VOLUME 17 • NUMBER 1 • 1984

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

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

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24.
H-1113 Budapest
Hungary

Technical Editor:
R. J. Valenti

Distributor for

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

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

For all other countries subscriptions should be sent to

VNU SCIENCE PRESS
P.O.B. 2073
3500 GB Utrecht, The Netherlands

Subscription price for volume 17

DM 279 incl. surface mail
DM 305 incl. air mail

VOLUME 17 · NUMBER 1

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

VNU SCIENCE PRESS, UTRECHT, THE NETHERLANDS

AKADÉMIAI KIADÓ, BUDAPEST

1984

Printed in Hungary
Akadémiai Nyomda, Budapest

Haematologia 17 (1), pp. 3–16 (1984)

Physiological and Pathological Effects of Activated Complement

M. P. DIERICH,[1] TH. SCHULZ[2]

Institut für Hygiene, Universität Innsbruck,[1] Fritz-Pregl Str. 3., A-6010 Innsbruck, Austria; Institut für Medizinische Mikrobiologie, Johannes Gutenberg-Universität,[2] Augustusplatz, D-6500 Mainz, FRG

(Received 20 January 1983; accepted 18 February 1983)

In this short review of our present understanding of the complement system the emphasis is on a synopsis of the biological aspects of the various complement components, their fragments and complexes. For this reason we decided to refer to a number of reviews where certain aspects are dealt with extensively.*

Keywords: alternative pathway, classical pathway, complement, complement receptors.

*

At the turn of the century complement was thought to consist of one heat labile factor. Today we know about 20 different complement components. They belong to one of the following groups:

1. The classical pathway of C activation (CPCA): C1q, r, s, C4, C2, C3.
2. The alternative pathway of complement activation (APCA): C3b, B, D.
3. The terminal complement sequence: C5, C6, C7, C8, C9.
4. The group of enhancing or inhibiting modulators of the complement sequence: e.g. P, H, I, C1INA and others.

In Table 1 standard physico-chemical characteristics of the components are listed.

CPCA is triggered by the 'activated' Fc portion of antibodies contained in aggregates of antibodies or in immune complexes. The CH_2 domain of IgG binds C1q by its globular heads. C1q is either part of an intact C1, which consists of C1r and C1s in addition to C1q, the whole complex being held together by Ca^{++} or the bound C1q fixes C1r and C1s sequentially. Following binding, C1r is cleaved and in turn cleaves and thus activates C1s [3, 4]. The resulting esterase activates C4 and C2 yielding a complex of C4b and C2a, the C3 convertase of the classical pathway. C4b binds C3 so that it can be cleaved by C2a into C3b and C3a. If C3a is deposited sufficiently close to C2a and C4b, the three components together form a complex, the C5 convertase, with C2a as the enzymatic centre. C5 is cleaved into C5a and C5b. It should be mentioned that the classical pathway can be started

* For the nomenclature of the components and their reaction products we follow the WHO recommendation [1, 2].

Table 1

Physico-chemical characteristics of factors of the complement system

	MW	Chains	Serum concentration µg/ml	Electrophorectic mobility pH 8.6
C1q	410 000	18	150	γ2
C1r	83 000	1	50	β
C1s	83 000	1	50	β1
C4	206 000	3	640	β1
C2	117 000	1	25	β1
C3	190 000	2	1200–1500	β1
C5	180 000	2	75	β1
C6	128 000	1	64	β2
C7	121 000	1	54	β2
C8	154 000	3	54	γ1
C9	79 000	1	58	α
B	93 000	1	240	β2
D	24 000	1	2	α
P	220 000	4	20	γ
H	150 000	1	450	β
J	93 000	2	35	β
C1 INA	105 000	1	240	α
S-protein	71 000	1	500	—

also by a direct interaction of certain membrane proteins of murine leukaemia viruses with C1q and C1s in the absence of antibody [5, 6]. The resulting C5b is the nucleus for the formation of the C5b-9 complex.

APCA is activated by bacteria, their lipopolysaccharides, dextransulfate and appropriate surfaces [7–9]. From C3 and factor B the complex C3b, B is derived. The mechanism which leads to the 'first' C3b in the 'first' C3b, B complex could not be explained until recently; the explanation will be discussed below. B is cleaved into Bb and Ba by factor D in a reaction depending on Mg^{++}. C3b, Bb, the alternative pathway C3 convertase is capable of splitting C3 into C3a and C3b, the latter being deposited on the activating surface close to the C3b, Bb complex. This gives rise to C3b, Bb, C3b formations which cleave C5 and thus trigger the terminal complement sequence. Like C2a in the convertase of CPCA, Bb functions as the enzymatic centre of the convertase built up by APCA. There exist striking biochemical similarities between C2a and Bb [10]. In addition both are coded for by genes of the major histocompatibility complex (MHC) [11, 12]. Apart from the genes for C2 and B only the genes for C4 are located in the MHC, while genes for the other components are found on other chromosomes [11, 12].

Activation of C3 by cleavage into C3a and the large fragment C3b can be brought about not only by C4b, C2a, the classical pathway C3 convertase, or C3b, Bb, the alternative pathway C3 convertase, but also by proteases like elas-

tase secreted by phagocytes [13–15]. C3 functions as a central mediator since the various activation pathways converge at this component (Fig. 1).

For years it was difficult to understand how C3b could be a cleavage product of C3 and at the same time be involved in generating the cleaving enzyme. Very recently a satisfactory answer has been given to the question from where the 'first' C3b originated. First, it was demonstrated that nascent C3b binds covalently to the cell surfaces [16] by means of an ester bond [17]. This bond is formed between

