPCM, and TPA 3 day cultures from patients with T-ALL

Patient	Surface phenotype	DPP IV positive lymphoid cells			
		Blood %	3 day cultures		
			Control %	HPCM %	TPA %
P. C.	OKT11+, UCHT1−, OKT4−, 8−	52	58	54	4
K. H.	OKT11+, OKT3+, OKT4−, 8−	0	0	0	0
V. Č.	UCHT1+, OKT4−, 8−	0	0	0	0
A. S.	UCHT1+, OKT4−, 8−	72	75	70	11
R. U.	UCHT1+, OKT4+, 8+	14	11	12	5

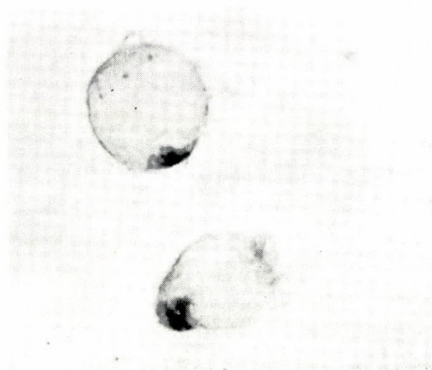

Fig. 2. Two DPP IV positive leukaemic cells from 3 day control culture of patient P.C. (magn.: × 1,300)

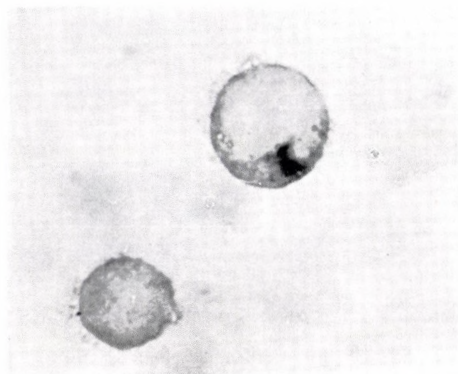

Fig. 3. One positive and one DPP IV negative leukaemic cell from 3 day HPCM culture of patient P.C. (magn.: × 1,300)

blast transformation of lymphocytes was induced by TPA. All other blood cells were negative before and after 3 days' cultivation in all three types of cultures.

Viability of leukaemic cells after 3 days' cultivation was greater than 80% as assessed by trypan blue dye exclusion. Leukaemic lymphoid cells in 3 of 5 patients with T-ALL were DPP IV positive in 14%, 52% (Fig. 1), and 72%, respectively (Table 1). The percentage of DPP IV positive cells remained unchanged in the control (Fig. 2), and HPCM (Fig. 3) cultures (Table 1), while it decreased in TPA cultures (Table 1). Leukaemic cells from two cases of T-ALL (V. Č., K. H. – Table 1) were originally DPP IV negative and remained negative in control, HPCM and TPA cultures. Thus no induction of DPP IV activity in these cases took place.

Leukaemic cells from null-ALL (7 patients), preB-ALL (1), chronic B-lymphatic leukaemia (3) and acute myeloid leukaemias (6) were DPP IV negative before cultivation, and remained negative in control, HPCM and TPA cultures.

Discussion

Investigations by Sannes [23] demonstrated DPP IV activity at ultrastructural level in rat alveolar macrophages. On the other hand, human cells of the mononuclear phagocyte system (monocytes, macrophages) in blood and bone marrow smears were DPP IV negative [1, 7]. In the present study macrophages originating in culture after the induction of human leukaemic myeloid blasts ($M_1 - M_5$) with TPA were shown to be also DPP IV negative. The only cells expressing DPP IV activity in culture were cells of normal lymphoid morphology or, in the case when leukaemic cells were cultured, malignant T-lymphoid leukaemic cells. The percentage of DPP IV positive normal lymphocytes remained unchanged in both control and HPCM cultures. This is in agreement with the results of Scholz

et al. [24], who have shown that the percentage of DPP IV positive cells did not change after 2 days' culture under conditions similar to our own control cultures. The likely explanation of this finding is that DPP IV production is typical only for a subset of T-lymphocytes [1, 7], probably to interleukin 2 producing cells [24]. The percentage of DPP IV positive cells was decreased in TPA cultures. This decrease could be attributed to the blast transformation of T-lymphocytes by TPA. A similar decrease was also observed in lymphocyte cultures induced with phytohaemagglutinin [1, 2].

Short-term cultivation of lymphoid cells with HPCM, the source of granulocyte-macrophage colony stimulating factors, as expected, did not influence their DPP IV reactivity.

DPP IV negative leukaemic T-cells of 2 T-ALL remained negative in control, HPCM as well as TPA cultures. Furthermore, the percentage of DPP IV positive leukaemic T-cells in 3 T-ALL decreased in TPA cultures and remained unchanged in control and HPCM cultures. For this reason an induction of DPP IV positivity in cells of DPP IV negative T-malignancies cultivated with TPA or HPCM is improbable.

Acknowledgements

We would like to express our thanks to Dr. V. Chudomel, CSc. (Institute of Haematology and Blood Transfusion, Prague), Prof. O. Hrodek, DrSc. and Drs. J. Starý and V. Komrska (2nd Pediatric Clinic, Pediatric Faculty Hospital, Charles University, Prague) for the blood samples of their patients.

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Haematologia 20 (3), pp. 171–178 (1987)

Age-Associated Sensitivity to Experimental, Drug-Induced Marrow Hypoplasia in Laboratory Rats

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(Received 13 February, 1986; accepted 13 August, 1986)

Treatment of adult rats of three age groups (8, 11 and 29 weeks) with haematotoxic doses of three different cytostatic agents (cyclophosphamide, 5-(2-dimethylaminoethoxy)-7-oxo-7H-benzo(c)fluorene, and lomustine) demonstrated a tendency to lower the sensitivity of elder animals to cytotoxic effects. The influence of age was especially distinct in changes of the reticulocyte or segmented neutrophil counts after all drugs tested. Changes in marrow nucleated erythroid cell counts were not significantly influenced by age. Reactions of other characteristics also depended on this factor but not following the administration of every drug.

Keywords: age, anticancer drugs, marrow hypoplasia, rats

Introduction

Many preclinical studies of various effects on the haemopoietic system were performed on laboratory rats aged 2–6 months. The intensity of rat myelopoiesis in this age interval seems to be stable [8] and that of erythropoiesis slightly decreased [4, 6]. As age may modify various toxic effects [9, 10] the question arose whether age influenced the sensitivity to stimuli which induce a depression of haemopoiesis: we could find no report on this subject in the literature although most drugs are examined for major toxicity on animal models before marketing [7].

Materials and Methods

Eighty-eight male Wistar rats (WIST/RIPB) of our institute's breeding were used after a two-week acclimatization. Three animals per one 15 l cage were housed under constant temperature (21–23 °C) and artificial light-dark cycle of 12 h/12 h. Pelletted diet VELAZ DOS 2b and water were available ad libitum.

Blood was drawn from the tail of which an about 1 mm long tip was cut off 50–70 min after the lights were switched on. Marrow specimens were taken immediately after blood sampling. Blood cells were counted with a Coulter Model ZF. Marrow femoral cells were counted using haemocytometer and Türk's solution [1]. Blood smears and femoral marrow imprints were stained by Pappenheim's panoptic method, reticulocytes were stained with brillant cresyl blue and counter-

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stained panoptically. Blood and marrow were taken 72 h after drug loading. This interval was chosen as it allowed to record deep myelo- and erythropoietic suppression [1, 2].

Cyclophosphamide (CY; VEB Jenapharm Ankerwerk, Rudolstadt, GDR) was dissolved in 0.9% saline and injected intraperitoneally in a dose of 50 mg/kg b.w., benflurone (BN; RIPB, Prague; 5-(2-dimethyl-aminoethoxy)-7-oxo-7H-benzo(c)fluorene) was administered orally suspended in 0.2% methylcellulose in a dose of 500 mg/kg, as well as lomustine (LN; RIPB, Prague) which was administered orally in a dose of 30 mg/kg. All drugs were administered in a volume of 5 ml per kg b.w.

Mean and standard error of mean were calculated from 6–18 animals. The data for the Kruskal and Wallis test were transformed by means of the equation $100 \times T/C$, where T = data from individual treated rats, and C = mean of the appropriate control group (cf. Table 1). Changes in per cent of each characteristic were expressed in Figs 1, 3, 4 and 6 as $100 \times (T\text{-}C)/C$.

The Mann-Whitney U-test for differences between experimental groups and controls, the Wilcoxon matched-pair signed rank test for differences between data before and after treatment, and the Kruskal and Wallis test for evaluation of the significance of age were used at the significance level of $2\alpha = 0.05$.

Results and Discussion

Changes in reticulocyte count (Fig. 1) as an expression of rat erythropoietic activity were significantly age-related following administration of all the used cytostatic drugs (Kruskal and Wallis test, $p < 0.05$). The fall in control reticulocyte counts (Table 1) between 8 and 11 weeks of age reflects the decrease of erythro-

Table 1

Data on controls

Age*	RET	RBC	MCV	WBC	SN	LY	E	M
8	213.4 ±9.7	5.63 ±0.16	61.5 ±0.6	16.9 ±1.1	2.25 ±0.52	14.49 ±1.05	51.1 ±2.9	86.7 ±7.0
11	84.9 ±10.8	6.37 ±0.13	58.0 ±0.7	12.9 ±0.6	1.99 ±0.21	10.54 ±0.49	41.6 ±2.2	112.2 ±7.5
29	63.4 ±7.8	6.02 ±0.09	55.8 ±0.1	10.9 ±0.9	1.82 ±0.21	8.38 ±0.50	41.5 ±3.3	120.1 ±12.6

RET — reticulocytes (10^9/l); RBC — red blood cells (10^{12}/l); MCV — mean cell volume (fl); WBC — white blood cells (10^9/l); SN — circulating segmented neutrophils (10^9/l); LY — blood lymphocytes (10^9/l); E — marrow nucleated erythroid cells (10^6/femur); M — marrow myeloid cells (10^6/femur)

* in weeks

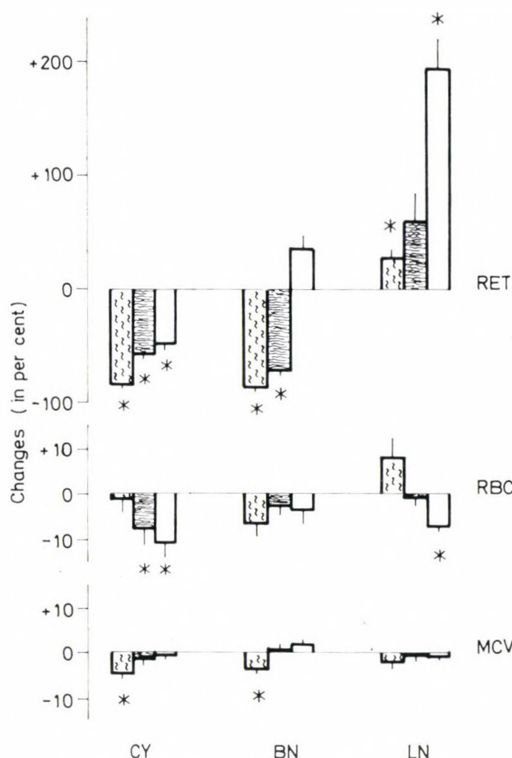

Fig. 1. Age-associated changes in sensitivity of MCV, reticulocyte and red blood cell counts to cytotoxic influences of CY, BN and LN. ▨ 8-wk-old, ▤ 11-wk-old, and □ 29-wk-old rats; * statistically significant as compared with controls (two-sided U test, $p < 0.05$); abbreviations as in Table 1

poietic activity in young adult rats. Only a slight fall in reticulocyte count was observed between the 11th and 29th week of age. Thus, the higher erythropoietic activity in young adult rats might be the cause of the increased sensitivity to the stimuli inducing erythropoietic depression.

Three days after LN in all age groups and after BN in 29-wk-old rats, an increment of the reticulocyte count was observed (Fig. 1). It seemed to be due to the shorter suppression of erythropoiesis as it was found in 29-wk-old rats after LN, which was similar as in rats after CY (Fig. 2).

Age-associated changes in marrow red nucleated cell counts after BN and LN (Fig. 3) were related to those in reticulocyte counts, but the influence of the age in the marrow nucleated erythroid cell counts was not significant. Reticulocytosis in 29-wk-old rats after BN and in all rats after LN was associated with a significant (after BN) and a non-significant (after LN) erythroid marrow hypoplasia. There was no relation between reticulocyte and nucleated erythroid femoral

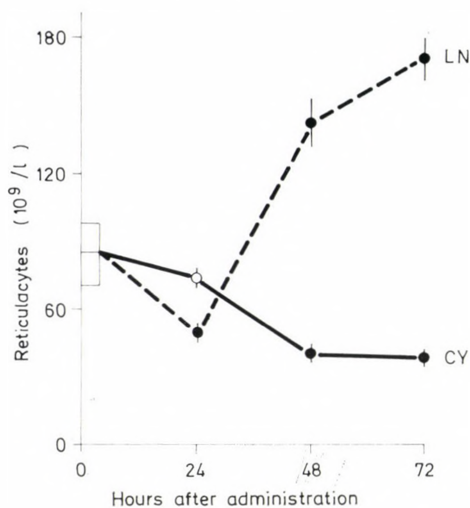

Fig. 2. Reticulocyte counts in 29-wk-old rats within 72 hours of LN (dotted lines) or CY (heavy ones) withdrawal. The column at the vertical axis represents the mean ± s.e.m. from animals examined before administration; black circles = statistically significant as compared with data obtained before drug administration (two-sided Wilcoxon test, $p < 0.05$)

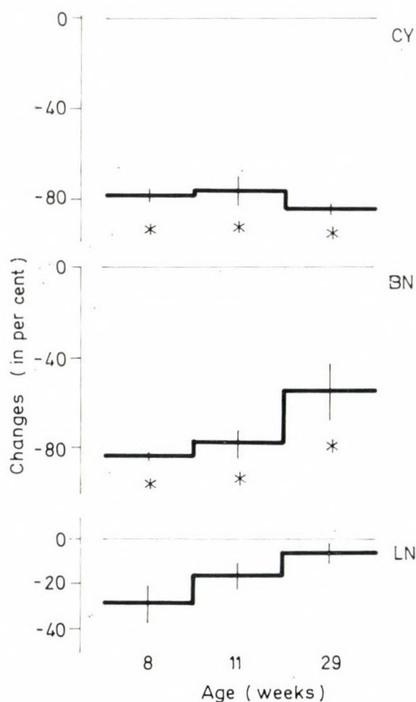

Fig. 3. Age-associated changes in marrow erythroid nucleated cell counts in 8, 11, and 29-wk-old rats 72 hours after drug administration. Symbols as in Fig. 1

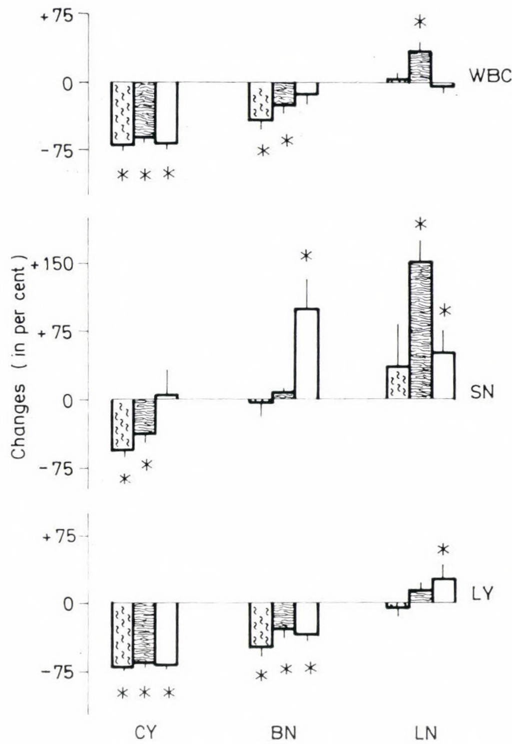

Fig. 4. Influence of CY, BN, and LN on white blood counts as related to age. Symbols as in Fig. 1

cell counts without CY. These findings seem to suggest that counts of marrow nucleated erythroid cells do not always reflect the intensity of rat erythropoiesis.

The age-related decrease of MCV in young rats was significant after CY and BN. The cause of these changes in MVC is obscure. The absence of age-related changes of MCV after LN may ensue from a lack of significant changes as compared with the controls.

Age-related changes in erythrocyte counts (Fig. 1) were significant after CY and LN and non-significant after BN. These changes after CY and LN were the inverse of reticulocyte counts.

Changes in circulating segmented neutrophil counts were significantly age-related (Fig. 4). Less myelotoxic effect seems to be present in the oldest rats after CY and BN, while changes after LN were irregular. Corresponding age-related changes in marrow myeloid cell counts (Fig. 6) were significant after BN and non-significant after LN (Kruskal and Wallis test) because only in 8-wk-old rats was the decrease significant as compared with controls (U test); no age effect was found after CY. As to the blood lymphocyte counts (Fig. 4) only LN induced significant

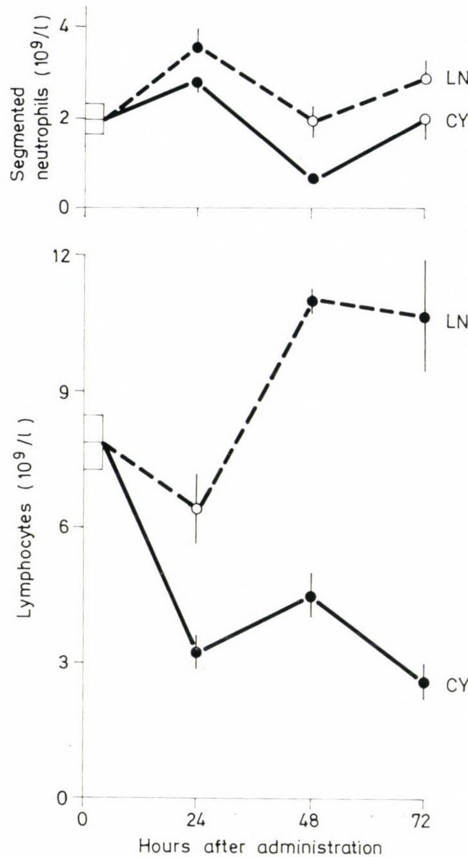

Fig. 5. Circulating segmented neutrophil and blood lymphocyte counts within 72 hours of LN or CY withdrawal. The same group of animals and symbols as in Fig. 2

age-related changes. The source of neutrophilia and lymphocytosis (Fig. 4) is apparent from Fig. 5 as a consequence of the recovery after slight and short term drug cytotoxic effects. Neutropenia did not develop after LN; this observation may be associated with the ability of redistribution of neutrophils among pools [3] as marrow myeloid cell counts slightly decreased (Fig. 6).

Thus, we found that the intensity of erythro suppression after cytotoxic drugs was age-related and weaker in elder rats. This age-associated sensitivity was more evident in the reticulocyte than in the marrow nucleated erythroid cell counts. Similar trends with several irregularities were observed in suppression of the marrow myeloid cell and circulating segmented neutrophil counts; these age-associated changes in neutrophil and myeloid cell counts were, however, less apparent and partially irregular.