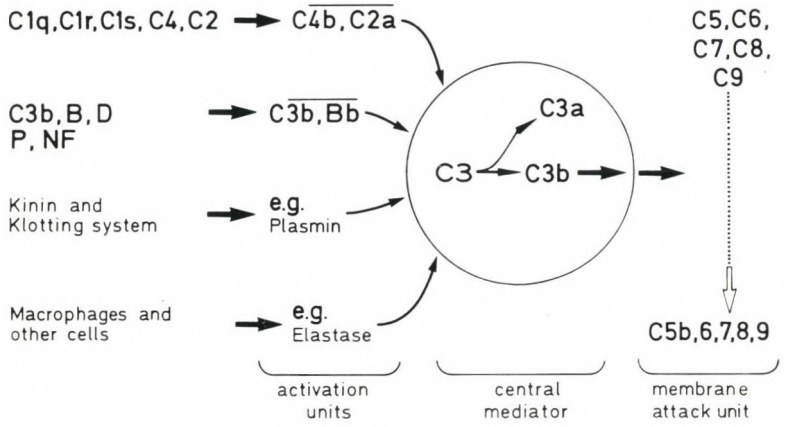


Fig. 1. Activation pathways of the complement system

the C3d part of the molecule and the cell surface [18]. Next it was shown that intact C3 contains in its α-chain an internal thioester bond [19–23]. Upon cleavage of C3 into C3b and the small fragment C3a, this thioester is exposed and becomes highly reactive to the interaction with OH-groups on cell surfaces or in the fluid phase. As a consequence C3b becomes covalently linked to the cell surface by transesterification (Fig. 2). The transitional state during which the exposed thioester is capable of reacting with OH groups on cell surfaces is of short duration. In the absence of accessible cell surfaces the exposed thioester will be hydrolysed by H_2O molecules and C3b will stay in the fluid phase [23, 24]. This thioester may, however, also react in the intact C3 with nucleophilic reagents such as primary amines or more importantly with H_2O [7, 21, 24–26] and from these reactions a 'C3b-like' C3 results [7, 24]. The 'C3b-like' C3 shares with C3b the capacity to react with factor B and to initiate the APCA [7, 23, 24]. Therefore in all likelihood 'C3b-like' C3 in plasma continuously supplies the 'first C3b' giving rise to the "initial" C3 convertase of the APCA.

Whether or not this initial trigger is successfully amplified on a surface depends largely on the special characteristics of the surface [27–32]. Surfaces carrying few neuraminic acid molecules allow the anchoring of metastable C3b in such a fashion that factor B is bound effectively and an AP convertase is generated

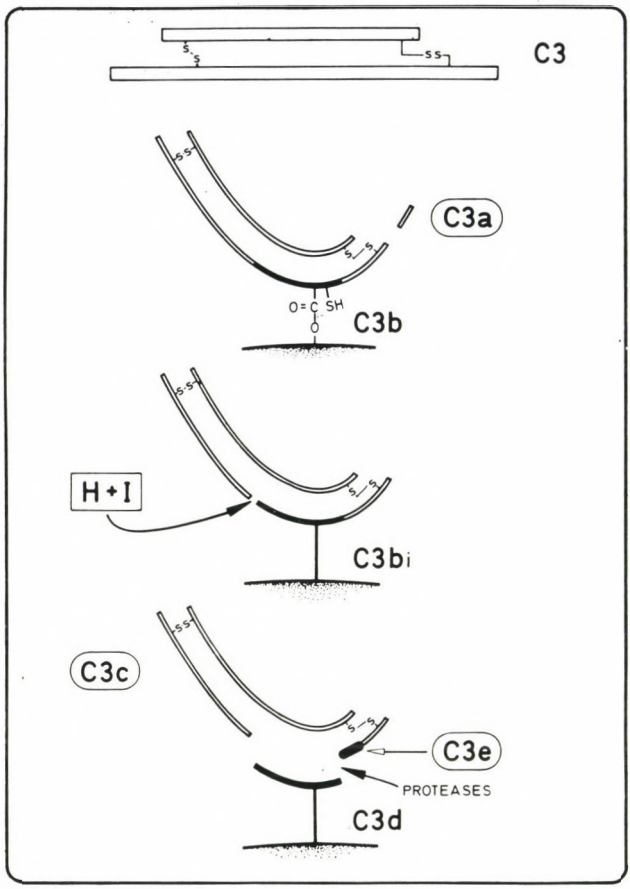


Fig. 2. Schematic drawing of C3, its binding to a cell surface and its processing. α chain = 110 000 dalton, β chain = 75 000 dalton. Upon cleavage of C3 into C3a and C3b, C3b can bind covalently to OH groups on cell surfaces. Cell-bound C3b is modified by factor H and I, the C3b inactivator, to C3bi. By the action of other proteases C3c, containing C3e, is cleaved off and C3d remains bound to the surface

on the surface continuing this activation process (Fig. 3). Although surfaces with a dense neuraminic acid stacking allow binding of C3b to surface OH groups, too, they also facilitate the binding of factor H to C3b so that the latter can be cleaved by factor I at certain sites in the α-chain [33]. The resulting iC3b is unable to bind factor B or to assist in activation of the terminal C-sequence. It is then further cleaved by proteases into C3d and C3c, the latter then being released (Fig. 2). From these facts it is obvious that the composition of cell surfaces is very critical. The relevance of these findings is underlined by the observation that viruses may transform a nonactivator surface into an activator surface probably by means of

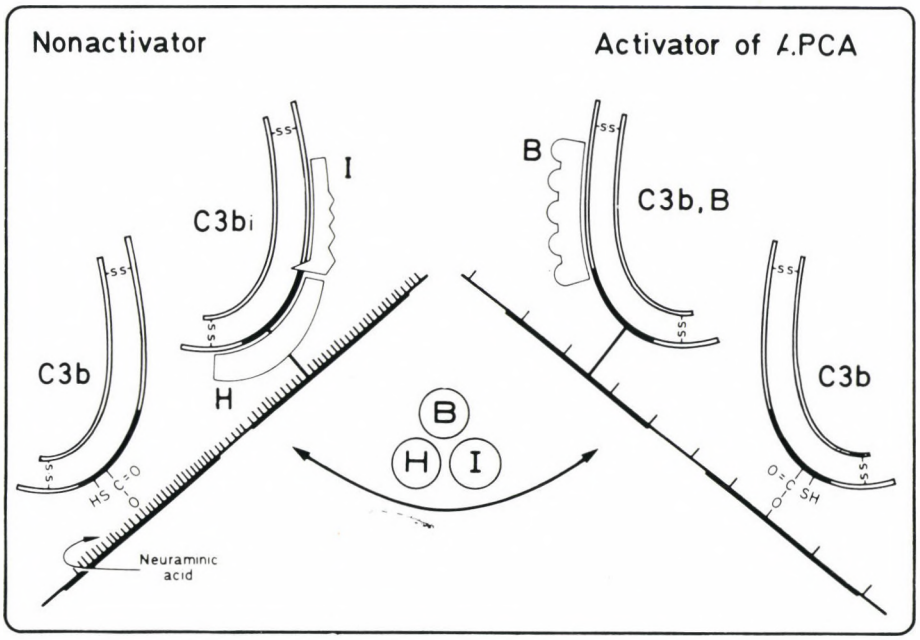


Fig. 3. Differential action of factors B, H and I on C3b deposited on surfaces containing many (non-activator) or few (activator) neuraminic acid molecules

their neuraminidase [34]. In addition, we have observed that certain lymphoid cells transformed by Epstein–Barr virus [35] and other cells infected with *Herpes simplex* virus [36] displayed proteolytic activity on their surface, allowing the deposition of C3b on these cells and subsequently their interaction with complement receptor cells [37].