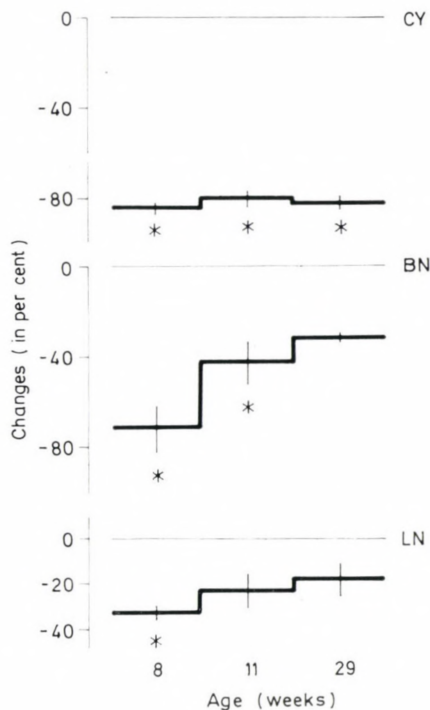

Fig. 6. Age-associated changes in sensitivity of marrow myeloid cell counts to cytotoxic effects of CY, BN, and LN. Symbols as in Fig. 1

Table 2

Role of age

Charac-teristic	Drug		
	CY	BN	LN
RET	+	+	+
RBC	+	–	+
MCV	+	+	–
WBC	–	–	+
SN	+	+	+
LY	–	–	+
E	–	–	–
M	–	+	–

+ statistically significant influence of age (Kruskall and Wallis test, $p < 0.05$)
– statistically not significant influence of age

We evaluated an influence of age by the Kruskal and Wallis test; more detailed information was obtained with the Mann-Whitney U test where the Kruskal and Wallis test revealed statistical significance. We found statistical (U test) differences between 8 and 11-wk-old rats only for reticulocyte counts after CY, BN and LN, for MCV after CY, for segmented neutrophil counts after LN, and absolute marrow myeloid cell counts after BN. It seems that

(i) the significance of age is evident in reticulocyte counts which reflect the fact that the intensity of rat erythropoiesis decreases after the age of 8 weeks, and
(ii) the influence of age became more evident when 29-wk-old rats were included into the comparison.

Thus, age may play a more significant role in haematological reactions during long-term use of cytotoxic xenobiotics rather than short-term ones, but the drugs affecting the intensity of erythropoiesis may induce erythrosuppression which greatly depends on the age of adult rats.

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Haematologia 20 (3), pp. 179–187 (1987)

A New Family with Congenital Factor XIII Deficiency Showing a Deficit of Both Subunit A and B. Type I Factor XIII Deficiency

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(Received 2 July, 1986; accepted 20 October, 1986)

In this study we present a new case of Factor XIII deficiency. The proposita, a 34 year old woman, showed a deficiency of both subunit a and subunit b, and a moderate bleeding tendency. Because of the concomitant decrease of subunits a and b the proposita is considered to be an example of Type I disease. Factor XIII levels were less than 10% both as activity and antigen. Several family members showed intermediate levels of both subunit a and b and were asymptomatic. They were considered to be heterozygotes. The hereditary pattern is autosomal incompletely recessive. Type I disease appears much less frequent than Type II.

Keywords: Factor XIII, subunits a and b, Type I deficiency

Introduction

Factor XIII, the fibrin stabilizing factor, is a β-globulin present in the plasma, platelets, monocytes, macrophages, fibroblasts, placenta [1, 35].

It has been demonstrated that Factor XIII is diminished in conditions in which hepatic disfunction is present. It has been hypothesized that it is synthesized in the hepatic parenchyma. Nevertheless, Fear et al., using an immunoperoxidase technique, have not been able to demonstrate Factor XIII in hepatocytes [20]. This suggests that Factor XIII might be produced in megacaryocytes and fibroblasts. The action of this factor consists in rendering the blood clot more rigid and resistent to lysis. The zymogen present in plasma has a molecular weight of 320,000 and is composed of two pairs of subunits a and b (a_2 and b_2) [10, 11, 30, 31, 37, 38]. Subunit a, which is the active portion with a molecular weight of 75,000, appears to be synthesized under the control of an autosomal locus composed of 2 alleles. Subunit b, which does not participate in the stabilization of the clot but probably prolongs the survival of the a_2 dimer in the circulation, has a molecular weight of 88,000 and appears to be synthesized under the control of an autosomal locus with 3 alleles [6, 7, 8]. The transformation of the zymogen in the active form occurs in two stages: first, by the action of thrombin which catalyzes a limited proteolysis and successively by way of Ca^{++}. The Ca^{++} work in two ways, 1) causing a dissociation of two subunits, and 2) by an unmasking of the active cysteine centers of the subunits. This brings about the formation of two different dimers, a_2 and b_2.

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The zymogen competes efficiently with the Ca^{++} in the unmasking of cysteine, but does not inhibit the dissociation of the a_2 and b_2 protein by the Ca^{++} (Calcium) [13].

Since 1960, the year in which Duckert [14] described the first case of Factor XIII deficiency, more than 100 cases have been recognized [5, 15, 16, 18, 38]. These data support the hypothesis that Factor XIII deficiency is a rare coagulation disorder. Two types of disease have been described. In Type I, both subunits a and b are markedly reduced or absent [27, 29]. In Type II disease, only subunit a is decreased or absent, whereas subunit b is normal or only slightly decreased [2, 3, 4, 17, 19, 21]. Little is known about the relationship existing between the two conditions [23]. It is only known that the Type II appears to be much more frequent [26]. The scope of the present research is to report data concerning a new family with Type I Factor XIII deficiency.

Case Report

The proposita is a 34 year old woman whose 1st hemorrhagic episode followed a tonsillectomy at the age of 8. Since that event, she has suffered from repeated hemorrhagic episodes after dental extractions, and a tendency for easy bruising. Her menstruations have always been prolonged and abundant. Patient has carried 2 pregnancies to completion without any particular problems. In both cases, however, she had shown profuse post-partum hemorrhages.

The family history appears negative in that neither her children, nor her brothers or sisters and their children have ever complained of hemorrhagic episodes (Fig. 1).

All routine coagulation tests have repeatedly yielded normal findings.

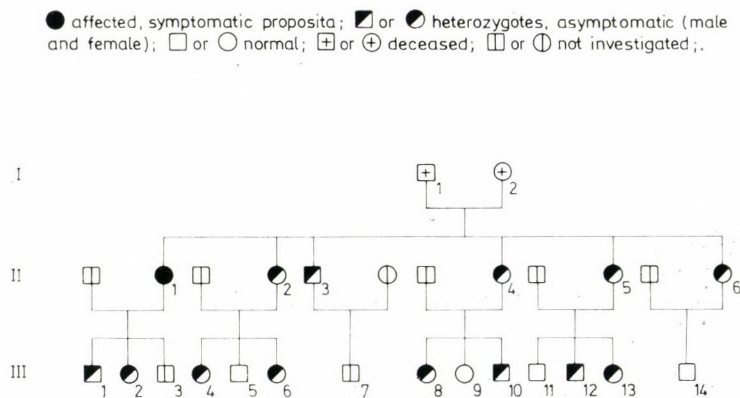

Fig. 1. Family pedigree. The children and several siblings of the proposita demonstrate slightly reduced levels of subunit a and b with respect to normal and were considered to be heterozygotes for the condition

Unfortunately, no reference can be made to the parents, both deceased prior to the beginning of our study. However, they were not consanguineous.

The patient was sent to us in January 1985 for a complete evaluation of the clotting system because of a life-long bleeding tendency.

At the time of study she had no bleeding manifestations.

Materials and Methods

In this work only the most pertinent data are presented, while those already described in former studies are omitted [24, 25].

The urea solubility test was performed by suspending a recalcified clot of whole plasma in a solution of 5M urea or 1% monochloro-acetic acid.

The clots were examined at intervals of a few minutes for the first hour, and then every 2 or 3 hours for at least 24 hours.

Factor XIII was evaluated employing the method of neutralization proposed by Bohn and Haupt [9], using the reagents furnished by the Behringwerke, Marburg, Germany. The antiserum was an anti-subunit a antiserum.

Furthermore, Factor XIII was evaluated by the method proposed by Josso et al. [32] using the reagents furnished by Boehringer, Mannheim, Germany. With this method one determines the greatest dilution of the plasma in which a fibrin clot, obtained by the addition of fibrinogen without Factor XIII and thrombin ($+Ca^{++}$), remains insoluble after adding monochloro-acetic acid.

The antigen evaluation was determined according to a modification of the method proposed by Laurell [33] using 1.2% agarose dissolved in a buffer of Na-Barbital (pH 8.2; μ 0.03) and 0.5% anti-a antiserum or 1% anti-b antiserum. Three 2.5 mm wells were punched out one above the other and filled with 16 μl of plasma. A potential difference of approximately 3.5 V/cm for 12 hours was applied to the system.

Bidimensional immunoelectrophoresis was performed following the method proposed by Clarke-Freeman [12] using 1.2% agarose and Na-Barbital (pH 8.2; μ 0.03). The wells were 4 mm in diameter and were filled with 20 μl of plasma. The first migration was obtained applying a potential difference of 6 V/cm for approximately 2½ hours to the system. The second migration was carried out using agarose containing 0.5% anti-a antiserum or agarose containing 0.9% anti-b antiserum and applying a potential difference of about 3 V/cm for 12 hours.

Results

Routine plasma and platelet tests were all within normal limits in the proposita with the exception of the thrombelastogram (TEG) which showed a reduced maximal amplitude and an increased post-maximal deflection.

The recalcified plasmatic clot showed complete dissolution in 5–10 min. when a solution of 5 M urea or 1% monochloro-acetic acid were added.

Table 1

Average Factor XIII levels observed in the proposita, the heterozygotes and controls

	n° of subjects	Average Factor XIII levels in % of normal	p (from normal)
Proposita	1		
activity in %		<10	
antigen, subunit a in %		<10	
antigen, subunit b in %		<10	
Heterozygotes	13		
activity in %		51.0 ± 8.7	0.001
antigen, subunit a in %		45.6 ± 8.8	0.0001
antigen, subunit b in %		44.5 ± 8.2	0.0001
Normal	10		
activity in %		81.7 ± 14.4	
antigen, subunit a in %		83.2 ± 17.1	
antigen, subunit b in %		91.1 ± 24.0	

Table 2

Results of immunoassay (Laurell's method) for subunit a and subunit b in the members of the family studied

	Subunit a	Subunit b	Comment
II_1, Proposita	$<10\%$	$<10\%$	homozygote
II_2	32%	28%	heterozygote
II_3	40%	40%	heterozygote
II_4	58%	55%	heterozygote
II_5	29%	33%	heterozygote
II_6	46%	49%	heterozygote
III_1	45%	45%	heterozygote
III_2	50%	50%	heterozygote
III_4	57%	57%	heterozygote
III_5	72%	75%	normal
III_6	56%	45%	heterozygote
III_8	45%	48%	heterozygote
III_9	83%	75%	normal
III_{10}	45%	43%	heterozygote
III_{11}	69%	70%	normal
III_{12}	49%	47%	heterozygote
III_{13}	41%	38%	heterozygote
III_{14}	76%	78%	normal
Normal values	70–120%	70–120%	

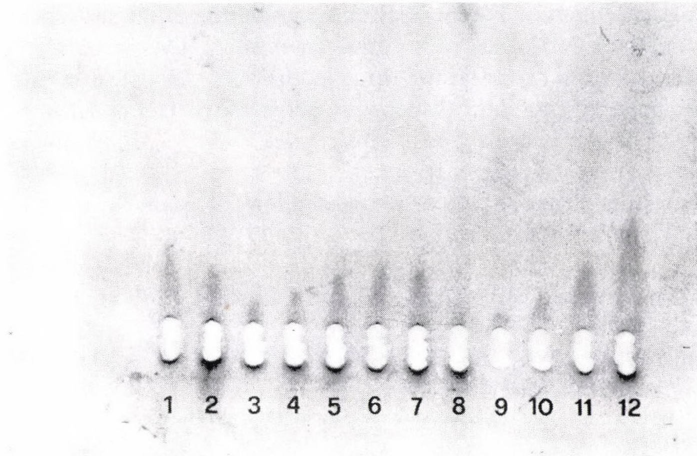

Fig. 2. Immunoassay of subunit "a" according to Laurell's method. See Fig. 1 and Table 2 for symbols. Well 1: patient II_4; well 2: patient II_6; well 3: patient II_5; well 4: patient II_2; well 5: patient II_3; well 6: patient III_2; well 7: patient III_1; well 8: proposita, II_1; wells 9 to 12: 1 : 8; 1 : 4; 1 : 2; 1 : 1 diluted pooled normal plasma. The proposita has subunit "a" levels lower than 10%. Some family members have reduced levels of this subunit and were considered as heterozygotes

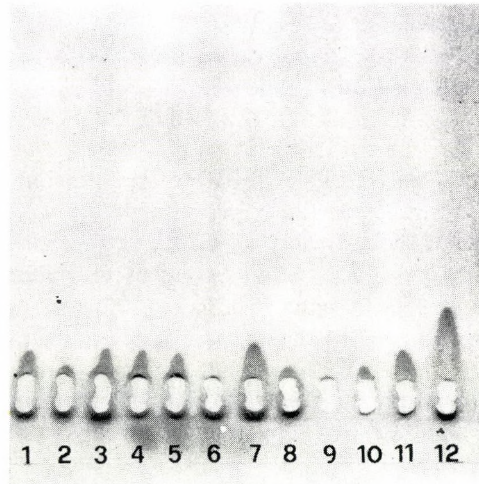

Fig. 3. Immunoassay of subunit "b" according to Laurell's method. See Fig. 1 and Table 2 for symbols. Well 1: patient II_3; well 2: patient II_2; well 3: patient II_6; well 4: patient III_2; well 5: patient III_1; well 6: proposita, II_1; well 7: patient II_4; well 8: patient II_5; wells 9 to 12: 1 : 8; 1 : 4; 1 : 2; 1 : 1 diluted pooled normal plasma. The proposita has subunit "b" levels lower than 10%. Some family members have reduced levels also for this subunit and were considered as heterozygotes

The measurement of Factor XIII using a neutralization test repeatedly showed levels of Factor XIII that were lower than 10%. This was also confirmed by Josso's method. Furthermore, using these methods, it was possible to discriminate between the heterozygous and the normal subjects. In fact, Factor XIII activity was 51.0 $\pm$ 8.7 in the heterozygous, while it was 81.7 $\pm$ 14.4 in the normal subjects (Table 1).

These results were confirmed by immunological methods, using anti-subunit a and antisubunit b antisera. In fact, the Laurell's immunoassay showed levels of $<$ 10% of subunits a and b in the proposita and also reduced levels of subunits a and b in her brother, her sisters and some of their children (Table 2, Figs 2 and 3). Their subunit a levels varied between 29% and 58%, while the subunit b levels varied between 28% and 57%. Others ($III_5 - III_9 - III_{11} - III_{14}$) had normal levels of both subunit a and b (Table 2).

Discussion

The main criteria for the diagnosis of Factor XIII deficiency are:

- normal routine clotting tests
- normal bleeding time
- normal platelet count and function
- altered thromboelastogram with reduced maximal amplitude and increased post-maximal deflection
- increased clot solubility in urea solution.

Our patient satisfies all these criteria. Both assays of Factor XIII activity and antigen have confirmed our suspicion.

The immunoassays have demonstrated that the patient is lacking both subunit a and subunit b.

On the basis of these results and of the classification of Factor XIII deficiency in 2 types of diseases, previously proposed by us, we can state that the patient represents Type I disease. In this condition, in fact both subunits are absent. In Type II disease, as previously demonstrated by us, subunit a is lacking while subunit b is normal or slightly reduced [26, 27, 29].

In terms of the incidence of the disease, Type I appears to be less frequently observed than Type II. In fact at the present moment, only 4 cases have presented a deficiency of both subunit a and b, while about 50 additional cases have demonstrated to have only an absence of subunit a and not of subunit b, which is normal or slightly reduced (Table 3).

The reasons for this marked discrepancy in incidence of the two forms is unknown. However it has to be remembered that most of the published immunological studies on Factor XIII defect were carried out using antisera supplied by Behringwerke (Marburg, Germany). At least one lot of this antiserum (anti-subunit b) was demonstrated by us to contain an anti-Factor VIII contamination [26, 27].

Table 3

Distribution of cases of Factor XIII deficiency studied by means of immunologic techniques reported in the literature

Type I disease	Homozygotes	Subunit a	Subunit b
Kiesselbach et al. 1972	1	absent	absent
Girolami et al. 1976–1977	2	absent	absent
Present report	1	absent	absent
Type II disease			
Duckert et al. 1970–1972	15	absent	sl.red. or near normal
Egbring et al. 1970–1975	3	absent	sl.red. or near normal
Soria et al. 1970	2	absent	sl.red. or near normal
Aguercief et al. 1971	1	absent	sl.red. or near normal
Bohn et al. 1973	2	absent	sl.red. or near normal
Israels et al. 1973	1	absent	sl.red. or near normal
Barbui et al. 1978	7	absent	sl.red. or near normal
Francis et al. 1979	1	absent	sl.red. or near normal
Board et al. 1980	1	absent	sl.red. or near normal
Endo et al. 1983	2	absent	sl.red. or near normal
Fear et al. 1983	6	absent	sl.red. or near normal
Berliner et al. 1984	10	absent	sl.red. or near normal

As a consequence, some of the investigated cases could have erroneously been considered to be Type II disease (absent subunit a and normal subunit b). As far as hereditary transmission is concerned, it was demonstrated that there are no differences between different races and that both males and females can be affected, even if there appears to be a slight predominance in the male.

In our family only a female is affected.

Concerning the mode of hereditary transmission Ratnoff and Steimberg [36] have suggested that it may be a recessive sex-linked trait on the basis of the studies of some families in which only the males were affected. Nevertheless, the fact that no maternal uncle ever demonstrated disease is against this hypothesis. Consanguinity is present in many families, and according to Lorand et al. [34] the incidence is 37%. The parents of our patient were not consanguineous.

In other families in which normals could be separated from heterozygotes, the mode of transmission seems to be of the autosomal incompletely recessive type [34].

The results we have obtained by carrying out an electroimmunoassay in the children and the siblings of the patient showed in the heterozygotes, levels between 28% and 57% for subunit b.

This lead us to favor an autosomal incompletely recessive type of transmission. In other families described in the literature [3, 4, 22] the transmission seems to be of autosomal recessive type. It is probable that both models of hereditary

transmission are present in congenital Factor XIII deficiency. The same has been shown to be the case for the congenital afibrinogenemia [28]. This could be interpreted as a different phenotypic expression of an abnormal gene.

The description of this family with type I Factor XIII deficiency confirms the existence of cases with a decrease of both subunit a and subunit b and confirms the validity of the classification proposed by us in 1978 [27].

It emphasizes also the need for a careful immunological investigation of all cases of Factor XIII deficiency using both anti subunit a and anti subunit b specific antisera.

Acknowledgement

This study was supported in part by grants from the MPI, Rome (Grant 1592—1985), by CNR, Rome (Grant 85.00612.56) and from the Veneto Region, Venice.

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Haematologia 20 (3), pp. 189–193 (1987)

Natural Killer Activity in Thyroid Cancer Patients

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(Received 20 June, 1986; accepted 26 August, 1986)

The natural killer activity of thyroid cancer patients has been studied. The cytotoxic capacity was found to be elevated in tumourless patients and in cases of highly differentiated histologic type compared to the results of patients with metastasis or anaplastic tumour. The values of patients in the progressive phase of the disease were significantly worse compared to the healthy controls. No connection was found between the natural killer activity and the time elapsed since the operation.