The second part of this survey is devoted to the complement dependent biological phenomena. We have grouped these phenomena under five headings: destruction, adherence, blockade, detergent-like action and activation.

Destruction (Fig. 4). Complement-dependent lysis of cells is brought about by complexes formed of the components C5, C6, C7, C8 and C9. These components become activated via activation of C3. Then the C5b-9 complex, the membrane attack complex (MAC), is gradually built up. This results in the formation of a channel through the membrane. Whether the membrane attack complex itself forms the hole in the membrane or whether a hole is induced by a disturbance of the membrane's lipid bilayer, is a matter of dispute among the expert groups. The pros and cons of the various arguments in this controversial matter have been described in a review [38]. Most recently it has been suggested that cell destruction is brought about by a C9 polymer [39]. The generation of C9 polymer is supposed to be induced by the C5b, 6, 7, 8 complex. The MAC may effect lysis of erythro-

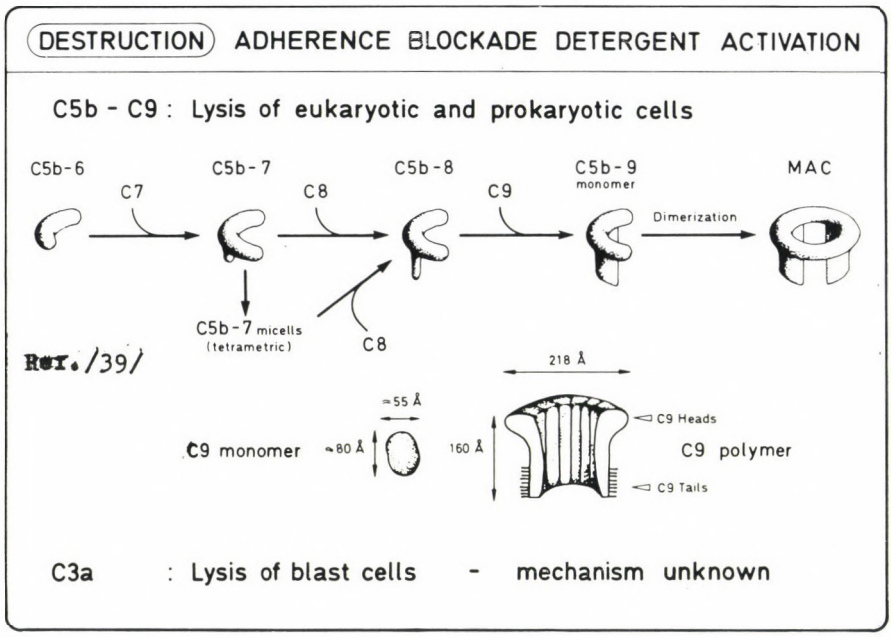


Fig. 4. Schematic drawing of formation of the membrane attack complex (MAC) and of a C9 polymer as designed by Podack et al. [39]

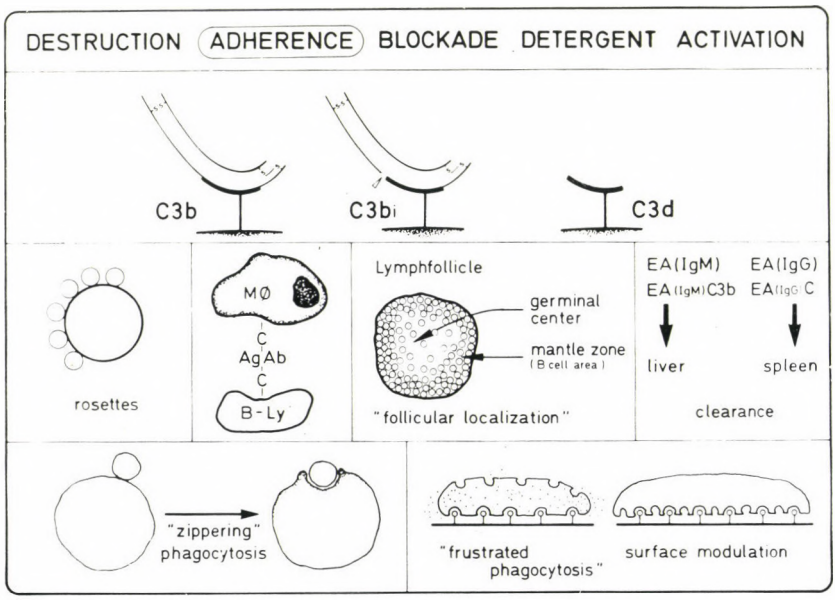


Fig. 5. Examples for complement mediated adherence phenomena

cytes or of nucleated cells. It may also destroy bacteria or viruses [40]. Recently C3a has been reported to affect the lysis of blast cells [41] by some unknown mechanism.

Adherence (Fig. 5). Particulate or soluble material coated by C3b, C3bi or C3d may adhere to certain cells by means of receptors specific for these three fragments of C3 [42–44]. All three types of C3 receptor (CR1 for C3b, CR2 for C3d, CR3 for C3bi) are represented on B lymphocytes and can be demonstrated by rosette formation between the CR-carrying cell and sheep erythrocytes coated with the respective C3 fragment. Phagocytic cells are endowed with the receptors for C3b and C3bi (CR1 and CR3). It is conceivable that immune complexes carrying appropriate C3 fragments bind to both macrophages and B-lymphocytes thus linking them together and that this might facilitate the cooperation between these two cell types that is a prerequisite for certain immunological process (e.g. antibody production). Furthermore, complement is instrumental in localizing antigenic material into lymph follicles [45]. Complement fragments on immune complexes also allow clearance of these complexes from the blood either into the liver or the spleen [46, 47]. On phagocytes C3 fragments mediate adherence and internalization by the zippering-mechanism [48]. If the complement-coated particle is too large to be internalized the phagocyte attaches itself by its complement receptors to the corresponding ligands on the particle and secretes its lysosomal content