Keywords: thyroid cancer, natural killer activity, clinical state

Introduction

The immunological aspects of thyroid cancer have only begun to be studied in the last decade and data are still scarce in the literature. Most of the reports deal with the immune response induced by the antigen associated with the tumour and with the recognition of the tumour antigen in the first place [1, 12, 13]. The latest results have proved the existence of this immune response [2], but there are no data whatever about the anti-tumour effector mechanisms in thyroid cancer. As the highly differentiated thyroid tumours can be ranged among the benign ones [3, 5, 15], it would be interesting to investigate these mechanisms and to compare them to the development of the disease.

The purpose of the present paper was to estimate the activity of natural killer (NK) cytotoxicity or capacity of large granular lymphocytes out of these effector mechanisms and to find a connection between the cytotoxic ability and the clinical phase of the disease.

Materials and Methods

Patients

The data of 25 patients were studied 3.8 (0.4 – 10.2) years after the operation. Histologically highly differentiated cancer was diagnosed in 20 cases, and anaplastic cancer in 5 cases. In 18 patients the tumour could not be demonstrated with routine

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VNU Science Press, Utrecht
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clinical examinations, while 7 patients were in a progressive phase of the disease as a result of a relapse or distant metastases. These patients had received local irradiation and/or iodine-isotope therapy which was discontinued at least 6 months before the investigation; at the time of the study the patients received only the usual hormone treatment/substitution. The data of 15 healthy donors served as controls.

Separation of effector cells

The mononuclear cells were separated on F/U* gradient from heparinized peripheral blood. Depletion of monocytes was carried out in plastic Petri dishes of 10 cm with the help of adherence at 37 °C for one hour. Non-adherent cells were removed with repeated washing and their concentration was set for 1×10^6 cell/ml in RPMI-1640 with 10% FCS content.

Preparation of target cells

1×10^6 K 562 cells in 1 ml RPMI-1640 containing 10% FCS were incubated at 37 °C for 1 hour with 100 μCi ^{51}Cr isotope. The cells were left to stand after being washed for 1 hour.

Determination of NK activity

In 50 : 1 and 25 : 1 effector : target cell ratio, the samples were incubated on Linbro microtest plate for 4 hours. After incubation the plates were centrifuged at 1500/min for 5 minutes, and the activity of the supernatants was measured with scintillation (gamma) counter [10]. The specific lysis is given in percentage and the calculation was carried out on the basis of the formula:

Specific lysis per cent =

$$= \frac{\text{activity in the given supernatant} - \text{spontaneous lysis}}{\text{the total activity observed in one sample.}} \times 100$$

In the experiments the spontaneous lysis was 8 – 12%.

Results

Table 1 shows the relation of the histological classes and the NK activity. There was no difference between the values measured in highly differentiated form, while the values of those suffering from anaplastic cancer were significantly lower

* F/U = Ficoll-Uromiro

Table 1

Natural killer activity of thyroid cancer patients

Patients	Specific lysis by 50 : 1 effector : target ratio	Specific lysis by 25 : 1 effector : target ratio
Highly differentiated types		
papillary (n = 10)	19.3 ± 2.4	16.5 ± 3.9
follicular (n = 7)	16.1 ± 2.8	14.1 ± 1.95
medullary (n = 3)	17.2 ± 3.8	16.6 ± 2.8
Mean value of highly differentiated types	16.9 ± 2.9	15.2 ± 2.6
Anaplastic (n = 5)	11.4 ± 1.1*	10.2 ± 0.8*
Healthy donor (n = 15)	20.6 ± 5.9	19.8 ± 5.3

* Significant difference between the two patient groups
$P < 0.05$

by both effector: target rates. In Table 2 the results of the patients with metastasis or recidive are compared independently of the histological type. The cytotoxic ability of the tumour-free patients was significantly higher than that of patients in the progressive phase. In Table 3 NK cytotoxicity and the time elapsed after operation is shown; no significant correlation was demonstrated.

Table 2

Correlation between the NK activity and present clinical stage

Patients	Specific lysis by 50 : 1 effector : target ratio	Specific lysis by 25 : 1 effector : target ratio
Tumourless patients (n = 18)	23.33 ± 8.3	17.4 ± 2.9
Metastasis or relapse (n = 7)	7.5 ± 1.96**	7.4 ± 0.93*

*, ** Significant difference between patient groups
*$P < 0.05$
** $P < 0.001$

Table 3

NK activity of patients' lymphocytes and time elapsed since the operation

Time elapsed since the operation		Specific lysis by 50 : 1	25 : 1
		effector : target ratio	
<1 year	(n = 5)	17.9 ± 2.9	16.88 ± 1.6
2–8 years	(n = 14)	15.6 ± 2.4	14.05 ± 2.65
>8 years	(n = 6)	14.8 ± 0.95	14.30 ± 0.78

Discussion

In recent years great interest has been shown towards the NK cells. It has been realized that the idea of the central role of T cells played in the defence against tumours cannot be maintained in every respect, and other mechanisms, for instance the NK activity, have come into the limelight [11]. This cell group which, according to numerous experimental data is heterogeneous in its phenotype, plays a significant role not only in the inhibition of the development of the primary tumour but also in the development of metastases and in influencing secondary tumour development [9].

On basis of the relative benignity and slow primary and metastatic tumour growth we may assume an effective operation of the immune apparatus of the donor organism as a clinical feature of the highly differentiated thyroid cancers. This may be concluded from the response measured with a certain immunological test in vitro, given to the antigen associated with the tumour. The migration inhibition and in vitro blastic transformation (applied by many authors) proved the existence of the immune response given by the cells in a considerable part of the patients [1, 2, 11, 13]. Especially important were of the present results those which proved the long-lasting antigen sensitization in some children where the cellular immune response could be demonstrated 10 years after the operation [4]. The expansion of the tumour also seemed to be important. In the case of tumour-free patients – and independently from the histological type – a significantly stronger NK activity in the peripheral blood and a significant weakening of the anti-tumour effector activity was observed. It cannot, of course, be concluded from these data that the weakened NK activity is the cause or the result of the development and expansion of the disease. It is known from animal experiments that the genetic or acquired disorders of the NK cells may lead to progression of the tumour in the same way [6, 7, 8, 14, 15], therefore both possibilities may account for the symptom.

The histological class also shows a connection with the NK activity measurable in vitro as in the patients suffering from the highly differentiated forms, a significantly higher value was observed. The anaplastic tumours amounted to 10%

of all the thyroid cancers, had a poor prognosis and their clinical development differed greatly from that of the previous group. According to the present results the tumour killer activity was weakened in these patients. No connection could be found between the time elapsed since the operation and the NK activity, and we were not able to prove changes that had taken place with the time passing after the operation in this relation [4].

The present results seem to be in accordance with the experience concerning the clinical development of thyroid cancers. The appearance of metastases/relapses seems to indicate a turning-point in the development of the disease, as proved by the immunological examinations as well as the NK activity measured in vitro.

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Abstracts

Modulation of erythrocyte membrane mechanical stability by 2,3-diphosphoglycerate in the neonatal poikilocytosis/elliptocytosis syndrome. W. C. Mentzer, Jr., T. A. Iarocci, N. Mohandas, P. A. Lane, B. Smith, J. Lazerson, and T. Hays (Department of Pediatrics and Laboratory Medicine, University of California, San Francisco, CA, USA). *J. Clin. Invest. 79*, 943 (1987).

To explain the transient anemia and poikilocytosis seen during infancy in hereditary elliptocytosis (HE), erythrocyte (RBC) ghosts were released from affected children or their elliptocytic parents with 2,3-diphosphoglycerate (DPG) (0–8 mM), a compound that dissociates membrane skeletons, then ghost mechanical stability was measured in the ektacytometer. Without added 2,3-DPG, ghost mechanical stability was subnormal in infantile poikilocytosis (IP) and HE but was even more abnormal in hereditary pyropoikilocytosis (HPP). Addition of 2,3-DPG (2.55 mM) to IP or HE ghosts, decreased their stability to that of HPP ghosts (without 2,3-DPG). Nonphysiological 2,3-DPG levels (6–8 mM) were required to elicit a similar effect in normal ghosts. The data suggest that free 2,3-DPG, present in neonatal RBC as a consequence of diminished binding to HbF, may render HE susceptible to in vivo fragmentation. The developmental switch from fetal to adult hemoglobin, by diminishing available free 2,3-DPG, may explain the abatement of poikilocytosis and hemolytic anemia that accompanies maturation.

G. Gárdos

The calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase in hemoglobin S erythrocyte membranes: effects of sickling and oxidative agents. L. Leclerc, F. Girard, F. Galacteros and C. Poyart (INSERM U 299, Suresnes, France). *Biochim. Biophys. Acta 897*, 33 (1987).

A decrease in the reactivity of erythrocyte membrane ($Ca^{2+} + Mg^{2+}$)-ATPase to calmodulin stimulation has been observed in aging red cells and in various types of hemolytic anemias, particularly in sickle red cell membranes. Unlike the aging process, the defect in the ($Ca^{2+} + Mg^{2+}$)-ATPase from SS red blood cells is not secondary to a decrease in calmodulin activity and is already present in the least dense SS red blood cells separated on a discontinuous density gradient. Deoxygenated AS red cells were forced to sickle by lowering the pH, raising the osmolarity of the buffer (sickling pulse). Under these conditions an inhibition of the calmodulin-stimulated enzyme was observed only if several cycles of oxygenation/deoxygenation were applied. No alteration of the enzyme could be detected after submitting AS red blood cells to other conditions or in AA red blood cells submitted to the same treatments. This suggests that oxidative processes are involved in the alterations of the ($Ca^{2+} + Mg^{2+}$)-ATPase activity. Treatment of membranes from AA erythrocytes by thiol group reagents and malondialdehyde, a by-product of auto-oxidation of membrane unsaturated lipids and a cross-linking agent of cytoskeletal proteins, led to a partial inhibition of the calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase. It is postulated that the hyperproduction of free

radicals described in the SS red blood cells and involved in the destabilization of the membrane may be also responsible for the $(Ca^{2+} + Mg^{2+})$-ATPase failure.

G. Gárdos

2,3-Diphosphoglycerate phosphatase activity of phosphoglycerate mutase: stimulation by vanadate and phosphate. P. J. Stankiewicz, M. J. Gresser, A. S. Tracey and L. F. Hass (Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, Canada). *Biochemistry 26*, 1264 (1987).

The binding of inorganic vanadate (V_i) to rabbit muscle phosphoglycerate mutase (PGM), studied by using ^{51}V nuclear magnetic resonance spectroscopy, shows a sigmoidal dependence on vanadate concentration with a stoichiometry of four vanadium atoms per PGM molecule at saturating $[V_i]$. The data are consistent with binding of one divanadate ion to each of the two subunits of PGM in a noncooperative manner with an intrinsic dissociation constant of 4×10^{-6} M. The relevance of this result to other studies which have shown that the V_i-stimulated 2,3-diphosphoglycerate (2,3-DPG) phosphatase activity of PGM has a sigmoidal dependence on $[V_i]$ with a Hill coefficient of 2.0 is discussed. At pH 7.0, inorganic phosphate has little effect on the 2,3-DPG phosphatase activity of PGM, even at concentrations as high as 50 mM. Similarly, 25 μM V_i has little effect on the phosphatase activity. However, in the presence of 25 μM V_i, a phosphate concentration of 20 mM increases the phosphatase activity by more than 3-fold. This behavior is rationalized in terms of activation of the phosphatase activity by a phosphate/vanadate mixed anhydride. This interpretation is supported by the observation of strong activation of the phosphatase activity by inorganic pyrophosphate. A molecular mechanism for the observed effects of vanadate is proposed, and the relevance of this study to the possible use of vanadate as a therapeutic agent for the treatment of sickle cell anemia is discussed.

G. Gárdos

Phospholipid outside-inside translocation in lymphocyte plasma membranes is a protein-mediated phenomenon. A. Zachowski, A. Herrmann, A. Paraf and Ph. F. Devaux (Institut de Biologie Physico-Chimique, Paris, France). *Biochim. Biophys. Acta 897*, 197 (1987).

The transbilayer diffusion of spin-labeled analogs of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in pig lymphocyte plasma membrane was measured. At 4 °C and 37 °C the aminophospholipids are rapidly transported from the outer to the inner leaflet of the membrane, whereas the choline-containing phospholipids experience a slower diffusion. This selectivity is abolished after cell treatment by SH-group reagents indicating that the aminophospholipid translocation is protein-dependent and must be driven by a system analogous to the one existing in the human red cell membrane. The fact that the selectivity exists at low temperature, that it does not depend on cytoskeleton integrity and that there is a competition between the two aminophospholipids show that this translocation is not purely an endocytic process.

Ilma Szász

Sodium ion influx in proliferating lymphocytes: an early component of the mitogenic signal. K. V. S. Prasad, A. Severini, and J. G. Kaplan (Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada). *Arch. Biochem. Biophys. 252 (No. 2)*, 515 (1987).

Stimulation of pig peripheral blood lymphocytes with concanavalin A (Con A) provoked a rapid increase (two- to threefold) in the rate of ouabain-inhibitable K^+ uptake observable within 3–10 min of stimulation with mitogen. At least two phases can be distinguished in the activation of the Na^+/K^+ pump: the early phase (till 3 h) is characterized by an unaltered number of ouabain binding sites and the later phase (noted at 5 h) by an increased number of such sites. Both K^+ efflux and influx increased to the same extent, thereby maintaining $[K^+]_i$ at the same level as in resting

cells (120 mM). Within 3 min of addition of mitogen, the rates of total and amiloride-inhibitable Na^+ uptake went up two- and fourfold, respectively, thus resulting in rapid increase in $[Na^+]_i$ from 20 to about 50 mM. Activation of the Na^+/K^+ pump was not observed when the cells were stimulated with Con A in low Na^+ medium (9 mM), nor did the usual rise in $[Na^+]_i$ occur. When monensin (30 μM), a Na^+/H^+ ionophore, was added to resting cells, an increase in both $[Na^+]_i$ and active K^+ uptake occurred in normal medium but not when cells were suspended in low Na^+ isotonic buffer. Amiloride (500 μM), on the other hand, prevented both the Con A-induced increase in $[Na^+]_i$ and the activation of the Na^+/K^+ pump. Despite complete inhibition of the Na^+, K^+-ATPase in the presence of ouabain (1 mM), Con A activated the amiloride-inhibitable Na^+ uptake in the usual way. In mouse splenocytes stimulated with ConA , there was also a parallel rise in both $[Na^+]_i$ and active K^+ uptake but this took considerably longer to occur than was the case in pig peripheral blood lymphocytes. Increase in both ionic fluxes, the former passive and the latter active is essential to the entry and maintenance of the cells in proliferative cycle.

Ilma Szász

Cytoplasmic [Ca^{2+}] and intracellular pH in lymphocytes. Role of membrane potential and volume-activated Na^+/H^+ exchange. S. Grinstein and S. Cohen (Department of Cell Biology, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada). *J. Gen. Physiol. 89*, 185 (1987).

The effect of elevating cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) on the intracellular pH (pH_i) of thymic lymphocytes was investigated. In Na^+-containing media, treatment of the cells with ionomycin, a divalent cation ionophore, induced a moderate cytoplasmic alkalinization. In the presence of amiloride or in Na^+-free media, an acidification was observed. This acidification is at least partly due to H^+ (equivalent) uptake in response to membrane hyperpolarization since: *(a)* it was enhanced by pretreatment with conductive protonophores, *(b)* it could be mimicked by valinomycin, and *(c)* it was decreased by depolarization with K^+ or gramicidin. In addition, activation of metabolic H^+ production also contributes to the acidification. The alkalization is due to Na^+/H^+ exchange inasmuch as it is Na^+ dependent, amiloride sensitive, and accompanied by H^+ efflux and net Na^+ gain. A shift in the pH_i dependence underlies the activation of the antiport. The effect of $[Ca^{2+}]_i$ on Na^+/H^+ exchange was not associated with redistribution of protein kinase C and was also observed in cells previously depleted of this enzyme. Treatment with ionomycin induced significant cell shrinking. Prevention of shrinking largely eliminated the activation of the antiport. Moreover, a comparable shrinking produced by hypertonic media also activated the antiport. It is concluded that stimulation of Na^+/H^+ exchange by elevation of $[Ca^{2+}]_i$ is due, at least in part, to cell shrinking and does not require stimulation of protein kinase C.

Ilma Szász

Substrate-induced conformational change of human erythrocyte glucose transporter: inactivation by alkylating reagents. A. L. Rampal and Ch. Y. Jung (Biophysics Laboratory, State University of New York at Buffalo, Buffalo, NY, USA). *Biochim. Biophys. Acta 896*, 287 (1987).

The glucose transport carrier in human erythrocyte membranes, when transporting glucose, undergoes a conformation change. In an attempt to delineate the extent of this substrate-induced conformational change, transport inactivation by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, *N*-ethylmaleimide, iodoacetamide, and 2,4,6-trinitrobenzenesulfonic acid was examined in the presence and in the absence of D-glucose. All these alkylating agents inactivated the carrier. With each of these reagents, with the exception of trinitrobenzenesulfonic acid, D-glucose modified the rate of inactivation as well as the activation enthalpy (ΔH^*) of the inactivation. The inactivation by trinitrobenzenesulfonic acid was not affected by the sugar. Based on these findings, it is suggested that the substra-

teinduced conformational change mostly occurs within the transmembrane hydrophobic domain while the hydrophilic extramembrane domains are largely outside of this change.

Mária Magócsi

Insulin causes insulin-receptor internalization in human erythrocyte ghosts. R. S. Kelleher, E. F. Murray and S. W. Peterson (Boston University, Biological Sciences Center, Boston, MA, USA). *Biochem. J. 241*, 93 (1987).

The effect of incubation with insulin on insulin-receptor internalization by erythrocyte ghosts was investigated. The number of surface insulin receptors decreased by 30–40% after incubation of ghosts with insulin. Total insulin-receptor binding to solubilized ghosts was the same in insulin-incubated and control ghosts, whereas insulin binding to an internal vesicular fraction was substantially increased in insulin-incubated ghosts. The findings suggest that erythrocyte-ghost insulin receptors are internalized to a vesicular compartment in response to incubation with insulin.

Mária Magócsi

Neutrophil-induced K^+ leak in human red cells: A potential mechanism for infection-mediated hemolysis. S. Claster, A. Quintanilha, M. A. Schott, D. Chiu, and B. Lubin (Children's Hospital Medical Center, Bruce Lyon Memorial Research Laboratory, Oakland, CA, USA). *J. Lab. Clin. Med. 109*, 201 (1987).

Activated neutrophils (AN) when incubated with red blood cells (RBCs) at a ratio 1 : 100 were shown to damage RBCs as reflected by an increase in passive potassium (K^+) permeability. Oxygenated sickle cells were more susceptible to this injury than normal (AA) RBCs. In both normal and sickle cells, the degree of K^+ leak was found to be linearly related to the amount of AN in the incubation mixture. Pretreatment of AA RBCs with low-dose H_2O_2 resulted in an increased K^+ leak after exposure to AN. Compared with patients with stable sickle cell anemia, those who were observed while infected or in crisis had notably more K^+ leak from their RBCs after AN exposure. Addition of activated neutrophils from a patient with chronic granulomatous disease resulted in K^+ leak from normal RBCs, despite a deficiency in production of toxic oxygen species. Cell-free supernatants from AN also mediated K^+ leak. Sickle cells were, again, leakier after exposure to these preparations. Scavengers of toxic oxygen species inhibited up to 40% of the leak, whereas the maximal inhibition obtained by using protease inhibitors was 60%. Addition of autologous plasma in low concentrations inhibited the leak but also resulted in hemolysis, probably via a different mechanism. These studies demonstrate that measurement of passive K^+ loss from RBCs allows discrimination between two separate mechanisms of AN-induced damage, an oxidant mechanism, as has been previously described, and a new pathway that appears to be mediated by granule-associated enzymes released from AN. The increased susceptibility of sickle RBCs, especially during periods of increased physiologic stress, suggests that previous membrane damage in vivo may render these RBCs more sensitive to the action of either oxidants or granules released from neutrophils.

Mária Magócsi

Inhibition of Na^+/H^+ exchange reduces Ca^{2+} mobilization without affecting the initial cleavage of phosphatidylinositol 4,5-biphosphate in thrombin-stimulated platelets. W. Siffert, G. Siffert, P. Scheid, T. Riemens, G. Gorter and J. W. N. Akkerman (Institut für Physiologie, Ruhr-Universität, Bochum, FRG). *FEBS Letters 212*, 123 (1987).

Stimulation of human platelets increases cytoplasmic pH (pH_i) via activation of Na^+/H^+ exchange. The effect of inhibiting Na^+/H^+ exchange in (i) thrombin-induced Ca^{2+} mobilization and (ii) turnover of ^{32}P-labelled phospholipids was determined. Blocking Na^+/H^+ exchange by removal of extracellular Na^+ or by ethylisopropylamiloride (EIPA) inhibited Ca^{2+} mobilization induced by 0.2 U/ml thrombin, whereas increasing pH_i by NH_4Cl enhanced the thrombin-in-

duced increase in cytosolic free Ca^{2+}. The effect of EIPA was bypassed after increasing pH_i by moneasin. The thrombin-induced cleavage of phosphatidylinositol 4,5-biphosphate (PIP_2) was unaffected by treatments that blocked Na^+/H^+ exchange or increased pH_i. It is concluded that activation of Na^+/H^+ exchange is a prerequisite for Ca^{2+} mobilization in human platelets but not for the stimulus-induced hydrolysis of PIP_2.

B. Sarkadi

Thrombin-induced phosphoinositide hydrolysis in platelets. Receptor occupancy and desensitization. E. M. Huang and Th. C. Detwiler (Department of Biochemistry, State University of New York Health Science Center at Brooklyn, Brooklyn, NY, USA). *Biochem. J. 242*, 11 (1987).

The relationship between occupancy of thrombin receptors on platelets and enhanced phosphoinisitide hydrolysis was analysed by examinations of the dose-response relationship, the effects of thrombin inhibitors and the contribution of secondary effects. Washed human platelets were labelled with [^{3}H] inositol, and agonist-induced accumulation of labelled inositol phosphates was measured. (1) The dose-response curves and the time courses for α-thrombin or γ-thrombin-induced accumulation of inositol phosphates were similar to those for dense-granule secretion. (2) Addition of the thrombin inhibitor hirudin to thrombin-activated platelets revealed that the continuous presence of active thrombin was required to maintain the accumulation of labelled inositol phosphates; the total production of inositol phosphates increased with longer periods of exposure to thrombin, reaching a maximum between 5 and 10 min. (3) After activation with thrombin, the ability of a second, greater, addition of thrombin to induce additional phosphoinositide hydrolysis decreased with time; it was absent within 10 min after the first addition. (4) The failure to sustain accumulation of labelled inositol phosphates or to respond to a second addition of thrombin beyond 10 min was not due to depletion of the pool of labelled precursors, because the platelets retained their ability to respond to collagen. (5) Addition of ADP-consuming enzymes decreased sensitivity to thrombin, but inhibition of cyclooxygenase with indomethacin did not impair the thrombin-induced hydrolysis of phosphoinositides. (6) It was concluded that thrombin-induced hydrolysis of phosphoinositides (i) has characteristics consistent with mediation by a receptor that is similar to that that triggers dense-granule secretion, (ii) requires continuous presence of active thrombin to be maintained, (iii) is mediated by a receptor that displays thrombin-induced desensitization, and (iv) is only partially enhanced by secondary agents.

B. Sarkadi

Lysosphingolipids inhibit protein kinase C: Implications for the sphingolipidoses. Y. A. Hannun and R. M. Bell (Department of Medicine, Duke University Medical Center, Durham, NC, USA). *Science 235*, 670 (1987).

Lysosphingolipids potently and reversibly inhibited protein kinase C activity and binding of phorbol dibutyrate in vitro and in human platelets. As with activation of protein kinase C by phosphatidylserine and *sn*-1,2-diacylglycerol, inhibition was subject to surface dilution. Accordingly, inhibition in mixed micelle assays was dependent on the molar percentage of lysosphingolipids rather than the bulk concentration. Lysosphingolipids inhibited protein kinase C activity at molar percentages similar to those required for activation by phosphatidylserine and *sn*-1,2-diacylglycerol. Since lysosphingolipids accumulate in Krabbe's disease, Gaucher's disease, and other sphingolipidoses, the hypothesis that lysosphingolipid inhibition of protein kinase C represents the missing functional link between the accumulation of sphingolipids and the pathogenesis of these disorders appears to unify existing data. The accumulation of lysosphingolipids would cause progressive dysfunction of signal transduction mechanisms vital for neural transmission, differentiation, development, and proliferation and would eventually lead to cell death.

B. Sarkadi

Book Review

Transfusionsmedizin aktuell — Infektionen, Thrombozyten, Granulozyten (Present Day Transfusion Medicine — Infections, Thrombocytes, Granulocytes. Symposium held in Marburg (FRG), September 26—28, 1985, by the German Society of Blood Transfusion and Immunhaematology. Eds.: V. Kretschmer (Marburg) and W. Stangel (Hannover), Karger-Basel, 1986. 87 figures, 87 tables, 293 pages.

The book is composed of a number of different topics.

The first chapter deals with the uses of autotransfusion, i.e. the intraoperative "cell saver" instruments. Blood transferable diseases: CMC, hepatitis and AIDS are also discussed.

The methods of thrombocyte processing and storage are described in detail. Thrombocyte substitution is of basic importance primarily in the intensive care of oncohaematologic patients. In treatment, the administering of platelet from a single donor is advantageous. It is essential that the platelet concentrate be contaminated by as few white blood cells as possible. In this field the use of blood cell separators has gained much importance.

Comparisons are made between platelet products processed by different blood cell separators. The function ability of the end products were characterized by in vitro laboratory tests.

It is possible to process rather pure (white blood cell poor) platelet concentrate not only with blood cell separators but also in the usual way: in plastic bags. Its precondition is the correct centrifuging and careful separation. The thrombocyte product can be freed of white blood cells by further centrifuging, but only with a loss of 20—30%.

Storage possibilities of platelet products were examined in plastic bags of different permeability.

A short chapter deals with the cryopreservation of thrombocytes.

Some chapters focus on the substitution of thrombocytes, namely in cases of bone marrow transplantation and children's cytostatic therapy.

A separate chapter is dedicated to the substitution of factor XIII in the case of leukaemic patients.

Interesting experiences are related as regards the administering of phospholipids to patients with thrombocyte disorders.

An in-depth description is given of the separation and function of polymorphonuclear leukocytes, and the changes during the storage of blood.

This book promotes to enlarge the knowledge of staff members at transfusion services in the field of substitution therapy. With its help they may work more efficiently in the relation of blood products.

Vera Harsányi, MD

VNU Science Press, Utrecht
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The 11th Jean Julliard Prize, which was established by the International Society of Blood Transfusion in memory of its first Secretary General, will be awarded during the XXth International Congress of Blood Transfusion to be held in London, UK from 10 to 15 July, 1988.

The prize is reserved for scientists under 40 years of age in recognition of recently completed scientific work on blood transfusion and related subjects.

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This prize will be awarded during the congress. The value of the prize is 3,000 Swiss Francs.

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VOLUME 20 • NUMBER 4 • 1987

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

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VNU SCIENCE PRESS, UTRECHT, THE NETHERLANDS

AKADÉMIAI KIADÓ, BUDAPEST

1987

Haematologia 20 (4), pp. 203–213 (1987)

Comparison of Efficiency of Complements from Various Species for T-Cell Depletion from Cercopithecus aethiops Bone Marrow with Campath-1 MoAb In Vitro

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(Received 15 September, 1986; accepted 20 November, 1986)

Mature T-cells were removed from Cercopithecus aethiops monkey bone marrow with Campath-1 MoAb plus complement from various species (man, rabbit and monkey). The T-cell depletion was more effective and stable with rabbit or human complement than with autologous (monkey) complement. The most complete and effective lytic function to T-cells was found in the case of rabbit complement. Rabbit complement can be used successfully to deplete bone marrow T-cells of C. aethiops with Campath-1 in vitro.

Keywords: monkey bone marrow, Campath-1, complement, T-cell depletion, CFU-GM, haemolysis of red cells

Introduction

One of the major obstacles of successful allogeneic bone marrow transplantation (BMT) is acute graft-versus-host disease (GVHD) due to the high rate of the incidence (50–70%) and mortality (10–20%) of GVHD even with intensive immunosuppressive treatment of the recipients [1]. Animal experiments and clinical studies have indicated that GVHD may be effectively prevented by removal of mature T lymphocytes from the bone marrow graft [2, 3, 4].

For this purpose several methods have been employed. Reproducibility of physical methods of separating human bone marrow cells by density gradient centrifugation was not sufficient in human BMT [2]. T-cells can be removed by rosette formation with sheep red blood cells (SRBC) [5] or by agglutination with soybean lectin in combination with SRBC rosetting technique [3, 4]. These methods required a number of complex manipulative steps, and the final yield of granuloid-macrophage progenitor cells (CFU-GM) was 4% and 10%, respectively.

More recently, monoclonal antibodies (MoAbs) generated against antigens expressed on immunocompetent T lymphocytes have been used for immunosuppression in clinical BMT. Studies with pan-T MoAb [6] or cocktail of eight monoclonal anti-T-cell antibodies [7] demonstrated that depletion of T-cells in vitro by

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incubating bone marrow cells with MoAb plus complement can prevent GVHD It was observed that complements from different species gave characteristic functional effectivity to combine with MoAbs.

The most available MoAbs, for example OKT3 and OKT11, were found to fix rabbit complement [8, 9]. Another MoAb, as designated Campath-1, has been described with the desirable property to fix human complement, particularly autologous complement [10]. Campath-1 recognizes lymphocytes and red cells in baboons, in Rhesus and some Cynomolgus monkeys, while in other Cynomolgus monkeys [10, 11] it recognizes only lymphocytes. These animals provide a suitable model for studying the consequences of in vivo administration of Campath-1 MoAb [12].

In this work we comparatively studied 1) the efficacy of elimination of T-cells from C. aethiops monkey bone marrow with Campath-1, and 2) the lytic effect on red cells in the presence of complement from various species (man, rabbit and monkey). The aim of our work was to find a complement optimal for depletion of T-cells from C. aethiops bone marrow in vitro and to develop an experimental model for in vivo administration of the antibody.

Materials and Methods

MoAbs: Monoclonal rat anti-pan-lymphocyte antibody (Campath-1) was the kind gift of Professor H. Waldman (Division of Immunology, Laboratory Block, Cambridge). The antibody concentration was 12.5 mg/ml. OKT series were obtained from the Ortho Diagnostic Systems, Raritan, New Jersey, USA. Crossreaction of these MoAbs with C. aethiops monkey lymphocytes has been demonstrated in our pilot experiments.

Complements: Human AB sera were prepared from normal donor blood. Pooled sera from 20 normal rabbits were stored at -180 °C. These sera were not toxic for human or monkey bone marrow cells. Autologous complement was prepared from the plasma of each studied monkey.

Mononuclear bone marrow cells: C. aethiops monkeys (from Van Der Bilj, The Netherlands, provided by the National Institute of Hygiene, Budapest) were used to prepare bone marrow cells. Cells in optimal concentration ($1-1.5 \times 10^7$ cells/ml) were layered over a Ficoll-Hypaque gradient (density = 1.077 g/ml) according to the method described by Boyum [13].

E rosette technique: Mononuclear cells (50 μl, 5×10^6 cells/ml) were added in equal volume to 1% SRBC treated with AET (2-aminoethyl-isothiouronium bromide, Calbiochem, USA) [14] and 50 μl of heat-inactivated fetal calf serum (FCS) absorbed with SRBC at 4 °C for 30 min. The suspension was centrifuged at 200*g* for 5 min, and left at 4 °C overnight. The pellet was resuspended and E rosettes were counted by microscope.

Immunfluorescence (IF) test: Target cells were incubated with 5% heat-inactivated pooled normal rabbit serum at 4 °C for 30 min to block the nonspecific

binding of MoAbs to Fc receptors [15]. Indirect IF test was performed as described previously [16].

Lysis of red cells: Reactivity has been examined on microtest plates. To 50 μl of Campath-1 diluted (1 : 2 – 1 : 8192) in PBS (phosphate buffered saline, pH 7.2) 5 μl of 10% suspension of washed red blood cells were added. The cell suspension was incubated at 37 °C for 30 min, then 20 μl of complement were added to each well and incubated at 37 °C for 45 min. The plates were centrifuged at 2000 rpm for 5 min. Cell lysis was scored visually.

Agglutination of red cells: To 50 μl of Campath-1 diluted in PBS 5 μl of 10% washed red blood cells were added. This suspension was incubated at 37 °C for one hour. After allowing the cells to settle, agglutination was scored visually.

Microcytotoxicity test of lymphocytes: The method described by Terasaki [17] was applied.

T-cell depletion: Removal of lymphocytes from monkey bone marrow with Campath-1 plus complement was performed as described by Waldman [18]. $50-100 \times 10^6$ mononuclear cells were incubated with 100 μg/ml Campath-1 serum for 30 min at room temperature. Free human AB or rabbit serum or autologous plasma (as complement) was added up to 25% final concentration. The mixture was incubated under continuous shaking at 37 °C for 45 min. The aggregates of dead cells were discharged and living cells were recovered from the residuum.

CFU-GM assay: colony forming units – granulocyte/macrophage (CFU-GM) were assayed by the method of Pike and Robinson [19]. As CSF source, human placental conditioned medium (HPCM) prepared according to Burges et al. [20] was used. Cell and CFU-GM yields were calculated from each experimental sample.

Results

The toxicity of Campath-1 MoAb in the presence of different complements:

Toxicity for human lymphocytes: Figure 1 shows the toxicity of Campath-1 plus different complements to human peripheral lymphocytes evaluated by the microcytotoxicity test. Titer of antibody based on border-line of toxicity was between 512 and 2048 with human AB complement, 2048 or 4096 with rabbit complement, between 256 and 1024 with autologous complement, and between 64 and 512 with monkey complement. Different titers (128, 256, 64 and 16, respectively) were characteristic of 90–100% cytotoxicity.

Toxicity for monkey peripheral lymphocytes and bone marrow mononuclear cells: Figure 2 shows that monkey peripheral lymphocytes and bone marrow mononuclear cells were also effectively killed with Campath-1 in the presence of AB, rabbit or autologous (monkey) complement. Different antibody titers were characteristic of the various complements. We observed that over 90% of bone marrow mononuclear cells were killed in the presence of either human (AB serum), rabbit or autologous complement at MoAb-titer of 16, 32 and 4, respec-

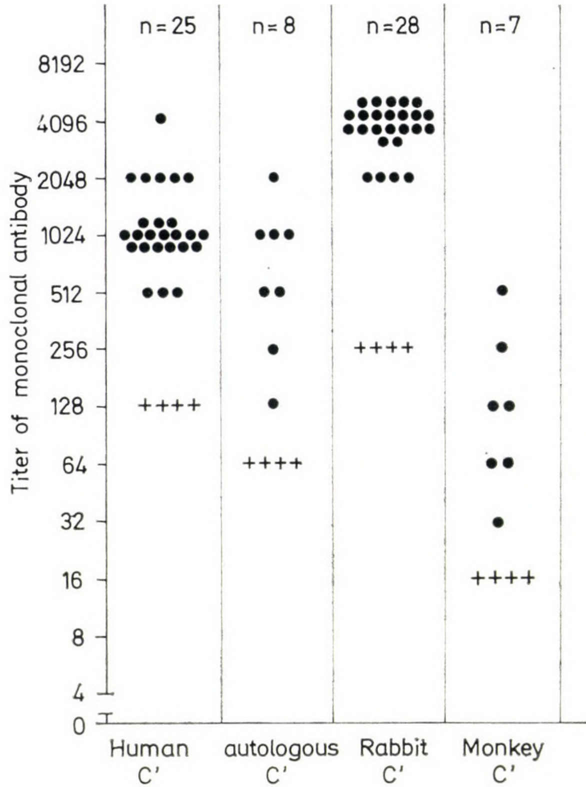

Fig 1. Toxicity of Campath-1 in the presence of complement from different species (man, rabbit and monkey) to human peripheral blood lymphocytes by microcytotoxicity test: ● titer of MoAb at which 10–20% of the cells were dead, ++++ titer of MoAb at which 90–100% of the cells were dead

tively. Thus, in this high antibody concentration, Campath-1 is toxic not only for lymphocytes but also for other bone-marrow cells (Fig. 2).

Reactivity with monkey red cells: Red blood cells from seven C. aethiops monkeys were tested as targets for lytic reactivity of Campath-1 in the presence of human, rabbit or autologous complement. Haemolysis occurred in all seven cases. The strongest lytic activity was found with rabbit complement (Table 1). In addition, Campath-1 antibody showed agglutination behaviour, thus red cells of these animals are agglutinated in similar titer as shown in Table 1 for haemolytic activity.