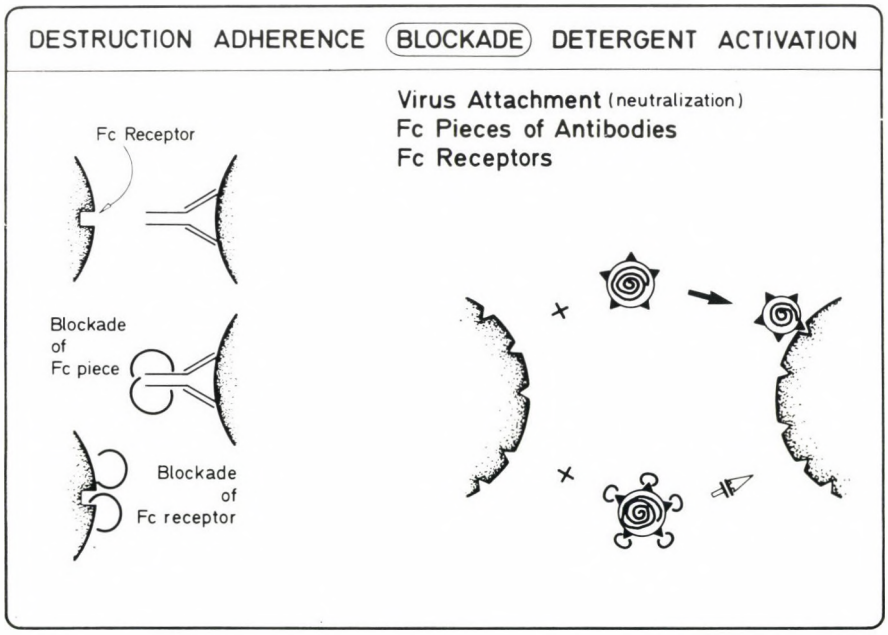


Fig. 6. Complement-dependent inhibition of virus attachment and of Fc/Fc receptor interaction as examples of complement mediated blockade of biological processes

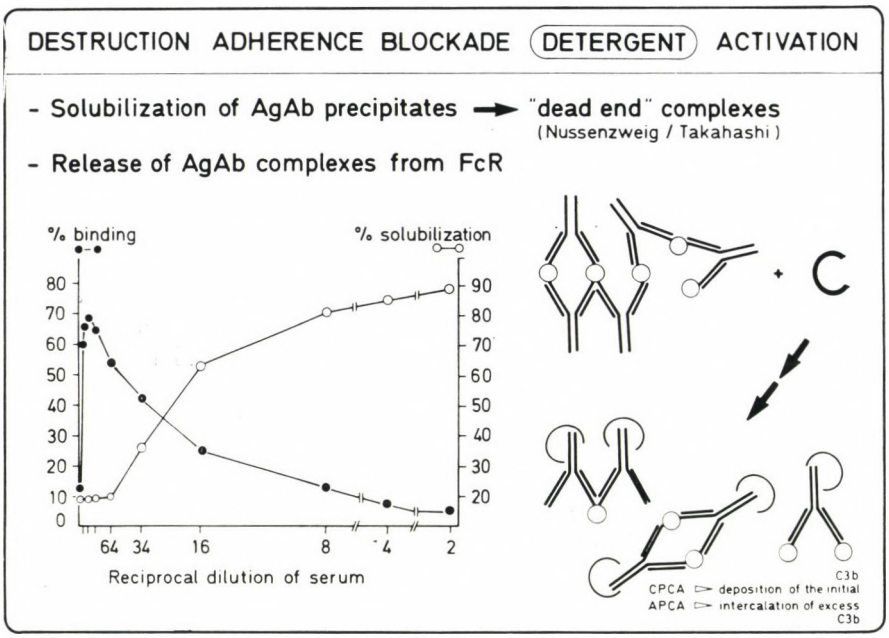
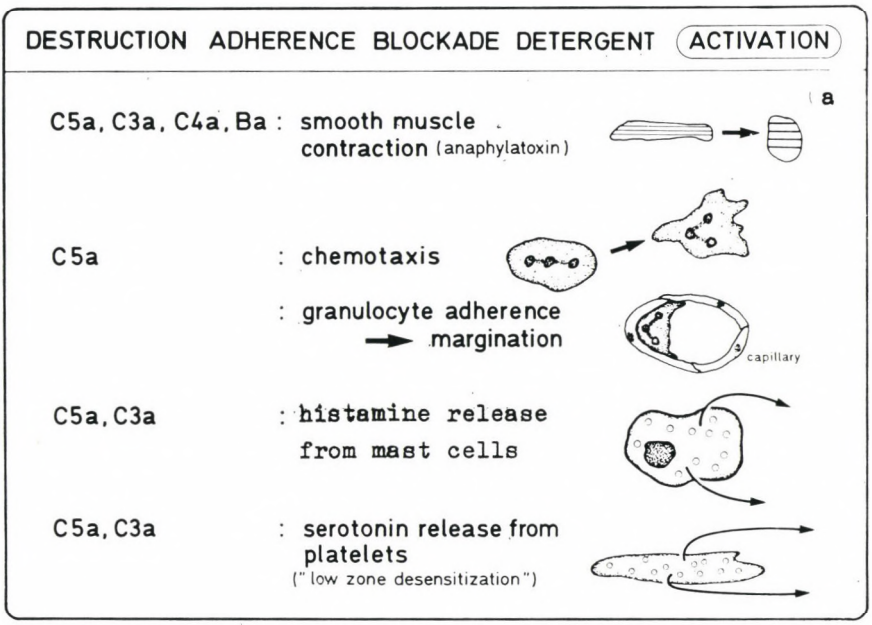


Fig. 7. Complement-mediated binding of immune complexes to cells and its loss due to solubilization. The graph in the left half of the figure is taken from Takahashi et al. [57]

into the surroundings, a process that could cause a local destruction of normal tissue. Under appropriate conditions phagocytes engaged in this kind of 'frustrated phagocytosis' [49] modulate [50] their receptors by moving them towards the surface area in contact with the particle which cannot be internalized [51].