Effectivity of T-cell removal from monkey bone marrow with Campath-1 + different complements:

Cell yield: Cell yield of monkey bone marrow after treatment with Campath-1 + human, rabbit or autologous complement are shown in Table 2. The

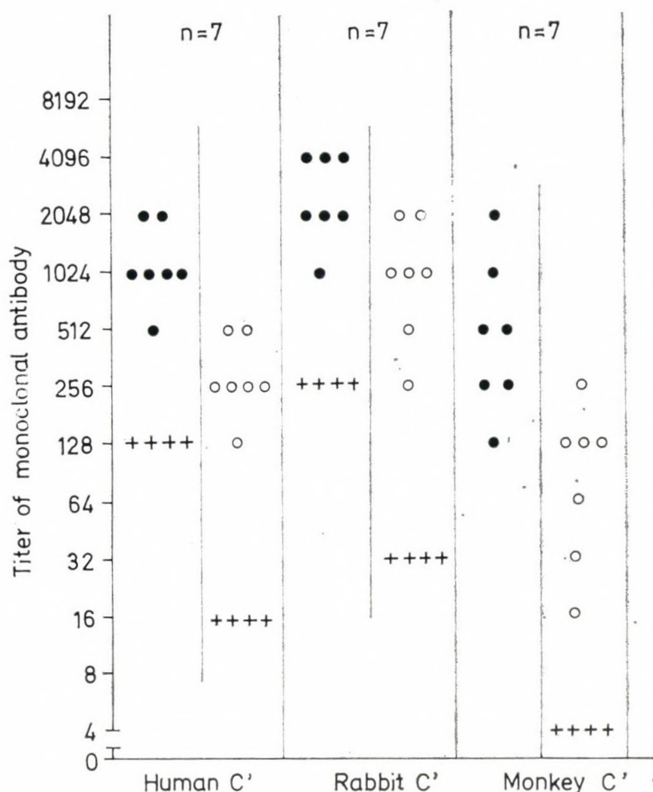

Fig 2. Toxicity of Campath-1 in the presence of complement from different species to C. aethiops blood lymphocytes (●) and mononuclear bone marrow cells (○) by microcytotoxicity test: ● ○ titer of MoAb at which 10—20% of the cells were dead, ++++ titer of MoAb at which 90—100% of the cells were dead

Table 1

Reactivity of Campath-1 with C. aethiops monkey red cells

RBC-donor monkey No.	Lytic titer*			Agglutination titer*
	Human C'	Rabbit C'	Autologous C'	
1	2048	4096	512	4096
2	2048	4096	512	4096
3	4096	4096	1024	4096
4	2048	4096	512	4096
5	2048	2048	512	2048
6	2048	4096	1024	4096
7	1024	2048	512	2048

* The antibody was diluted from 1 : 2 to 1 : 8192. The tests were performed on microtest plates

Table 2

Cell yields of monkey bone marrow after treatment with Campath-1 plus different complements in vitro

Monkey No.	Nucleated bone marrow cells (× 10^5) before treatment	Nucleated bone marrow cells after treatment with MoAb plus					
		Human AB serum		Rabbit serum		Autologous plasma	
		number (× 10^5)	yield %	number (× 10^5)	yield %	number (× 10^5)	yield %
1	600	334	55.6	202	33.6	272	45.3
2	508	261	51.3	235	46.2	256	50.3
3	516	396	76.0	240	46.5	272	52.7
4	400	250	62.5	192	48.0	286	71.5
5	930	550	59.1	430	46.2	500	53.7
6	270	166	61.4	88	32.5	135	50.0
7	256	205	80.0	168	65.6	188	73.4
8	171	128	75.2	87	51.1	140	82.3
Mean ± SD			61.1 ± 10.5		46.2 ± 10.3*		59.9 ± 13.6

* Statistically significant value ($P < 0.01$)

mean cell yield was 61.1 ± 10.5%., 46.2 ± 10.3% and 59.9 ± 13.6%, respectively (Table 2).

CFU-GM yields: CFU-GM recovery of T-cell depleted bone marrow was investigated and the following two parameters were comparatively studied:

– Concentration of CFU-GM per 10^5 T-cell depleted bone marrow cells with Campath-1 + human, rabbit or autologous complement was 192.0 ± 22.4, 273.3 ± 76.0 and 196.8 ± 72.3, respectively, in comparison to the pretreatment concentration of CFU-GM: 155.1 ± 24.1 per 10^5 mononuclear bone marrow cells (Table 3).

– The yield of CFU-GM of T-cell depleted bone marrow by the same experimental circumstances were 78.3 ± 13.3, 76.9 ± 16.7 and 68.6 ± 15.4 per cent, respectively (Table 4).

Residual T-cells: Residual T-cells of T-cell depleted bone marrow were detected by the E rosette technique, indirect IF test with anti-human T-cell monoclonal antibodies (OKT3 – 8 – 11), and microcytotoxicity test with Campath-1 plus complement. The results are demonstrated in Table 5. Before treatment E-rosette forming cells were 6 – 13 per cent, T-cells detected by a mixture of OKT3 + 8 + 11 MoAbs were 8 – 18 per cent and Campath-1 positive cells were 35 – 47 per cent. After treatment, however, the ratio of residual T-cells decreased significantly. Mature T-cells could not be detected in the T-cell depleted bone marrow, particularly if marrow suspension had been treated with Campath-1 supplemented with rabbit complement.

Table 3

Concentration of CFU-GM per 10^5 bone marrow cells before and after treatment with Campath-1 plus different complements

Monkey No.	CFU-GM/10^5 bone marrow cells before treatment	CFU-GM/10^5 bone marrow cells after treatment with		
		Human AB serum	Rabbit serum	Autologous plasma
1	172.0 ± 7.5	198.0 ± 9.0	244.6 ± 26.5	146.0 ± 8.1
2	155.6 ± 15.5	173.3 ± 20.4	214.3 ± 19.5	140.6 ± 13.6
3	262.6 ± 16.7	230.5 ± 14.7	431.6 ± 10 4	357.3 ± 17.1
4	197.6 ± 12.5	nd	341.0 ± 34.4	202.0 ± 13.1
5	128.6 ± 15.9	nd	217.0 ± 20.5	205.6 ± 23.8
6	132.3 ± 18.8	199.6 ± 11.5	261.3 ± 16.0	209.0 ± 7.5
7	162.6 ± 9.0	179.3 ± 10.6	217.0 ± 31.4	187.0 ± 13.0
8	129.6 ± 8.1	171.3 ± 11.9	260.0 ± 4.5	127.3 ± 2.8
Mean ± SD	155.1 ± 24.1	192.0 ± 22.4	273.3 ± 76.0*	196.8 ± 72.3

* Statistically significant value ($P < 0.01$)
nd: not determined

Table 4

CFU-GM yields of bone marrow depleted T cells with Campath-1 plus different complements

Monkey No.	CFU-GM number before treatment	CFU-GM number after treatment with Campath-1 plus					
		Human AB serum		Rabbit serum		Autologous plasma	
		abs. no.	yield (%)	abs. no.	yield (%)	abs. no.	yield (%)
1	103 200	66 132	64.0	49 288	47.7	39 712	38.4
2	79 044	56 669	71.6	63 004	79.7	43 726	55.3
3	135 501	91 278	67.3	103 384	72.2	98 614	72.7
4	79 040	nd	nd	65 472	82.8	57 773	73.0
5	11 959	nd	nd	9 331	79.2	10 030	85.3
6	35 648	33 200	80.2	22 968	64.4	28 215	79.1
7	41 472	36 695	88.0	36 456	87.9	35 156	65.0
8	22 161	21 888	98.7	22 620	102.0	17 780	80.2
Mean ± SD			78.3 ± 13.3		76.9 ± 16.7		68.6 ± 15.4

nd: not determined
abs. no.: absolute number

Table 5

Detection of residual T-cells in bone marrow treated with Campath-1 plus different complements in vitro

	Before treatment	After treatment with Campath-1 plus:		
		Human AB C'	Rabbit C'	Autologous C'
E rosette (%)	6–13	0–1.5	0	0–1.5
Cytotoxic test with Campath-1 (% dead cells)	35–47	0–1	0	0–1
IF with OKT-3 + 8 + 11 (% positive cells)	8–18	0–1.5	0	0–2

Discussion

T lymphocytes can be eliminated from bone marrow allografts by incubation with monoclonal antibodies plus complement [10, 21, 22]. T-cell depletion significantly decreases the incidence and severity of GVHD [3, 6, 11, 23] without impairing bone marrow stem cells [10, 24, 25]. On the basis of these promising results, it has been attempted to extend indication of allogeneic BMT to HLA-mismatched donor-recipient combinations.

The role of complement in the T-cell depletion from bone marrow with MoAbs has been discussed by many authors. Some of them emphasized that from human bone marrow T-cells could be eliminated by MBG-6 + RFT-8 [21] pan-T MoAb combination [22, 26, 27] in the presence of xenogeneic (rabbit) complement. Pre-incubation of donor marrow with combination of anti-T MoAbs without complement did not prevent GVHD [7]. However, the treatment of donor bone marrow with T-cell-specific MoAbs and xenogeneic complement has not been uniformly accepted as really effective combination for the prevention of severe GVHD [28]. Certain batches of xenogeneic complement themselves may be toxic to stem cells [10]. Recently, Campath-1 has been described as an effective MoAb to deplete T-cells from human and Rhesus or Cynomolgus monkeys, furthermore, Campath-1 has the desirable property to fix human complement [10, 11, 23, 29]. In the present studies we observed that T-cells can be successfully removed by in vitro treatment with Campath-1 plus complement from bone marrow cells of C. aethiops, a species not examined in this respect previously. We used human, rabbit and autologous monkey complement in removing T-cells from bone marrow with Campath-1. Each of these complements can be used effectively in eliminating T-cells from C. aethiops bone marrow with Campath-1. No significant differences were observed ($P > 0.1$) in the CFU-GM yields of the bone marrow T-cell depleted with Campath-1 plus any of the studied complements (Table 4). In contrast to the observation of others [10, 28], in our experiments

rabbit complement (as xenogeneic complement) did not inhibit the growth of monkey haemopoietic stem cells. Higher CFU-GM per 10^5 values were obtained with C. aethiops marrows depleted by Campath-1 plus rabbit complement than with the untreated original bone marrow suspension ($P < 0.01$) (Table 3). However, higher concentrations (> 125 μg/ml cell-suspension) of Campath-1 MoAb are toxic to other bone marrow cells of C. aethiops monkeys, too (Fig. 2). Rabbit complement displayed a superior lytic function on target cells than did the human and C. aethiops complement (Figs 1, 2). We found no nonspecific toxicity of rabbit complement for C. aethiops bone marrow. It should be noted, however, that our rabbit complement was a mixture of 20 rabbit sera, which had been previously screened and controlled on human lymphocytes in microcytotoxicity test. Hale et al. found that Campath-1 reacts with both lymphocytes and red cells in baboons, Rhesus and in some Cynomolgus monkeys, it reacts, however, only with lymphocytes in other Cynomolgus monkeys. Our results show that Campath-1 recognizes lymphocytes and red cells in C. aethiops monkeys. Reaction of Campath-1 with red cells from seven C. aethiops was found with either of two techniques (haemolysis and agglutination) (Table 1).

In conclusion we supported earlier findings that Campath-1 fixes not only human complement, but also rabbit and monkey complement. These complements are not toxic for haemopoietic stem cells. High and uniform rate of depletion of mature T-cells together with high yields of CFU-GM in the T-cell depleted bone marrow cell suspension were found with Campath-1 + rabbit complement.

The results indicate that C. aethiops monkeys can be used as a model for in vitro studying lysis of T-cells from bone marrow with Campath-1 plus rabbit complements, but it cannot be a model for in vivo administration because Campath-1 recognizes both lymphocytes and red cells in C. aethiops.

Acknowledgement

We wish to express our special thanks to Professor H. Waldman (Div. of Immunology Lab. Block, Cambridge, UK) for the Campath-1 MoAb serum. We thank Dr. L. Nemes, Head of the Department of Experimental Animals of the National Institute of Hygiene, Budapest, Hungary, for access to monkey specimens.

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Haematologia 20 (4), pp. 215—220 (1987)

Deficiency of C2, the Second Complement Component, in the Family of a Patient With SLE-like Syndrome: The First Case of Hereditary C2 Deficiency in Czechoslovakia

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(Received 21 October, 1986; accepted 23 October, 1986)

A family with hereditary C2 deficiency was discovered in Czechoslovakia. The proband is a 47-year-old female with a SLE-like syndrome and zero activity of the classical complement pathway. Functional CH50, C1, C2, and C4 estimations for all family members revealed a homozygous C2 deficiency in both the proband and her elder sister, and several heterozygotic C2-deficient individuals. The defect segregates with haplo-type HLA A25, B18, DR2.

Keywords: C2 deficiency, SLE-like syndrome, HLA A25, B18, DR2

Introduction

Of the components which comprise the classical complement system, C2 is one of the most frequently affected by genetic deficiency. Since 1960 when Silverstein (1960) described the first case of C2 deficiency, more than 60 cases of homozygous deficiency of this serum protein have been reported. Hereditary C2 deficiency is transmitted to the progeny as an autosomal recessive trait (Klemperer et al., 1966) and is associated with the occurrence of immune-complex diseases, particularly SLE, with recurrent infections, or it occurs in healthy subjects (Agnello, 1978). In 1974 Fu et al. (1974) first demonstrated the association of the C2 locus with the HLA system and the segregation of the C2 defect with the haplotype HLA A25, B18. The present paper describes the case of a patient with a homozygous C2 defect who suffers from a SLE-like syndrome. The defect was suspected on the basis of repeated laboratory findings of the absence of classical pathway activity. Further tests aimed at the exact determination of the defect, carried out not only in the proband but also in her relatives, revealed a further homozygote and several heterozygotes for the C2 defect.

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Case report

Case history. The patient S. A. (II, 2), female, born in 1938, gave no history of childhood diseases, yet in adulthood she frequently suffered from influenzae-like disorders mostly in the winter season, presenting with increased temperature, malaise, fatigue, dry cough, and headaches. Five years ago she complained of metrorrhagia and on gynecological examination myoma was diagnosed. From 1980 to 1982, the patient suffered from recurrent episodes of arthritis accompanied by a marked loss of hair. In the spring of 1982 edema of her feet and lower legs appeared, she developed subcutaneous nodules on the extremities, and the surface of the extremities was of bluish-purple coloration. She also developed high temperatures. Antibiotics were ineffective and the symptoms disappeared only after prednisone administrations: initial dose was 40 mg daily lasting for three weeks. In May 1982 she underwent hysterectomy with a satisfactory postoperative course. In June 1982 she developed signs of hemorrhagic syndrome (petechiae, bleeding from the oral mucous membrane and gingivae) and marked thrombocytopenia was established. The patient was given long-term prednisone treatment (initial dose 60 mg with gradual decline to maintenance doses). In October 1982, on examination at the Research Institute of Rheumatic Diseases in Piestany, depressed hematopoiesis along with an absence of classical complement pathway activity, and mild positivity of antinuclear antibodies were found. The finding first evaluated as bicytopenic syndrome was later diagnosed as oligosymptomatic systemic lupus erythematosus, or SLE-like syndrome.

Family history. The father (I, 1) and the mother (I, 2) of the patient had no history of susceptibility to infections and did not suffer from systemic disorders, but in childhood the father had pneumonia twice. The proband has three sisters. The oldest sister H. A. (II, 1) has suffered throughout her life from frequent inflammations of the respiratory tract and has been treated for chronic bronchitis for over 10 years. She has two children, and both have been susceptible to infections from childhood. Hodgkin's disease was diagnosed in her son when he was 23 years old. Neither of the children were available for further study. The middle sister of the proband (II, 4) underwent tonsillectomy at the age of 17 for chronic tonsillitis with frequent relapses. The youngest sister (II, 5) had transient anemia two years ago, and although she complains of recurring headaches, she appears to be healthy. The proband has three children. Her daughter (III, 1) is not susceptible to infections, her second daughter (III, 2) suffers from rather frequently recurring tonsillitis, and her son (III, 3) had pneumonia at pre-school age, but has not had frequent illnesses. The husband of the proband (II, 3) is a healthy adult.

Materials and Methods

Blood was allowed to clot for 30 min and then centrifuged for 10 min. to separate the serum. The serum was frozen at −70 °C until assayed. Buffers, veronal buffered saline (VBS) with EDTA or sucrose, the cell intermediates (EA, EAC14, etc.), the CH50 assay and the molecular titration of C1, C2 and C4 have been described previously (Rapp and Borsos, 1970).

Results of the titrations were expressed as CH50 units/ml or effective molecules/ml respectively. Functionally pure human and guinea pig complement components were purchased from Cordis, Miami, Fl., USA. All complement titrations were done by the microliter modification method (Ringelmann et al., 1969).

HLA-A, B, C and DR typing were performed by microtoxicity tests on unseparated lymphocytes and separated B lymphocytes isolated from peripheral blood as described previously (Terasaki and McCleland, 1964; Niks et al., 1982). VIII International Histocompatibility Testing Workshop and equivalent local antisera were used for antigen estimation.

Results

The results of the titrations of whole complement levels, C1, C2, and C4 that were carried out in the proband and in members of her family are given in Table 1. The proband (II, 2) has a zero level of the classical pathway complement activity expressed in CH50 and also zero functional activity of C2. Her sister (II,

Table 1

Functional CH50, C1, C2 and C4 estimations in a family with C2 deficiency

Family member		CH50 units/ml	C1 $\times 10^{13}$	C2 $\times 10^{10}$	C4 $\times 10^{12}$
			effective molecules/ml		
Proband	(1938)	0	5.0	0.02	11.6
Father	(1911)	18.4	3.6	5.2	8.1
Mother	(1914)	26.3	1.2	7.1	12.9
Husband	(1931)	17.0	3.0	6.5	7.4
Sister	(1936)	6.8	3.6	0.2	12.9
Sister	(1944)	31.4	6.6	22.0	15.5
Sister	(1949)	26.8	2.8	6.4	16.6
Daughter	(1958)	26.8	3.2	0.9	12.2
Daughter	(1963)	25.8	4.8	4.3	6.4
Son	(1968)	25.8	5.2	1.0	28.2
NHS		29.9	1.4	16.0	7.3

NHS: Normal human serum

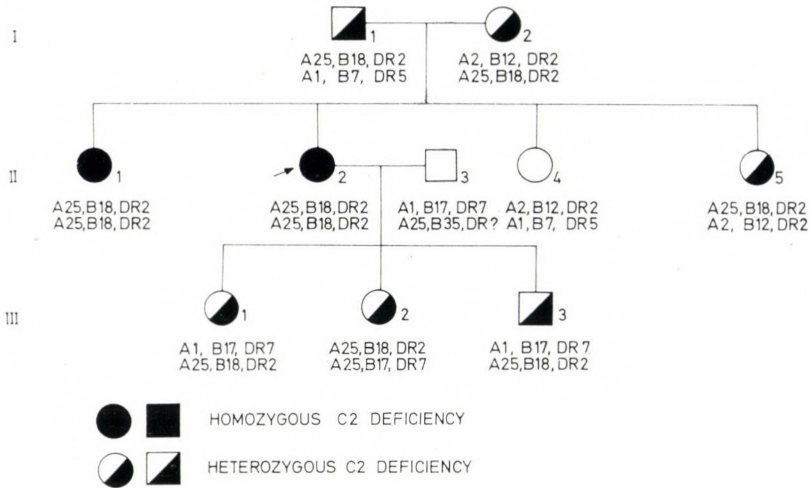

Fig. 1. Pedigree of a family with C2 deficiency

1) has an excessively decreased CH50 level and virtually no C2 functional activity. The parents of the proband (I, 1 and I, 2), her youngest sister (II, 5), and her three children have their C2 levels (number of effective molecules C2/ml of serum) diminished by over 50%. Only one sister of the patient (II, 4) has a normal CH50 level and a normal C2 level. The husband of the patient (II, 3) has decreased CH50 and C2 levels. The values of other components were normal or even increased.

Tests of the HLA system in the patient's family showed that the C2 defect segregated with the haplotype A25, B18, DR2 (Fig. 1). The pedigree shows an autosomal recessive type of inheritance, with the haplotype A25, B18, DR2 present in heterozygous state in all heterozygotes for the C2 defect, and in homozygous expression in the proband (II, 2) and her sister (II, 1). In addition, recombination between HLA-A and HLA-C loci were demonstrated in this family.

Discussion

Many individuals with a homozygous C2 defect have been described who are highly susceptible to diseases associated with deposition of immune complexes. The most frequently occurring clinical manifestations accompanying this defect are SLE, discoid LE, vasculities and anaphylactoid purpura. This can be accounted for by the inability of the defective complement system to interact with antigen-antibody complexes, which is one of the main biological functions of the complement system in the organism (Miller and Nussenzweig, 1975; Colten et al., 1981). It is interesting that in affected subjects, increased susceptibility to recurrent bacterial infections is rather rare (Agnello, 1978; Thong et al., 1980). According

to published reports, the defect of C2 is the most frequent defect of the components of the classical pathway (Agnello, 1978; Glass, et al., 1976). The incidence rate in the population is approximately one homozygote per 10,000 blood donors. Heterozygous deficiency of C2 occurs in about 1.2% of the population at large, whereas in patients with SLE the rate is about 6%, with juvenile rheumatoid arthritis about 4%, and with rheumatoid arthritis, 1.4%.