Blockade (Fig. 6). Complement-mediated blockade of functions may occur very often. We want to present only a few examples. Coating of viruses with complement may cover surface elements of the virus which would otherwise mediate attachment of the virus-to-virus receptors. Thus the virus is neutralized, i.e. it is unable to infect the cell [52, 53]. Another example of blockade is the covering of the Fc portion of antibodies [51] or covering of the Fc receptor by complement [54]. Considering this phenomenon one wonders whether complement dependent blockade renders Fc/Fc receptor mediated processes less frequent in vivo than is generally thought.

Detergent (Fig. 7). The 'detergent-like' effect describes the potency of complement to effect solubilization of immune complexes (IC) and to release IC from binding to Fc receptors [55, 56]. Probably in both instances, the first C3 molecule is activated and deposited on the IC via the CPCA. Then a large number of C3 molecules is intercalated via the APCA thus breaking larger IC nets into small soluble complexes. Takahashi et al. have shown that the interaction of an antigen



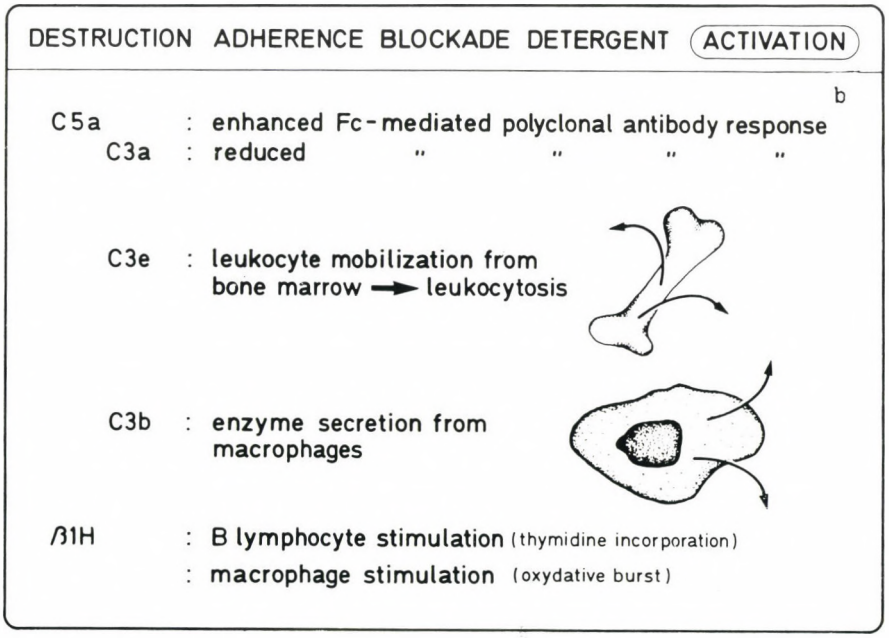


Fig. 8a., 8b. Complement-mediated activation: Examples of biological process in which components as well as fragments are involved

antibody complex with serum at low concentration enhances the binding of these complexes to B lymphocytes while incubation in concentrated serum yields soluble complexes which do not bind to cells [57]. These complexes were therefore called 'dead end' complexes. Such in vitro observations might suggest that insoluble IC in the tissue could be solubilized by complement and then appear in the blood stream. But a redistribution of IC from blood to tissue could also be imagined. Which of the two ways is more important under conditions in vivo remains to be clarified.

Activation (Fig. 8a,b). Finally we want to outline the capacity of various fragments of the C system to activate a series of cellular processes. The small fragments C5a, C4a, C3a and Ba act as anaphylatoxins and cause contraction of smooth muscle [15], C5a being by far the most potent reagent. C5a also causes directional movement of phagocytes and modifies granulocytes in such a fashion that they adhere to each other [58, 59] and to capillary cell walls [60, 61]. Due to this alteration granulocytes are trapped in the pulmonary capillary bed after having been modified in peripheral blood. This could be a critical mechanism occurring during the early phases of an acute respiratory distress syndrome following a polytrauma [62, 63]. Furthermore, C5a and C3a both trigger release of histamine from mast cells and of serotonin from platelets [64]. In addition both fragments modulate a polyclonal Fc mediated antibody response: C5a enhances and C3a reduces this response in an unknown way [65–67]. C3e, a part of C3c (Fig. 2), mobilizes leukocytes from the bone marrow [68, 69]. C3b triggers enzyme secretion from macrophages [70]. Apart from these fragments of C3 another complement component, factor H, has been shown to stimulate B lymphocytes and trigger the oxidative burst in human macrophages [71, 72].

From these in vitro data it is clear that activation products of the complement system may induce a broad spectrum of biological effects. These effects are prerequisites for physiological processes and at the same time they may contribute to the development of pathological conditions. To which degree these phenomena are essential in vivo has yet to be determined. Very informative in this respect are patients suffering from deficiencies of certain complement components [73]. Lack of C1 inactivator leads to angioneurotic oedema. A deficiency in C2 is typically associated with connective tissue disease. There is, however, no explanation of how a lack of C2 might be responsible for such a disease. Possibly the altered handling of IC is critical. It is easy to deduce from what has been mentioned above why patients with C3 deficiency suffer from numerous and severe bacterial and fungal infections. In patients with C8 deficiency frequent infections with pathogenic neisseria have been observed. A deficiency in C8 inhibits formation of the membrane attack complex. Opsonization and phagocytosis are apparently insufficient for the elimination of neisseria. To underline the role of complement in vivo one might list the vast literature documenting the concentration of complement components in various diseases [74]. The relevance of these findings is not clear in most instances and we shall have to await more data from the continuously expanding research in the complement field to be able to fully appreciate all

the functions which the complement may fulfil in vivo. For additional reading on complement and complement related aspects we refer to the surveys mentioned and to further reviews [75–79].