In the family described here, a homozygous C2 deficiency was found in the proband (II, 2). She suffers from oligosymptomatic SLE, or lupus-like syndrome, as has been reported to occur in patients with C2 deficiency. Lupus-like syndrome is considered to be a variant of SLE (Agnello, 1978). Contrary to classical SLE, this form is characterized by a more benign course. In addition, the kidneys are less frequently affected and discoid lesions are more frequent. The disease established in the proband reported here resembles this variant to a considerable extent. Her oldest sister (II, 1) is the other homozygote showing the C2 defect in the family. Her clinical symptomatology is completely different from that of the proband, presenting recurrent infections of the respiratory tract and resulting in chronic obstructive bronchitis. Of the several heterozygotes in the family, two are susceptible to respiratory tract infections, and the son of the proband's sister, who unfortunately was not available for examination, suffers from Hodgkin's disease. We do not know the significance of the decreased levels of whole complement and C2 in the proband's husband (II, 3).

The structural locus for C2 is on the small arm of chromosome 6, where five alleles alternate: C2–1 (frequent), C2–2, C2A and C2A1 (rare) and the zero allele C2-Q0 (Hauptmann et al., 1982). The locus for C2 occurs with the locus for factor B and loci for C4, and is situated in the HLA system between the loci B and D (Olaisen et al., 1983). Fu and coworkers were the first to discover that an association exists between the C2 defect and the haplotype HLA A25, B18. Later an association with the antigen DR2 and the complotype BF-S, C4A4B2 was established (Hauptmann et al., 1982; Allen, 1974; Alper, 1981). According to published data, approximately 50% of subjects with the haplotype HLA A25, B18 have a low functional activity of C2 (Mortensen et al., 1980). In the family described here, in homozygotes the given antigens displayed the homozygous state, and in heterozygotes the haplotype was always present in their genotype.

Genealogic studies confirmed that the C2 defect was transmitted as an autosomal recessive trait, which was in agreement with the clinical findings: the homozygotes presented clearly marked clinical symptoms, in heterozygotes the clinical picture was milder or the family members were normal. With regard to the relatively rare occurrence rate of the C2Q0 allele, the parents of homozygotes are presumed to be in consanguineous marriage. Although in our case we were unable to confirm this situation, there was some evidence for a familiar inter-relationship.

This study has confirmed the importance of screening for defects of the complement system in patients with connective tissue disorders and recurrent infections. The data aid the clinician in making an accurate diagnosis, in elucidating the etiopathogenesis, in determining the therapy and in taking preventive

measures. Only genealogic studies can provide the necessary data for differentiating inborn defects of the complement proteins and diminished complement levels due to excessive consumption by immune complexes.

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OPEN FORUM

A selection from the lectures held at the WHO, ISH, ICSH sponsored
International Workshop on "Appropriate Iron Stores"
(November 7–9, 1986; Pilisszentkereszt, Hungary)

Introduction

Iron is indispensable for many metabolic processes in living cells and organisms, including transport and storage of oxygen, electron transport and cell proliferation and differentiation. Iron has to be taken up from the environment where it is present in the biologically unavailable forms of ferric hydroxide. In the course of evolution iron complexing agents have been developed. These serve to solubilize iron in the surrounding media of living cells and organisms and to deliver iron to the cell as well as to transport and store it within the organism. Such physiological iron chelators are the transferrins, lactoferrins and the ferritins in mammals, the ovotransferrin in birds, the siderochromes in bacteria and the low molecular weight chelators in the cell sap and roots of plants.

Under physiological conditions, minimal amounts of iron are being absorbed and excreted daily. During evolution, highly sophisticated regulatory mechanisms have been developed to protect the living cells and organisms from iron toxicity. The body has no physiological route to eliminate excessive iron. Still during the last two decades the attention of physicians, paediatricians and obstetricians is mainly focused on iron deficiency and the majority of general practitioners are unaware of the risk of iron overload.

Studies of the incidence of infections and activation of preexisting malaria brucellosis and tuberculosis have shown definite adverse effects of iron repletion in previously iron deficient Somali nomads. These data are in contradiction with former statements that individuals with iron deficiency anaemias are more prone to bacterial infections. Well controlled international studies are needed to resolve this contradiction since it is of utmost importance to clarify whether hypoferraemia in different populations is an adoptive host defence mechanism or a pathological condition with adverse effects. Since the diagnostic tests which are presently in widespread use and cannot reliably differentiate the amount of stored iron and especially the distribution of iron in the organism, well planned and standardized international clinical and laboratory research work is needed. Since excess iron may seriously affect different organs, amongst them the cardiovascular system, a challenging hypotheses has been raised that the sex difference in the risk of heart disease between man and premenopausal women may be due to the higher iron stores of the former. The validity of this hypothesis could also be studied by an international group in men who are regular blood donors.

Haematologia 20 (4), pp. 225—228 (1987)

Maintenance of Normal Iron Balance

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Iron balance depends on the matching of absorption with requirements. Replacement is needed for desquamated losses from gut and skin, physiologic bleeding from the gut, the needs for essential body iron during growth and requirements of menstruation and pregnancy in the female [1]. Physiologic losses in the adult male can be modified by the state of iron balance, but within a relatively narrow range from 0.5 to 2.0 mg, and presumably such changes occur primarily through altered plasma iron levels

Absorption is more important than loss in regulating iron balance, and maximum absorption in the iron-depleted otherwise normal individual amounts to 3—4 mg/day [2, 3]. However, such a level of absorption is believed to depend on high food iron availability [4]. Absorption in the economically underprivileged on a limited diet may be considerably less [5].

Normal iron balance for purposes of this paper will be defined as the preservations of essential body iron. In trying to understand how this may be accomplished, a good starting point is the nature of the absorptive mechanism. Iron is taken into the mucosal cell in three ways, as ionized iron, as transferrin iron and as hemoglobin iron. Once in the mucosal cell, there is a common pathway that can be blocked by an intracellular chelator such as desferrioxamine.

It is well known that absorption is regulated in a way to maintain body iron balance. There are proposed explanations for the way that the mucosal cell accomplishes this. Some of us grew up with the Hahn-Grannick concept of mucosal block in which ferritin was regarded as an intracellular iron chelator which trapped and held iron until the mucosa was exfoliated [6]. Perhaps saying the same thing, Crosby suggested that regulation might be determined by the amount of iron already in mucosal cells [7]. Attempts to prove this gave variable results. More recently, there is somewhat better evidence of the inverse relationship between mucosal cell ferritin or ferritin iron and absorption in experimental animals [8] and in man [9]. One can also demonstrate an increase in ferritin and suppression of absorption by exposing the mucosal cell to increased amounts of iron [8, 10]. Thus the ferritin content of the mucosal cell would seem to predict the ab-

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sorptive capacity of the gut. From this one cannot conclude that the role of ferritin is simply to block absorption since there is also evidence that ferritin may facilitate absorption [11]. It may be that ferritin acts as a buffer and is in equilibrium with other cellular non-ferritin iron in the mucosal cell and that it is the latter iron fraction which most directly determines the metabolic capacity for moving iron across the cell.

If it is assumed that the important determinant of absorption is the amount of mucosal cell iron, can we further assume that this amount is the product of the iron entering the cell from the lumen and the amount exchanged with the body interior. Increased iron requirements within the body would be expected to reduce the iron content of the mucosal cell and increase absorption. This unloading from the mucosal cell into the body is not a function of plasma iron concentration, but would appear to result from some message from the body interior [12].

There are three different situations where internal conditions cause an increased iron release from the mucosal cell into the body. The first of these occurs with expansion of the erythroid marrow, whether in response to bleeding or hemolysis [13]. This specifically increases the transferrin receptor number in the erythroid marrow and, thereby, changes the balance between receptors and iron supply, leaving the marrow with the need for increased iron. A second way to increase absorption is through the production of true iron deficiency as may be produced by a diet inadequate in available iron. This has the result of increasing the receptor population, but this time in all tissues.

The most subtle regulator is store depletion. Cavil et al. have postulated that stores are reduced, the donation of iron to transferrin from internal tissue is decreased and, as a result, more iron will be procured from the gut [14]. This is supported by relationships between serum ferritin, mucosal ferritin and absorption. For example, there is a direct relationship between ferritin in plasma and ferritin in the mucosal cell [9], and between ferritin in plasma and of the absorption [15]. This relationship can be manipulated acutely not only by parenteral administration of iron as previously shown [8], but also by high iron diet [10]. In a more chronic form, our study shows the continued effect of store alteration on absorption over several years [16].

It is not at all clear how store regulation is accomplished. Crosby's suggestion that such regulation might be due to a variable iron loading of the mucosal cell from the mucosal side, if valid [7] cannot be due to an altered plasma iron supply since the plasma iron is usually not decreased. Alternately the iron balance of the crypt cell could be changed by a greater movement of iron back into the plasma, but if so, the way in which this is accomplished is still unclear.

Faced with increased iron requirements or decreased available dietary iron, absorption will accomodate up to a point at the price of decreased stores. For example, donating one unit of blood a year for four or five years will reduce iron stores in an adult man by at least half as judged by plasma ferritin [17]. Stores are buffering the amount of iron which must be absorbed, but as they decrease, a larger and larger proportion of iron must obtained from the mucosal cell.

If one examines the effect of a decrease in iron requirement when the adolescent boy attains his adult size, one finds it takes 10 or 15 years for the serum ferritin to build up to the adult level. This would represent an increase in storage iron at a rate of only about 0.1 to 0.2 mg/day. This is less than 1/10 the amount which can be absorbed by a patient with polycythemia vera treated by phlebotomy or that seen with blood donors who give 3 to 6 units per year [2, 3]. It seems possible that the much greater absorption in these latter instances is due to a depletion of essential body iron, that is, iron deficiency as compared to iron depletion. Perhaps this is also the effect of an inadequate iron supply for receptors, but at a much lower level.

What can the oral intake of additional iron do to the iron balance of the normal individual? One way of examining this is by increasing the availability of food iron through fortification with ascorbic acid. Isotope studies suggest that absorption should greatly increase [18]. However, monitoring the ferritin over 6 or more months of such fortification showed change in two studies on normal subjects [19, 20]. Also, in highly preliminary studies of iron fortification in normal subjects, we have seen surprisingly little effect on plasma ferritin. While there seems to be a discrepancy between isotopic absorption studies and ferritin balance raising additional questions, these studies would agree with the general assumption that the normal individual with iron stores is most resistant to any substantial increase in body iron. Presumably, in the absence of increased internal needs, there is mucosal iron overload with its suppressive effect on absorption. In the context of the relationships between stores, requirements and available oral iron, it would be of interest to see the extent to which donors giving several units of blood a year could protect their stores by continuous intake of medicinal iron. From a practical standpoint, there appear to be two levels of absorptive response, a maximal one associated with depletion of essential body iron and a minimal response relating to modest changes in stores. We need a more detailed description of the behaviour between these two extremes.

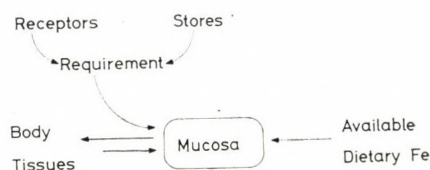

Fig. 1

The iron content of the mucosal cell is proposed to be the determinant of iron absorption (Fig. 1). The amount and availability of dietary iron affects this from the luminal side, influencing mucosal cell intake. It is proposed that an iron exchange with plasma transferrin also occurs, in which the dominant influence is the removal of iron from the mucosa according to the need of transferrin receptors.

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Haematologia 20 (4), pp. 229–235 (1987)

The Diagnostic Value of Serum Ferritin Determinations for Assessing Iron Status

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Introduction

The iron storage protein ferritin consists of an apoprotein shell (mol. wt. 480,000) which encloses an iron core in the form of ferric hydroxy-phosphate, which may contain up to 4,500 iron atoms [1]. Soluble ferritin is degraded to insoluble haemosiderin, which accumulates in lysosomes and is the "stainable iron" beloved of pathologists and haematologists. Normally most of the storage iron in the body (approx. 1 g in men and less in women) is in the form of ferritin but with increasing iron overload more and more is present as haemosiderin.

The ferritin in human tissues is composed of two types of subunit which are called H and L [2]. The well studied ferritins of liver and spleen are mostly composed of L subunits and are the more basic isoferritins having isoelectric points in the range 5.3 – 5.8. H subunits have a molecular weight of 21,000 and are found in the more acidic isoferritins (pI. 4.5 – 5.0) in heart, red blood cells, lymphocytes and monocytes and the HeLa cell, as well as other tissues [3 – 8]. Variation in the ratio of H to L subunits explains the heterogeneity of charge of ferritin, which is most readily demonstrated by isoelectric focusing [3]. The iron content of ferritin does not significantly affect its isoelectric point. The iron content of ferritin varies from tissue to tissue and with the iron content of the particular tissue. H and L subunits are only 55% homologous [9] and are coded for by genes on chromosomes 11 and 19q 13.3-qter respectively [10, 11]. The H rich isoferritins are immunologically distinguishable from the L rich forms and both polyclonal and monoclonal antibodies which are specific for heart ferritin have been raised. Although only a single expressed gene has been described for the H and L subunits, there are multiple gene copies of H sequences [12, 13] and H genes have been found on at least nine different chromosomes [14]. Presumably some of these sequences will be found to be pseudogenes. L genes are found on chromosome 20 and the X chromosome as well as chromosome 19 [15].

The major function of ferritin would appear to provide a store of iron which may be used for haem synthesis when necessary. Iron uptake has been studied extensively in vitro and requires an oxidizing agent whereas iron release requires a reducing agent [1]. The function of iron storage appears to be largely carried out by the L rich isoferritins such as those found in liver and spleen. Other biological activities for ferritin have been proposed: vasopressor activity [16], inhibition of

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granulocyte-macrophage colony formation in vitro [17], inhibition of lymphocyte transformation in vitro [18]. In addition to the tissue ferritins there may be a glycosylated, secreted form in the plasma (see later) and there may be membrane bound ferritin which differs from ferritin found in the cell cytosol [19, 20].

Ferritin in plasma

It was only after the development of a sensitive immunoradiometric assay for ferritin that it was detected in normal serum or plasma [21]. Ferritin concentrations in normal subjects (15 – 300 μg/l) were found to be related to the amount of storage iron in the body [22], with low concentrations in patients with iron deficiency anaemia (< 15 μg/l) and high concentrations in patients with iron overload [23]. This relationship with the level of storage iron is what makes the serum ferritin assay useful in the clinical laboratory.

The original immunoradiometric assay and radioimmune assays have been supplemented by commercial kits which may also employ ELISA techniques and, increasingly, newer methods such as those involving luminescence. The International Committee for Standardization in Haematology (Expert Panel on Iron) has prepared a reference reagent of human liver ferritin which provides a standard for the serum ferritin assay (80/602). This reagent is now a WHO standard.*

Biochemistry and physiology of plasma ferritin

The following observations refer largely to studies with normal serum or serum from patients with iron overload. Immunologically serum ferritin resembles liver or spleen ferritin. Only low concentrations of ferritin are usually measurable with antibodies to heart or HeLa cell ferritin [24, 25]. Plasma ferritin from patients with iron overload has a low iron content (0.02 – 0.07 μg Fe/μg of protein [26] compared with 0.2 μg of Fe/μg of protein in the liver and spleen of such patients [4]). Little is known about the iron content of normal serum. On isoelectric focusing both native and purified serum ferritin display a wide range of isoferritins encompassing the isoelectric points found in both liver and heart [27, 28], yet on anion exchange chromatography it behaves as a relatively basic isoferritin [29, 30]. This seems to be due to glycosylation rather than variation in the ratio of H to L subunits. About 60% of ferritin from normal serum binds to the lectin concanavalin A [31], whereas tissue ferritins show little binding. On incubation with neuraminidase the acidic isoferritins of serum are converted to the more basic isoferritins, whereas the pI of tissue isoferritins is unaffected. It has been shown that ferritin from plasma of patients with iron overload has an additional subunit (which has been called the G subunit), which stains for carbohydrate and has an apparent molecular weight of 23,000 [26].

* For further details please write to the author.

The origin of plasma ferritin is not known. The presence of glycosylation suggests secretion of ferritin, possibly from phagocytic cells degrading haemoglobin. In liver disease another mechanism is clearly important, that of direct release of cellular ferritin through damaged cell membranes [33]. In patients with ferritinaemia due to necrosis of the liver very little of the plasma ferritin binds to concanavalin A [31]. Various isoferritins may also be cleared from the circulation at different rates. On injection of ^{131}I-labelled spleen ferritin into two normal subjects it was found that clearance was very rapid ($T^1/_2$ approx. 9 min), with uptake and degradation of ferritin by the liver [34]. ^{131}I-labelled plasma ferritin was removed only slowly from the plasma and radioactivity had only decreased by 50% after 30 h [35]. Rapid clearance may be initiated by interaction with another plasma component. Heart ferritin labelled with ^{125}I – and to a lesser extent spleen ferritin – interacted with serum to form a complex which precipitated with polyethylene glycol whereas purified plasma ferritin labelled in the same way did not form a precipitate [36]. It is not known how such findings relate to the demonstration of ferritin receptors on liver parenchymal cells [37]. It is possible that many isoferritins are released into the plasma but the only ones which accumulate are L_{24} molecules and glycosylated molecules which are also rich in L subunits and contain little iron. It should be noted that clearance studies with labelled purified ferritins described above have not confirmed estimates of plasma ferritin turnover derived from measurements of plasma ferritin during exchange transfusions. In these studies plasma ferritin half-lives ranging from 3–95 min have been estimated [38, 39].

Clinical use of the serum ferritin assay

Many reports have confirmed the first demonstration of a relationship between serum ferritin concentration and iron storage levels (for a review see ref. 40). In adults serum ferritin concentrations are usually between 15 and 300 μg/l. Mean values are lower in women than in men reflecting their lower iron stores caused by the menstruation and childbirth. The changes during development also reflect changes in storage iron levels. The low levels of ferritin and iron storage in children should be noted. The relationship between level of storage iron and serum ferritin concentration has been shown directly by quantitative phlebotomy and examination of stainable iron in bone marrow and indirectly by the inverse relationship between serum ferritin concentration and iron absorption from a dose of radioactive iron. In patients with simple iron deficiency anaemia serum ferritin concentrations are almost always less than 15 μg/l. In patients with homozygous idiopathic haemochromatosis serum ferritin concentrations are usually in the range of 500–5000 μg/l. However, concentrations may be in the normal range in patients presenting early in the course of the disease. When screening for idiopathic haemochromatosis it is important to measure serum iron concentration and total iron binding capacity, as a rise in the percentage saturation of transferrin is one of the earliest signs

of the disease and usually occurs before a significant rise in serum ferritin concentration [41].

In patients who have received multiple blood transfusions and have iron overload, ferritin concentrations are high [42–44]. Cazzola et al. [43] found that much of the iron was deposited in parenchymal tissues and concluded that most of the serum ferritin entered the circulation by leakage from the cyosol of iron-loaded liver parenchymal cells. In ascorbic acid replete patients, there was an excellent relationship between the number of units of blood received, or liver iron concentration, and the serum ferritin concentration. In patients deficient in ascorbic acid, the relationships were much weaker [44]. In guinea-pigs ascorbic acid deficiency reduces both serum iron and ferritin concentrations [45].

Many hospital patients are anaemic but the anaemia is usually secondary to another disease process, for example infection, inflammation or malignancy. Such patients usually have a low serum iron concentration but the total iron binding capacity will be either depressed or normal. The serum ferritin should increase with breakdown of haemoglobin and increased iron stores, but a low serum ferritin concentration is a valuable indicator of depleted iron stores. Patients with an absence of stainable iron in the bone marrow may have serum ferritin concentrations may be up to 100 μg/l instead of < 15 μg/l. This may be because of the difficulty of assessing stainable iron in the bone marrow, the presence of storage iron elsewhere in the body and the direct response of apoferritin synthesis to inflammation or infection, which appears to make it one of the 'acute phase response' proteins [46]. In practice ferritin concentration of < 100 μg/l in anaemic patients with chronic disease should be taken to indicate depleted iron stores (there may be insufficient storage iron for haemoglobin regeneration). Despite these limitations serum ferritin has proved to be a useful way of assessing iron stores in hospital patients. In patients with renal failure undergoing treatment by dialysis, the serum ferritin assay alerted physicians to the danger of inducing iron overload by over-use of oral and parenteral iron therapy to compensate for iron losses during the dialysis procedure.

There are, however, other important causes of ferritinaemia, liver damage being the best understood [33]. The liver normally contains much of the body ferritin so that cellular damage causes ferritinaemia. If liver damage is suspected, the only safe interpretation of a serum ferritin concentration is that a concentration in the normal range indicates that iron stores are not raised [47]. Before an elevated concentration can be ascribed to iron overload, consideration of liver function tests, serum iron concentration or, finally, liver biopsy may be needed.

Serum ferritin and cancer

High concentrations of serum ferritin are seen in most patients with pancreatic carcinoma, lung cancer, hepatoma and neuroblastoma, although in many cases of cancer of the gastrointestinal tract, serum ferritin concentrations are within the normal range. Although serum ferritin concentrations are normally

elevated in patients with breast cancer with metastatic disease, the assay has proved to be of little value in predicting the development of metastatic disease. Patients with untreated acute leukaemia generally have elevated serum ferritin concentrations but concentrations are lower in patients with chronic leukaemia. In Hodgkin's disease ferritin concentrations increase from stages 1 to 4 but are not related to the histological type.

The idea that tumours may produce high concentrations of acidic isoferritins or possibly abnormal ferritins has not been supported in practice. The conflicting reports resulting from the application of a number of assays for isoferritins rich in H subunits have recently been reviewed [48]. The high serum ferritin concentrations in malignancy may therefore be due to the associated anaemia with accumulation of iron in cells of the phagocytic system, to tissue necrosis, causing direct release of cytosolic ferritin and to changes in liver function causing changes in the rate of ferritin clearance. The effects of therapy must also be remembered.

Use of the serum ferritin assay

The use of the serum ferritin assay in the hospital laboratory or in population surveys requires an understanding of both iron metabolism and of the other factors such as liver damage or inflammation which may influence serum ferritin concentrations. The assay has proved to be valuable for studying the incidence of iron deficiency at least in North America and in European countries [49] but malnutrition and chronic disease limit its value in many parts of the Third World. It is important to use a well standardized method and to employ the international standard preparation 80/602 for calibrating the method. The variation in serum ferritin concentration with age means that controls should be matched for both age and sex. Perhaps the most useful studies are "intervention" studies in which serum ferritin concentrations are measured before and after a trial of iron therapy.

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Haematologia 20 (4), pp. 237—246 (1987)

Iron and Infection

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Introduction

The outcome of any infectious process depends upon the ability of the invading microorganisms to establish themselves and proliferate within the host, and on the ability of the host's innate and acquired defence mechanisms to eliminate the organisms. Any factor which can influence either of these processes could therefore tip the balance in favour of infection rather than recovery, or vice versa.

Iron can play a role in both these mechanisms. On the one hand, iron is essential for the growth of almost all bacteria, but sequestration of this metal by various host iron-binding proteins makes it relatively unavailable to invading microorganisms. The ability of microorganisms to overcome the obstacle and changes in the efficacy of the host iron-binding mechanisms can therefore affect bacterial growth. However, iron is also necessary for some of the events that take place during immune and inflammatory responses, and these may be compromised if iron status is abnormal. In this article the dual role played by iron in infectious disease will be discussed, with particular reference to the effect of abnormal iron status.

Iron and Microbial Growth

This section will, in effect, deal with bacterial growth. Viral replication is dependent upon the metabolism of the host cell, and therefore does not have any independent requirement for iron acquisition. On the other hand the iron requirements of parasites, such as trypanosomes, schistosomes and plasmodia are only just beginning to be studied. There is, however, some evidence that iron is important for growth of plasmodia and trypanosomes [1, 2].

Bacteria entering host tissues are faced with the problem of acquiring iron, which is bound either to haem compounds (mainly haemoglobin), or to the iron storage and transport proteins ferritin and transferrin. It should be emphasised that rarely is the problem one of *quantity;* it is the lack of *availability* of iron that potentially restricts microbial growth.

The best studied mechanism of microbial iron acquisition under such circumstances is that of siderophore production. Siderophores are low molecular weight iron chelators of high affinity [3], which in many cases are capable, at

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least in theory, of removing iron from transferrin and ferritin, or perhaps more importantly, of competing for any iron that may become available during normal iron recirculation. Most siderophores are derivatives of catechol or hydroxamic acid. Enterochelin, which is a cyclic trimer of 2,3-dihydroxybenzoyl serine, is produced by many enterobacteriaceae, and is one of the best studied members of the former group. Desferrioxamine, which is produced by streptomyces and is used clinically for iron chelation therapy, is a member of the latter group.

Synthesis of siderophores by microorganisms is normally not constitutive, but induced by the absence of readily available iron. Outer membrane siderophore receptors for the iron-siderophore complex are induced simultaneously, and it is through these receptors that the microorganism acquires the siderophore-bound iron [4]. Some siderophores are recycled by the bacteria, but others are broken down and need to be resynthesised [5].

It is evident, therefore, that the ability of a microorganism to produce siderophores will contribute towards it ability to gain a foothold within the host and, if it is an organism capable of causing damage, increase its pathogenicity. It is known that certain plasmids associated with increased virulence encode the components of siderophore-mediated iron uptake, such as the aerobactin system in invasive strains of *E. coli* [6, 7]. Furthermore, there is now good evidence that bacteria growing in vivo produce siderophores: enterochelin has been isolated from the peritoneum of guinea pigs lethally infected with *E. coli* [8], and *Pseudomonas aeruginosa* organisms recovered without subculture from the lungs of patients with cystic fibrosis possess siderophore receptors [9].

It is generally assumed that the source of iron scavenged by siderophores is transferrin, as this represents the main pool of extracellular iron. However, when desferrioxamine is used for chelation therapy it acquires iron mainly from the liver [10], suggesting that intracellular iron is acquired, rather than transferrin-bound iron. Possibly the chelator competes with transferrin for iron liberated by the cells during normal iron exchange. Further studies are required in this area.

Some bacteria, notably *Neisseriae*, appear to acquire iron by a mechanism which does not involve siderophores, but instead requires a direct interaction of proteins of the transferrin class with the bacterial surface [11, 12]. Full details of the nature of this interaction have not yet been reported, but it seems possible that a type of microbial transferrin receptor might be involved.

While current research has focussed on the microbial iron acquisition mechanisms described above, in which induction by lack of available iron is an important feature, it should be remembered that there are additional methods of microbial iron acquisition. Some bacteria can utilise haem iron [13], and their growth can be increased as a result of haemolysis. Finally, damage to host cells by invading microorganisms may result in the liberation of intracellular compounds whose iron is more readily available.

Iron and the ImmuneSystem

While innate defence mechanisms such as iron sequestration by host iron-binding proteins provide an important first line of resistance, the resolution of an established infection will require the participation of the immune system. The immune response is a complex process, involving activation and proliferation of lymphocytes, synthesis of soluble proteins such as lymphokines and antibodies, and the recruitment and activation of phagocytic and cytotoxic cells. The increased metabolic activity involved, and the requirement for iron as a co-factor for many enzymes suggests that iron may also play a role in the immune response. Research over the past decade has highlighted two areas in which iron seems to be particularly important, namely lymphocyte proliferation and phagocytic activity.

It is now well established that all proliferating cells need to acquire iron [14]. In particular, the enzyme ribonucleotide reductase which is involved in DNA synthesis is iron dependent, and if cells are deprived of iron DNA synthesis is impaired [15]. To obtain iron, cells express receptors for transferrin, which bind Fe-transferrin molecules and are internalised by receptor-mediated endocytosis. The iron is released intracellularly, and the receptor-apotransferrin complex returned to the cell membrane, from which the apotransferrin then dissociates [16].

Studies with mitogen-stimulated lymphocytes have shown that this mechanism of iron acquisition operates when lymphocytes proliferate [17, 18]. If the cells are cultured in serum-free (and hence transferrin-free) medium, little proliferation occurs unless Fe-transferrin is added [19]. Little proliferation occurs when apotransferrin is added if care is taken to exclude iron from the culture system. On the other hand, iron salts and hydrophilic iron chelates such as nitrilotriacetate and citrate are ineffective at promoting lymphocyte proliferation [19], although lipophilic iron chelators are able to replace transferrin [20]. It is now known that in T-lymphocytes the expression of transferrin receptors, and subsequent uptake of iron, is dependent upon prior binding of interleukin-2 (IL-2) to the Tac antigen (IL-2 receptor) [21], an event which leads to activation of the transferrin receptor gene [22]. However, it has recently been reported that desferrioxamine blocks Tac expression, which suggests that iron may also be needed at an earlier stage of the cell cycle [23]. An inadequate supply of transferrin-bound iron may therefore impair the important step in the immune response of lymphocyte proliferation. In iron-deficient mice, it has been shown that the level of transferrin-bound iron does indeed become inadequate to support optimal proliferation, even though the lymphocytes themselves are potentially able to respond normally [24].

Lymphocyte proliferation in vitro may also be reduced when the transferrin saturation approaches 100% [19], suggesting that excessive iron uptake by lymphocytes may be toxic. In consequence, this stage of the immune response might also be impaired by iron overload.

The activity of phagocytic cells is also closely linked with iron, and once again both inadequate and excessive amounts of the metal can be deleterious. There is evidence that the respiratory burst which occurs when phagocytic cells experience a membrane triggering event, for example during phagocytosis, is iron dependent. In clinical or experimental iron deficiency, the ability of neutrophils to reduce nitro-blue tetrazolium (NBT) is lowered [25, 26], indicating an impairment of superoxide production. In macrophages, hydrogen peroxide production is impaired if the cells are rendered iron-deficient in vitro by incubation with desferrioxamine [27]. The basis of these observations may be the fact that cytochrome b_{559} and NADPH oxidase, which are both involved in superoxide production, contain iron [28, 29]. Furthermore, iron is required for the catalytic production of hydroxyl radicals from hydrogen peroxide and superoxide [30, 31]. Hydroxyl radicals are thought to be the most potently bactericidal of the reactive oxygen species produced by phagocytic cells. However, the source of this catalytic iron within the cell is not clear. It has been proposed that in neutrophils iron bound to lactoferrin could fulfill this role [32], but recent evidence indicates that lactoferrin-bound iron is not catalytic [33].

Excessive iron can inhibit phagocyte activity in two ways. Firstly, exposure of neutrophils to iron salts, or to iron chelates formed at low chelate : iron ratios results in a lowered ability of the cells to ingest bacteria, apparently because of iron-induced lipid peroxidation of the phagocyte membrane [34]. Secondly, excessive uptake of iron by neutrophils may saturate lactoferrin, which is present in the secondary granules, with iron. The contribution of lactoferrin to the intracellular armoury of antimicrobial activities is thus lost, and killing of ingested bacteria is consequently impaired [35].

Iron Status, Immunity, and Susceptibility to Infection

In the preceding sections the various mechanisms by which iron is capable, at least in theory, of influencing infection and immunity have been reviewed. It must therefore be asked whether individuals whose iron status is abnormal show decreased immune responsiveness and/or increased susceptibility to infection.

Iron deficiency. Clinical studies of the effect of iron deficiency on infection and immunity are notoriously difficult to carry out due to the problems of finding adequate controls, and ensuring the absence of additional nutritional deficiencies, preexisting infections, etc. Nevertheless, some patterns can be established. A number of studies, reviewed in [36], indicate that cell-mediated immunity, in particular, seems to be depressed in iron deficiency, as assessed by lymphocyte proliferative responses and skin tests. Lymphocyte transfer from primed iron deficient donors to normal recipients, and vice versa, have shown that the defect is at the level of effector mechanisms rather than sensitisation [37, 38], a conclusion which is in accord with the crucial role of iron in lymphocyte proliferation. How-

ever, mitochondrial abnormalities in lymphocytes from iron-deficient patients have been reported [39, 40]. Possibly these intrinsic defects become apparent when iron deficiency is prolonged. There is little information on whether iron deficiency preferentially impairs any particular T-cell subsets, although it has been reported that the proportion of Ly1+ and Ly2+ subsets in mouse lymph nodes was unaffected by iron deficiency [24]. Further work in this area is desirable.

There is less evidence for impairment of humoral immunity in iron deficiency, the majority of clinical studies reporting normal immunoblobulin levels or antibody responses to vaccination [36]. These observations are in accord with in vitro studies showing that antibody production by B-lymphocytes can occur even if transferrin binding is blocked by an anti-receptor monoclonal antibody [41], and that proliferation of B cells is less susceptible to inhibition by desferrioxamine than is that of T-cells [42]. However, in animal studies in which severe iron deficiency was induced, lowered antibody levels or numbers of IgG and IgM plaque-forming cells were found [43–45].

Clinical studies of phagocyte (normally neutrophil) function in iron deficiency suggest that reduced intracellular killing of bacteria may occur but that ingestion is normally unaffected [46–48]. Reduction in the production of reactive oxygen species, as discussed above, or in the activity of the peroxidase-halide-H_2O_2 bactericidal system, may be responsible.

Despite these clear indications that iron status can adversely affect the immune system, evidence that this leads to increased susceptibility to infection is limited. Chronic mucocutaneous candidiasis is associated with iron deficiency, and is alleviated if iron status is improved [49, 50]. There is also evidence that iron-deficient infants show increased susceptibility to a variety of infections [51]. However, the lack of any strong evidence linking tuberculosis with iron deficiency would suggest that defective cell-mediated immunity associated with iron deficiency may not in itself be sufficient to cause increased susceptibility to infection and that other predisposing factors are involved.

It is theoretically possible that iron deficiency might actually be protective as a result of making iron less available to invading microorganisms. Some evidence for such an effect has been provided in experimental systems [52, 53]. However these workers used mice, in which transferrin saturation is normally about 80%, and drops to about 10% in iron deficiency [24]. In man the drop is much less pronounced, as normal saturation is only about 30%, and there is no good evidence that iron deficiency is protective. One would suspect that any marginal effect on bacterial growth would be more than outweighed by impairment of immune and phagocytic function.

Iron Overload. Increased susceptibility to infection has been reported in patients suffering from idiopathic haemochromatosis, or from transfusional iron overload in thalassaemia. Much of the evidence has been summarised by Weinberg [54], and there appear to be two mechanisms that cause predisposition to infection. Firstly, serum transferrin in these patients is often fully saturated with iron. As a result an abnormal serum iron fraction appears which consists of iron non-speci-

fically bound to serum proteins other than (or perharps including) transferrin [55, 56]. This iron is more accessible to bacteria than is transferrin-bound iron, as is shown by the large number of reports, summarised in [54], demonstrating that the in vitro bacteriostatic effect of serum is lost when sufficient iron is added to saturate the transferrin present. The other factor that commonly predisposes to infection is liver damage, which can increase the risk of infection even in patients whose serum transferrin saturation is $< 100\%$. Release of intracellular iron compounds from damaged cells may again provide additional sources of iron to bacteria. However, impairment of liver phagocyte activity may also be important, and it has been shown that monocytes from patients with haemochromatosis have impaired bactericidal activity [57], as also do neutrophils from iron-overloaded patients on haemodialysis [58].

Depression of specific immune responses in iron overload is less well documented than in iron deficiency, although the poor in vitro response of lymphocytes to mitogens in the presence of highly-saturated transferrin would suggest that impairment may occur. In inflammatory disease local accumulation of iron occurs and may cause damaging free-radical reactions [59], which may affect the immune system, as free radicals appear to be more toxic to T-lymphocytes than to B-lymphocytes [60].

Changes in Iron Metabolism Associated with Inflammation

So far this review has discussed how changes in host iron status may affect susceptibility to infection. However, infectious or inflammatory disease can itself cause changes to occur in the pattern of iron recirculation. Acute inflammation causes a rapid drop in serum iron [61], an event which can be brought about experimentally by the injection of endotoxin [62]. This is due to a reduction in the amount of iron released from the reticuloendothelial system to the circulation. If the inflammatory process is prolonged, there is a significant increase in reticuloendothelial iron, and anaemia (the so-called anaemia of chronic disease) may develop [61]. In local inflammatory conditions such as rheumatoid arthritis, accumulation of iron occurs in the vicinity of the joint [59].

The underlying mechanisms causing these changes are imperfectly known. The hypoferraemic response is caused by a soluble factor released from phagocytic cells and known previously as leucocyte endogenous mediator (LEM) [63], which is now thought to be identical with interleukin-1 (IL-1) [64], although rigorous proof using recombinant material has yet to be provided. The most popular theory proposes that LEM/IL-1 acts directly upon cells of the reticuloendothelial system, causing them to release less iron than normal [65]. In vitro studies showing that inflammatory macrophages release less iron than resident macrophages support this theory [66, 67]. However, it has also been proposed that the primary effect is to decrease erythropoiesis, and that reduced liberation of iron from reticuloendothelial cells is simply a consequence of reduced erythropoietic needs [68].

The physiological role of these changes is, like the mechanisms involved, unclear. It is often suggested that hypoferraemia enhances the antimicrobial activity of transferrin, and thus impedes the infectious process [54]. Although there is much circumstantial evidence in favour of this theory, it is often not possible to exclude the intervention of other protective effects of inflammation. Other studies have involved introduction of exogenous iron, either in vivo or in vitro, to nullify the hypoferraemic response, a procedure which is liable to give rise to artefacts such as the transient appearance of non-transferrin-bound iron. In any case, it is difficult from a biochemical standpoint to see how reduction of transferrin saturation from a normal level (in man) of 30% to around 15% greatly alters the availability to bacteria of transferrin-bound iron; even at the lower figure there is still an adequate amount of the metal potentially available to the bacteria. It seems more plausible that decreased levels of metabolically-available iron within cells, or decreased release of iron from reticuloendothelial cells is of more significance in relation to reduced availability to microbial iron-scavenging systems.

Conclusion

Evolution has ensured that under normal conditions, man possesses sufficient iron to allow metabolic activity, including immunological functions, to proceed optimally, but at the same time prevents the metal being readily acquired by invading microorganisms. Deviations in iron status either side of normal are potentially harmful; iron deficiency may impair the immune system without significantly improving the already highly effective antimicrobial properties of host iron-binding proteins, while in severe iron overload, the latter system may be impaired, and at the same time excessive iron may damage phagocytic and lymphoid cells. Changes in iron metabolism associated with inflammation may play a role in combatting infection, but proof is lacking. While it would be an exaggeration to say that abnormal iron status is a prime cause of infection, it seems likely that it can be a predisposing factor.