References

1. WHO Report, Nomenclature of complement. *Bull. WHO 39*, 935 (1968).
2. WHO Report. Nomenclature of the alternative activating pathway of complement. *Europ. J. Immunol. 11:* 668 (1981).
3. Cooper, N. R., Ziccardi, R.: In: *Proteolysis and Physiological regulation.* D. W. Ribbons, K. Brew (eds). Academic Press, New York 1976, p. 167.
4. Loos, M.: The classical complement pathway: Mechanism of activation of the first component by antigen-antibody complexes. *Progr. Allergy 30*, 135 (1982).
5. Bartholomew, R. M., Esser, A. F., Müller-Eberhard, H. J.: Lysis of oncorna viruses by human serum — isolation of the viral complement (C1) receptor and identification as p15E. *J. Exp. Med. 147*, 844 (1977).
6. Bartholomew, R. M., Esser, A. F.: Mechanism of antibody-independent activation of the first component of complement (C1) on retrovirus membranes. *Biochemistry 19*, 2847 (1980).
7. Müller-Eberhard, H. J., Schreiber, R. D.: Molecular biology and chemistry of the alternative pathway of complement. In: *Advances in Immunology*. H. G. Kunkel, F. J. Dixon (eds). Academic Press, New York 1980, Vol. 29, p. 2.
8. Kazatchkine, M. D., Nydegger, U. E.: The human alternative complement pathway: Biological and immunopathology of activation and regulation. *Progr. Allergy 30*, 193 (1982).
9. Bitter-Suermann, D.: Mediators of inflammation: Contribution of complement factors and reaction pathways to inflammation. In: *Agents and Actions Supplements*, M. J. Parnham, J. Winkelmann (eds). 1982, Vol. 11, p. 159.
10. Reid, K. B. M., Porter, R. R.: The proteolytic activation systems of complement. *Ann. Rev. Biochem. 50*, 433 (1981).
11. Rittner, Ch.: Genetic loci of components of the classical and alternate pathway of complement activation: A new dimension of the immunogenetic linkage group (HLA) on chromosome 6 in man. *Hum. Genet. 35*, 1 (1976).
12. Lachmann, P. J., Hobart, M. J.: Complement genetics in relation to HLA. *Brit. Med. Bull. 34*, 247 (1978).
13. Bokisch, V. A., Müller-Eberhard, H. J., Cochrane, C. G.: Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. *J. Exp. Med. 129*, 1109 (1969).
14. Johnson, U., Ohlsson, K., Olsson, J.: Effects of granulocyte neutral proteases on complement components. *Scand. J. Immunol. 5*, 421 (1976).
15. Hugli, T. E.: Chemical aspects of the serum anaphylatoxins. In: *Contemporary Topics in Molecular Immunology*. R. A. Reisfeld, F. P. Juman (eds). Plenum Press, New York, 1978, Vol. 7, p. 181.
16. Law, S. K., Levine, R. P.: Interactions between the third complement protein and cell surface macromolecules. *Proc. Nat. Acad. Sci. USA 74*, 2701 (1977).
17. Law, S. K., Lichtenberg, N. A., Levine, R. P.: Evidence for an ester linkage between the labile binding site of C3b and receptive surfaces. *J. Immunol. 123*, 1388 (1979).
18. Law, S. K., Fearon, D. T., Levine, R. P.: Action of the C3b-inactivator on cell-bound C3b. *J. Immunol. 122*, 759 (1979).
19. Janatowa, J., Tack, B. F., Prahl, J. W.: Third component of human complement: structural requirements for its function. *Biochemistry 19*, 4479 (1980).

20. Janatowa, J., Lorenz, P. E., Schechter, A., N. Prahl, J. W., Tack, B. F.: Third component of human complement: Appearance of a sulfhydryl group following chemical or enzymatic inactivation. *Biochemistry 19*, 4471 (1980).
21. Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L., Prahl, J. W.: Evidence for presence of an internal thiolester bond in third component of human complement. *Proc. Natl. Acad. Sci. USA 77*, 5764 (1980).
22. Howard, J. B.: Methylamine reaction and denaturation-dependent fragmentation of complement component 3. *J. Biol. Chem. 255*, 7082 (1980).
23. Pangburn, M. K., Müller-Eberhard, H. J.: Relation of a putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. *J. Exp. Med. 152*, 1102 (1980).
24. Müller-Eberhard, H. J.: Complement reaction pathways. In: *Immunology '80*, M. Fougereau, J. Dausset (eds). Academic Press, New York 1981, p. 1001.
25. Twose, T. M., Sim, R. B., Paterson, D. S.: The acceptor-binding reaction of C3. 4th Int. Congr. Immunology, Abstr. No. 15, 1.22 (1980).
26. Isenmann, D. E., Kells, D. J. C., Müller-Eberhard, H. J., Pangburn, M. K.: Nucleophilic modification of human complement protein C3: correlation of conformational changes with acquisition of C3b-like functional properties. *Biochemistry 20*, 4458 (1981).
27. Fearon, D. T., Austen, K. F.: Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms. *Proc. Natl. Acad. Sci. USA 74*, 1683 (1977).
28. Pangburn, M. K. Schreiber, R. D., Müller-Eberhard, H. J.: Human complement C3b inactivator: Isolation, characterization, and demonstration of an absolute requirement for the serum protein β1H for cleavage of C3b and C4b in solution. *J. Exp. Med. 146*, 257 (1977).
29. Fearon, D.: Regulation by membrane sialic acid of β1H-dependent C3 convertase of the alternative complement pathway. *Proc. Natl. Acad. Sci USA 75*, 1971 (1978).
30. Kazatchkine, M. D., Fearon, D. T., Austen, K. F.: Human alternative complement pathway: membrane associated sialic acid regulates the competition between B and β1H for cell-bound C3b. *J. Immunol. 122*, 75 (1979).
31. Fearon, D. T.: Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocytes membrane. *Proc. Natl. Acad. Sci. USA 76*, 5867 (1979).
32. Pangburn, M. K., Morrison, D. C., Schreiber, R. D., Müller-Eberhard, H. J.: Activation of the alternative complement pathways: recognition of surface structures on activators by bound C3b. *J. Immunol. 124*, 977 (1980).
33. Ross, G. D., Lambris, J. D., Cain, J. A., Newmann, S. L.: Generation of three different fragments of bound C3 with purified factor I or serum. I. Requirements for factor H versus CR_1 cofactor activity. *J. Immunol. 129*, 2051.
34. Lambré, C. R., Kazatchkine, M. D., Maillet, F., Thibon, M.: Guinea pig erythrocytes after their contact with influenza virus acquire the ability to activate the human alternative complement pathway through virus-induced desialation of the cells. *J. Immunol. 128*, 629 (1982).
35. Schulz, T., Dierich, M. P., Yefenof, E., Klein G.: C3 activating proteases on human lymphoblastoid cells superinfected with Epstein–Barr virus. *Cell. Immunol. 51*, 168 (1980).
36. Dierich, M. P., Landen, B., Schulz, T., Falke, D.: Protease activity on the surface of HSV-infected cells. *J. Gen. Virol. 45*, 241 (1979).
37. Dierich, M. P., Landen, B.: Complement bridges between cells: Analysis of a possible cell-cell interaction mechanism. *J. exp. Med. 146*, 1484 (1977).
38. Esser, A. F.: Interactions between complement proteins and biological and model membranes. In: *Biological Membranes*. D. Chapman (Ed.). 1982, Vol. 4., p. 277.
39. Podack, E. R., Tschopp, J.: Polymerization of the ninth component of complement (C9):