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Haematologia 20 (4), pp. 247—252 (1987)

Iron Loading and Chelation as Studied in a Heart Cell Culture System

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Iron is one of the most common substances in nature and yet, iron deficiency is the most frequent cause of anemia in man. This apparent contradiction is explained by the very low solubility of inorganic iron. In order to provide sufficient iron for their metabolic needs, living organisms have developed efficient mechanisms for the transfer and storage of iron. However, evolution did not provide us with protective mechanisms against iron excess. Hemochromatosis, the final clinical expression of abnormal iron accumulation is associated with serious damage to a number of organs, the most critical of these being the functional derangement of the myocard. This is the reason that in recent years we have concentrated on the toxic effects of iron on myocardial cells, and the possible reversibility of such toxicity by iron chelating therapy.

In the following, I would like to focus on two aspects of iron toxicity: (a) the mechanism of impaired myocyte contractility, and (b) the modification of iron toxicity by ascorbic acid and alpha-tocopherol.

In a previous study [1], we have shown that cultured, beating myocardial cells obtained from newborn rats can be used as a model of iron toxicity; that the uptake of large amounts of iron provided in the form of ferric ammonium citrate in serum-free culture medium is followed by a sharp increase in cellular malonyldialdehyde (MDA) concentrations indicating increased lipid peroxidation; that most of the iron deposited in these cells may be removed within 24 hours by in vitro treatment with deferoxamine (DF), and; that the removal of iron is followed by normalization of cellular MDA concentrations.

The purpose of the first part of the present studies was to characterize the effect of in vitro iron loading on the contractility and spontaneous beating rate of cultured rat myocardial cells and to evaluate the reversibility of these effects by washout, and by various pharmacological interventions employing calcium, adrenaline, ouabain and caffeine.

Measurements of contractility: Changes in the contractile state of individual cells in the monolayer were assessed by the use of an optical-video system. A glass coverslip with attached cell monolayer was continuously perfused in a chamber

Supported by Grant No. HL 34062-01A1 of the National Heart, Lung and Blood Institute and Grant No. 032-4133 of the Israel Academy of Sciences and Humanities awarded to C. H.

VNU Science Press, Utrecht
Akadémiai Kiadó, Budapest

provided with inlet and exit ports. The chamber was placed on the stage of an inverted phase contrast microscope (Leitz Diavert), and constant temperature and pH were maintained. The cells were magnified by a 40X objective and the image was monitored by a low light level TV camera connected to a videomotion detector. The composition of the various solutions used during these experiments is given in Table 1.

Table 1

Solutions employed

Culture medium:	HAM F-10 (Biomedical Industries, Kibbutz Beth-Haemek, Israel) with $CaCl_2 \cdot 2H_2O$ 135 mg/L, $NaHCO_3$ 1.2 g/L, penicillin 200,000 g/L, Streptomycin 0.2 mg/L, 10% horse serum (Sera Lab., Sussex, England), and 10% fetal calf serum (FSC Gibco, Grand Island, New York)
Solution H:	Ca^{++} and Mg^{++}-free HAM F-10 medium
Control solution:	Culture medium with 1% FCS only
Iron solution:	20 μg ferric ammonium citrate (FeAC) per ml of control solution
Citrate solution:	350 μmol sodium citrate in control solution
Adrenaline:	10^{-6} M adrenaline in iron solution
Ouabain:	10^{-7} M ouabain in iron solution
Caffeine:	20 mM caffeine in iron solution
Calcium:	7.2 mM Ca^{++} in iron solution

Control experiments: The stability of the system was documented by the absence of significant changes in the ACM or spontaneous beating rate during superfusion of the cells with control solution for 75 minutes, or following exposure to 350 μmol citrate for 60 min. *Effect of iron:* There was a gradual reduction in the amplitude of cell contractions during the first 45 – 50 minutes of continuous exposure to iron solution. Later, the cell motion stabilized at a new steady state. There was also a decrease in the rate of spontaneous beating. This change developed earlier, after 20 minutes of exposure to the iron solution. We assume that the negative chronotropic effect of iron indicates membrane dysfunction since the spontaneous beating rate in the myocytes is a membrane function and the development of the negative chronotropic effect with a new steady state at 20 minutes correlates well with the kinetics of both iron uptake and increased MDA concentrations which were shown to be doubled by that time in our previous study. *Reversibility of iron effect:* The negative inotropic and chronotropic effects induced by iron were reversible. After 30 minutes of superfusion of iron-loaded myocytes with control solution, the AMC and spontaneous beating rate recovered completely. We next wished to examine whether agents with a known positive inotropic effect may reverse the depressant effect of iron on cultured heart cells. After the development of steady state iron effect, the cells were exposed to various pharmacologic agents in the continued presence of the iron solution. Exposure to [Ca^{2+}], adrenaline and ouabain in the presence of iron showed only a transient, non-significant tendency to reverse the iron effect. Only caffeine was able to

completely reverse the negative inotropic effect of iron. In contrast to caffeine, $[Ca^{2+}]$, and ouabain were unable to improve the rate of beats, and only adrenaline showed a positive inotropic effect. The observed dissociative effect of adrenaline on contractility and spontaneous beating rate suggests that the negative chronotropic effect induced by iron is readily overcome by beta-stimulation, but this is not sufficient to correct the impaired contractility.

The ability of caffeine to overcome the negative inotropic effect of iron may be explained by a number of possible mechanisms:

(a) Caffeine inhibits phosphodiesterase and increases the level of cyclic AMP which, in turn, activates sarcolemmal $[Ca^{2+}]$ channels.
(b) It attenuates $[Ca^{2+}]$ reuptake and promotes $[Ca^{2+}]$ release from the sarcoplasmic reticulum.
(c) It increases the sensitivity of myofilaments to $[Ca^{2+}]$ [2–5].

In view of these alternative effects of caffeine, is it still possible to identify a more specific mechanism of reduced cellular contraction in iron toxicity? In the following the various arguments for a sarcolemmal vs intracellular mechanism are summarized (Fig. 1): The late timing of reduced contractility favours an intracellular effect. Similarly, the failure of both adrenaline and ouabain, both of which are active at the sarcolemmal level, to improve contractility, support an intracellular effect of iron. Finally, the fact that cultured neonatal heart cells lack a well developed SR system implies that reduced sensitivity of myofilaments to Ca is the most likely mechanism of reduced myocyte contractility in acute iron toxicity. Obviously further studies are required to verify such a hypothesis.

What are the implications of these in vitro observations to the clinical effects of acute or chronic iron overload in man? In vivo conditions are very different from those of the artificial in vitro system employed in the present studies. Nevertheless, it may be assumed, that the basic mechanism of iron toxicity involving free radical formation and membrane lipid peroxidation is probably

	Sarcolemmal membrane	Intracellular effect
Late timing	no	yes
Failure of (a) adrenaline (b) ouabain	no	yes
Poor development of *SR* in neonatal myocytes		Reduced sensitivity of *myofilaments* to $[Ca^{2+}]$

Fig. 1 Tentative mechanism of reduced contractility

similar at all conditions of iron excess. Our in vitro model of iron toxicity in cultured myocardial cells is the only experimental model available at present in which the correlation between the biochemical and functional effects of iron toxicity may be studied. A unique advantage of such a model is the ability to control the extracellular environment throughout such studies.

The objective of the second part of our studies was to explore the capacity of ascorbic acid, alpha-tocopherol and hypoxia to modify iron toxicity as manifested in the generation of MDA by iron loaded cultured rat myocardial cells.

Iron in cell cultures was supplied as ferric ammonium citrate. Iron uptake was rapid within the first few hours, and tended to level off after 24 h. With increasing concentrations of iron, increasing amounts of iron were deposited. The highest % uptake was observed at 20 μg/ml, and this concentration was used in all subsequent studies.

The toxic effects of iron are based on its role in the conversion of superoxide into the active free hydroxyl radicals. Ferric iron is converted by superoxide into ferrous iron, which in turn catalyses the conversion of H_2O_2 into the hydroxyl radical in the Haber–Weiss reaction. This reaction is accelerated by ascorbic acid, a reducing agent. Hydroxyl radicals initiate a chain reaction of lipid peroxidation and membrane injury terminating in the production of malonyl dialdehyde. Alpha-tocopherol (vitamin E) is an antioxidant capable of inhibiting this reaction [6].

Our interest in the effect of hypoxia on iron uptake and lipid peroxidation arose from the unique observation of Necheles et al. in congenitally anemic mice [7] indicating that coexistent iron loading and hypoxia resulted in increased rates of myocardial iron accumulation. Our in vitro studies are entirely in line with these observations. Coexistent iron loading and hypoxia resulted in a 2 to 3-fold increase in myocardial iron uptake in cultured cells. This increased uptake, however, did not cause a corresponding enhancement of MDA production. We assume that the failure to increase MDA production is probably the result of a temporary reduction in free hydroxyl radical formation under hypoxic conditions.

In the following studies we examined the effects of ascorbic acid and alpha-tocopherol on the rate of cellular iron uptake and MDA production. Previous studies by Wills and O'Connell in liposomes and in a microsomal system have shown that both ascorbic acid and alpha-tocopherol may modify iron-induced lipid peroxidation [8]. Both compounds were used at a concentration of 1 mg/ml added at time 0, simultaneously with the addition of radioiron-labelled ferric ammonium citrate. Ascorbic acid and alpha-tocopherol had opposing effects on iron uptake and on MDA production. Thus, the addition of ascorbate resulted in a significant reduction in iron uptake. This effect became more pronounced with time, so that by 24 hours iron uptake with ascorbate was only 27% of the control culture of iron loaded cells. In contrast, the addition of alpha-tocopherol resulted in a small but significant increment in iron uptake.

The effect of ascorbate and alpha-tocopherol on cellular MDA concentrations was the opposite of their effect on iron uptake. Thus, despite the reduced iron

uptake observed in the presence of ascorbate, there was a more than twofold increase in cellular MDA. Likewise, in spite of the moderate increase in iron uptake observed with alpha-tocopherol, cellular MDA concentrations were markedly reduced between 1 and 24 hours of incubation. Comparison of MDA measurements following ascorbate or alpha tocopherol with, or without iron indicated that their combined effect was clearly not additive, but synergistic enhancement or inhibition.

In order to characterize further the ability of hypoxia, ascorbate, or alpha-tocopherol to modify the effect of acute iron loading on MDA formation, the ratio of cellular MDA to per cent radioiron uptake in treated cells (iron loading and ascorbate, tocopherol or hypoxia) was divided by the corresponding ratio in iron loaded cells without additional treatment. By definition, this "lipid peroxidation ratio" was 1 in iron loaded cells without additional treatment. Hypoxia had a modest inhibitory effect on the lipid peroxidation ratio. Ascorbate and tocopherol had opposing effects increasing with time. Thus, 24 hours of combined iron-loading and ascorbate treatment resulted in a 7-fold increase in MDA as compared to iron loading only, whereas alpha-tocopherol resulted in an 8-fold reduction in the lipid peroxidation ratio – a more than 50-fold difference between the 2 extremes.

In view of in vivo evidence for the ability of ascorbic acid to enhance the iron chelating ability of desferrioxamine [9], and a possible inhibitory effect of alpha-tocopherol on in vivo iron chelation [10], we were interested in examining the ability of these two compounds to modify the effect of desferrioxamine in vitro. Neither one of these compounds had any effect on the ability of desferrioxamine to remove iron from myocardial cells. In contrast, both ascorbic acid and alpha-tocopherol had a profound effect on the ability of desferrioxamine to alter cellular MDA concentrations. Combined ascorbic acid and desferrioxamine treatment resulted in a sharp increase in MDA concentrations in contrast to the reduction in MDA observed with desferrioxamine treatment alone. Conversely, simultaneous alpha-tocopherol and desferrioxamine treatment resulted in a further reduction in cellular MDA as compared to that observed with desferrioxamine only. These observations suggest that ascorbic acid or alpha-tocopherol treatment may exert a profound modifying effect on the ability of desferrioxamine to prevent, or reverse iron-induced myocardial membrane lipid peroxidation.

One should be aware of the fundamental differences between in vitro conditions and of the significant difference in the clinical manifestations of acute, as against chronic iron toxicity. Nevertheless, these observations on the modifying effects of hypoxia, ascorbate, or alpha-tocopherol treatment on myocardial iron uptake, toxicity and chelation may be of some relevance to the management of clinical iron overload in transfusional siderosis.

The accelerated rate of myocardial iron deposition in hypoxia may be regarded as a further argument in favour of maintaining near-normal hemoglobin levels in patients with thalassemia major and other chronic iron loading anemias requiring chronic transfusion therapy. The slight increase in transfusional require-

ments needed to maintain adequate hemoglobin levels is more than balanced by the beneficial effects of correcting anemia and avoiding further organ damage.

The demonstration of increased myocardial lipid peroxidation and prevention of the beneficial effects of ascorbate therapy, underlines the controversy regarding ascorbate supplementation in thalassemia [11].

Finally, the direct demonstration of a protective effect of alpha-tocopherol against iron-induced lipid peroxidation in myocardial cells, underlines the need to explore the therapeutic potential of this compound in transfusional iron overload.

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Abstracts

Binding of quinine- and quinidine-dependent drug antibodies to platelets is mediated by the Fab domain of the immunoglobulin G and is not Fc dependent. M. E. Smith, D. M. Reid, Ch. E. Jones, J. V. Jordan, C. A. Kautz, N. R. Schulman (Clinical Hematology Branch, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA). *J. Clin. Invest. 79*, 912—917 (1987).

The antibody domain controlling reactions between platelet membranes and drug-dependent (dd) antibodies from patients with thrombocytopenia induced by cinchona alkaloids was studied using $F(ab')_2$, Fab, and Fc fragments made from purified dd-IgG. By direct binding radioimmunoassay (RIA) measurements, 20,000 to 50,000 antibody molecules bound per platelet equivalent of purified platelet membranes at apparent saturation with three different antibodies. $F(ab')_2$ and Fab fragments bound to platelet membranes drug dependently but Fc fragments did not. The ability of dd-IgG fragments to compete with intact IgG was quantitatively measured by RIA and by complement fixation. $F(ab')_2$ and Fab competed with intact IgG at an 8:1 and >50:1 molar ratio, respectively, in RIA, and at a 1.6—3:1 and 44—75:1 ratio, respectively, by complement fixation assays. Fc did not compete with IgG in either assay. The conclusion is that the Fab domain supports attachment of dd antibody to the platelet surface.

G. Gárdos

Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. M. Kuno, P. Gardner. (Department of Medicine, Stanford University School of Medicine, Stanford, California 94305, USA). *Nature 326*, 301—304 (1987).

Hydrolysis of membrane-associated phosphatidylinositol 4,5-bisphosphate (PtdIns-$(4,5)$-P_2) to water soluble inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) is a common response by many different kinds of cells to a wide variety of external stimuli. Ins $(1,4,5)P_3$ is a putative second messenger which increases intracellular Ca^{2+} by mobilizing internal Ca^{2+} stores, a hypothesis which has been substantiated by studies with chemically permeabilized cells and with isolated microsomal membrane fractions. But the possibility that $Ins(1,4,5)P_3$ could induce in intact cells an influx of external Ca^{2+} through transmembrane channels, originally hypothesized by Michell in 1975, has never been directly tested. Single-channel recordings of an $Ins(1,4,5)P_3$-activated conductance in excised patches of T-lymphocyte plasma membrane is reported. The $Ins(1,4,5)P_3$-activated transmembrane channel appears to be identical to the recently described mitogen-regulated, voltage-insensitive Ca^{2+} permeable channel involved in T-cell activation. It is suggested that $Ins(1,4,5)P_3$ acts as the second messenger mediating transmembrane Ca^{2+} influx through specific Ca^{2+}-permeable channels in mitogen-stimulated T-cell activation.

G. Gárdos

Antileukemic agent alkyllysophospholipid regulates phosphorylation of distinct proteins in HL60 and K562 cells and differentiation of HL60 cells promoted by phorbol ester. Z. Kiss, E. Deli, W. R. Vogler, J. F. Kuo (Departments of Pharmacology and Medicine, Hematology and Oncology, Emory University School of Medicine, Atlanta, GA 30322, USA). *Biochem. Biophys. Res. Comm. 142*, 661—666 (1987).

The tumor-promoting 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulated phosphorylation of several proteins in block I (including protein Ia) and protein 3 in HL60 cells. The antileukemic agent alkallysophospholipid (ALP) inhibited the TPA-stimulated phosphorylation of these proteins and the TPA-induced differentiation of the cells. In comparison, TPA only stimulated phosphorylation of protein 3 in K562 cells which, in contrast, were not induced to differenciate by TPA and lacked protein Ia and had a very high basal phosphorylation of protein B. ALP inhibited phosphorylation of protein 3 as well as protein B in K562 cells. The data suggest that the presence of distinct phosphoproteins and regulation of their phosphorylation may be related to the selective susceptibility of the two leukemia cell lines to the maturating effect of TPA and cytotoxicity of ALP.

G. Gárdos

Cell volume regulation in hemoglobin CC and AA erythrocytes. L. R. Berkowitz, E. P. Orringer (Division of Hematology, Department of Medicine, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514, USA). *Am. J. Physiol. 252*, C300—C306 (1987).

Swelling hemoglobin CC erythrocytes stimulates a ouabain-insensitive K flux that restores original cell volume. This volume regulatory pathway was characterized for its anion dependence, sensitivity to loop diuretics, and requirement for Na. The swelling-induced K flux was eliminated if intracellular chloride was replaced by nitrate and both swelling-activated K influx and efflux were partially inhibited by 1 mM furosemide or bumetanide. K influx in swollen hemoglobin CC cells was not diminished when Na in the incubation medium was replaced with choline, indicating Na independence of the swelling-induced flux. Identical experiments with hemoglobin AA cells also demonstrated a swelling-induced increase in K flux, but the magnitude and duration of this increase were considerably less than that seen with hemoglobin CC cells. The increased K flux in hemoglobin AA cells was likewise sensitive to anion replacement and to loop diuretics and did not require the presence of Na. These data indicate that a volume-activated K pathway with similar transport characteristics exists in both hemoglobin CC and AA red cells.

G. Gárdos

Putitative role for lithium in human hematopoiesis. R. D. Barr, M. Koekebakker, E. A. Brown, M. C. Falbo (Department of Pediatrics, McMaster University, Hamilton, Ontario, Canada). *J. Lab. Clin. Med. 109*, 159—163 (1987).

Ingestion of lithium salts increases production of neutrophil granulocytes from the bone marrow in human subjects when the concentration of the ion in blood is within the range 5 to 10×10^{-4} mol/l. Results of preliminary dose-response experiments appeared to indicate that nanomolar levels of lithium stimulated clonal proliferation of granulocyte precursors from normal bone marrow in vitro, suggesting the possibility that this element may contribute to the physiologic regulation of blood cell formation in humans. The present studies confirm that the influence of lithium on hematopoiesis is evident in vitro at concentrations equivalent to that demonstrable in normal blood (2 to 4×10^{-7} mol/l). Furthermore, such effects are not cell lineage specific, being observed also in clonogenic cultures of erythroid and eosinophil granulocyte progenitor cells, and the phenomenon attributed to lithium is a property shared with rubidium and cesium salts. These findings point to a role for lithium and its elemental relatives in the biophysical mechanisms involved with the control of human blood cell production.

B. Sarkadi

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