Formation of poly (9) with a tubular ultrastructure resembling the membrane attack complex of complement. *Proc. Natl. Acad. Sci. USA 79*, 574 (1982).
40. Cooper, N. R., Jensen, F. C., Welsh, R. M., Oldstone, M. B. A.: Lysis of RNA tumor viruses by human serum: direct antibody-independent triggering of the classical complement pathway. *J. Exp. Med. 144*, 970 (1976).
41. Schorlemmer, H. U., Hadding, U., Bitter-Suermann, D., Allison, A. C.: The role of complement cleavage products in killing of tumour cells by macrophages. In: *The Macrophage and Cancer*. K. James, B. McBide, A. Stuart, (eds). Econoprint, Edinburgh 1977, p. 68.
42. Schmitt, M., Mussel, H.-H., Dierich, M. P.: Qualitative and quantitative assessment of C3-receptor reactivities on lymphoid and phagocytic cells. *J. Immunol. 126*, 2042–2047 (1981).
43. Dierich, M. P.: Complement receptors: biological and biochemical characterization. In: *Immunopathology. VIIIth Intern. Symposium 1980*. F. J. Dixon, P. A. Miescher (eds). Academic Press, New York 1982, p. 421.
44. Dierich, M. P., Scheiner, O., Mussel, H.-H., Hamman, K. P., Schopf, R. E., Schulz, T.: Characterization of complement receptors. *Molec. Immunol. 19*, 1255 (1982).
45. Dukor, P., Dietrich, F. M., Gisler, R. H., Schuman, G., Bitter-Suermann, D.: Possible targets of complement action in B-cell triggering. In: *Progress in Immunology II*. L. Brent, J. Holborrow (eds). North-Holland, Amsterdam 1974, Vol. 3., p. 99.
46. Jaffé, C. J., Vierling, J. M., Jones, E. A., Lawly, T. J., Frank, M. M.: Receptor specific clearance by the reticuloendothelial system in chronic liver diseases. *J. Clin. Invest. 62*, 1069 (1978).
47. Frank, M. M.: In vivo studies of Fc receptor dependent clearance: defects in autoimmune disease and associated with the HLA B8 DRw3 haplotype. In: *Immunopathology VIIIth Int. Symposium*. F. J. Dixon, P. A. Miescher (eds). Academic Press, New York 1982, p. 387.
48. Griffin, F. M., Griffin, J. A., Leider, J. E., Silverstein, S. C.: Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. *J. Exp. Med. 142*, 1263 (1975).
49. Henson, P. M.: Interaction of cells with immune complexes: adherence, release of constituents and tissue injury. *J. Exp. Med. 134*, 114s (1971).
50. Rabinovitch, M., Manijas, R. E., Nussenzweig, V.: Selective phagocytic paralysis induced by immune complexes. *J. Exp. Med. 142*, 827 (1975).
51. Michl, J., Pieczonka, M. M., Unkeless, J. C., Silverstein, S. C.: Effects of immobilized immune complexes on Fc- and complement-receptor functions in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med. 150*, 607 (1979).
52. Mills, B. J., Cooper, N. R.: Antibody-independent neutralisation of vesicular stomatitis virus by human complement. I. Complement requirements. *J. Immunol. 121*, 1549 (1978).
53. Cooper, N. R.: Humoral immunity to viruses. In: *Comprehensive Virology*. H. Fraenkel-Conrat, R. Wagner (eds). Plenum Press, New York 1979, Vol. 15, p. 123.
54. Füst, G., Molenaar, J. L.: Inhibition by nascent C3b of the immune complex binding to lymphocytes. *Immunol. Lett. 1*, 61 (1979).
55. Takahashi, M., Czop, J. Ferreira, A., Nussenzweig, V.: Mechanism of solubilization of immune aggregates by complement. Implications for immunopathology. *Transplant. Rev. 32*, 121 (1976).
56. Nussenzweig, V.: Interaction between complement and immune complexes: role of complement in containing immune complex damage. In: *Immunology '80, Progress in Immonology IV*. M. Fougereau, J. Chausset. Academic Press, New York 1980, p. 1044.
57. Takahashi, M., Takahashi, S., Hirose, S.-I.: Solubilization of antigen-antibody complexes: a new function of complement as a regulator of immune reactions. *Progr. Allergy, 27*, 134 (1980).

58. O'Flaherty, J. T., Kreutzer, D. L., Ward, P. A.: Neutrophil aggregation swelling induced by chemotactic agents. *J. Immunol. 119*, 232 (1977).
59. O'Flaherty, J. T., Kreutzer, D. L., Showell, H. J., Ward, P. A.: Influence of inhibitors of cellular function on chemotactic factor induced neutrophil aggregation. *J. Immunol. 119*, 1751 (1977).
60. Craddock, P. R., Hammerschmidt, D. E., Moldow, C. F., Yamada, O., Jacob, H. S.: Granulocyte aggregation as a manifestation of membrane interactions with complement: possible role in leukocyte margination, microvascular occlusion, and endothelial damage. *Semin. Haemat. 16*, 140 (1979).
61. Jacob, H. S., Craddock, P. R., Hammerschmidt, D. E., Moldow, C. F.: Complement-induced granulocyte aggregation. *New Engl. J. Med. 302*, 789 (1980).
62. Hosea, S., Brown, E., Hammer, C., Frank, M. M.: Role of complement activation in a model of adult respiratory distress syndrome. *J. Clin. Invest. 66*, 375 (1980).
63. Skubitz, K. M., Craddock, P. R.: Reversal of hemodialysis granulocytopenia and pulmonary leukostasis. *J. Clin. Invest. 67*, 1383 (1981).
64. Meuer, S., Zanker, B., Hadding, U., Bitter-Suermann, D.: Low zone desensitization: a stimulus-specific control mechanism of cell response: Investigation on anaphylatoxin-induced platelet secretion. *J. Exp. Med. 155*, 698 (1982).
65. Morgan, E. L., Weigle, W. O., Hugli, T. E.: Anaphylatoxin-mediated regulation of the immune response. I. C3a-mediated suppression of human and murine humoral immune responses. *J. Exp. Med. 155*, 1412 (1982).
66. Goodman, M. G., Chenoweth, D. E., Weigle, W. O.: Induction of interleukin 1 secretion and enhancement of humoral immunity by binding of human C5a to macrophage surface C5a receptors. *J. Exp. Med. 156*, 912 (1982).
67. Weiler, J. M., Ballas, Z. K., Needleman, B. S., Hobbs, M. V., Feldbusch, T. L.: Complement fragments suppress lymphocyte immune responses. *Immunol. Today 3*, 238 (1982).
68. Rother, K.: Leukocyte mobilizing factor: a new biological activity from the third component of complement. *Europ. J. Immunol. 2*, 550 (1972).
69. Ghebrehiwet, B., Müller-Eberhard, H. J.: C3e: an acidic fragment of human C3 with leukocytosis-inducing activity. *J. Immunol. 123*, 616 (1979).
70. Schorlemmer, H.-U., Davies, Ph., Allison, A. C.: Ability of activated complement components to induce lysosomal enzyme release from macrophages. *Nature 261*, 48 (1976).
71. Hamman, K. P., Raile, Astrid, Schmitt, M., Mussel, H. H., Peters, H., Scheiner, O., Dierich, M. P.: β1H stimulates mouse spleen B lymphocytes as demonstrated by increased thymidine incorporation and formation of B cell blasts. *Immunobiology 160*, 289 (1981).
72. Schopf, R. E., Hamman, K. P. Scheiner, O., Lemmel, E.-M., Dierich, M. P.: Activation of human monocytes by both human β1H and C3b. *Immunology 46*, 307 (1982).
73. Lachmann, P.: Complement defects, immunological health, and immunological disease. In: *Immunopathology. VIII Int. Symposium.* Academic Press, New York 1982, p. 365.
74. Fine, D. P.: *Complement and Infections Diseases.* CRC Press, Boca Raton, Florida 1981.
75. Bitter-Suermann, D.: Die Funktionseinheit Makrophage-Komplement. G. Dhom (ed.). *Verh. Dtsch. Ges. Path.* 1980, p. 63.
76. Hugli, T. E.: The structural basis for anaphylatoxin and chemotactic functions of C3a, C4a, C5a. *Crit. Rev. Immunol. 1*, 321 (1981).
77. Porter, R. R., Reid, K. M. B.: Activation of the complement system by antibody-antigen complexes: the classical pathway. *Adv. Prot. Chem.* C. B. Anfinsen, J. T. Edsall, F. M. Richards (eds). Academic Press, New York 1979, Vol. 33. p. 1.
78. Pepys, M. B.: Role of complement in the induction of immunological responses. *Transplant. Rev. 32*, 93 (1976).
79. Theofilopoulos, A. N., Dixon, F. J.: The biology and detection of immune complexes. *Advanc. Immunol. 28*, 89 (1979).

Haematologia 17 (1), pp. 17—24 (1984)

Iron Absorption in Non-Transfused Iron Loading Anaemias: Prediction of Risk for Iron Loading, and Response to Iron Chelation Treatment, in β Thalassaemia Intermedia and Congenital Sideroblastic Anaemias

M. J. PIPPARD, D. J. WEATHERALL

Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford, OX3 9DU United Kingdom

(Received 15 February 1983; accepted 15 March 1983)

A variable rate of iron loading, reaching toxic levels in some patients, was seen in a series of non-transfused patients with β thalassaemia intermedia or sideroblastic anaemia. The degree of anaemia was a poor guide to the risk of iron overload. However the extent of erythroid hyperplasia, judged by ferrokinetic studies or more simply by bone marrow aspiration, was useful in predicting both the rate of iron loading and the need for iron chelation therapy.

Keywords: desferrioxamine, ferrokinetics, iron absorption, sideroblastic anaemia, thalassaemia intermedia.

Introduction

In patients with congenital anaemias requiring regular blood transfusions iron overload eventually damages the heart, liver and endocrine organs. This problem is seen most commonly in the major thalassaemia syndromes where, in the absence of measures to remove excess iron, cardiac iron damage can be expected to prove fatal by early adult life [1, 2]. Increased food iron absorption may also contribute to iron overload in these disorders, but is kept to a minimum by transfusion regimens that maintain a high circulating haemoglobin level [3]. Iron loading from the parenteral administration of blood therefore occurs at a predictable rate. By contrast, patients with the clinical picture of thalassaemia intermedia, who maintain a haemoglobin concentration above 5–7 g/dl without regular blood transfusions, show a variable degree of excessive food iron absorption [4] which may lead to serious iron loading by middle life [1, 5, 6]. These patients, in whom the genetic basis of the disease is heterogeneous [1] form a major public health problem in populations in which thalassaemia is common. A similar picture is encountered in other congenital anaemias characterised by ineffective erythropoiesis and massive erythroid expansion, for example the sideroblastic disorders [7, 8]. Because the increase in iron absorption is so variable and the mechanism by which this is maintained in the face of increased body iron stores is poorly understood, it has been difficult to predict the risk of serious iron-loading in individual non-transfused patients. This study examines the factors influencing

the risk in a series of non-transfused patients with either β thalassaemia intermedia or congenital sideroblastic anaemia. In addition, the potential role of iron chelating agents in reducing iron absorption and/or removing established iron overload is discussed.

Patients and Methods

Patients

Seven patients had sideroblastic anaemia with haemoglobin levels ranging from 5.6 to 12.8 g/dl. Five cases were clearly familial and in two, disease had been present at least since childhood. No patient had received blood transfusions. Further details of these patients are to be published elsewhere [9]. Of 25 patients with β thalassaemia intermedia, 17 had received no, or only very occasional, transfusions in the past. In this group, haemoglobin levels ranged from 5.1 to 11.5 g/dl and the haematological features were characteristic of homozygous β thalassaemia.

Methods

Haemoglobin concentrations and red cell indices were measured using a Coulter S counter. Standard methods were used to determine the plasma iron and iron binding capacity [10, 11]. Plasma ferritin was determined by immunoradiometric assay, by courtesy of Dr. Mark Worwood, University Hospital of Wales. Radioiron absorption was measured as the total body retention at 14 days of a test dose of 5 mg of oral iron ($^{59}FeSO_4$ with 50 mg ascorbate) [12]. Plasma iron turnover (PIT) and erythroid iron turnover (EIT) were determined after i.v. injection of 2 μCi of ^{59}Fe in 5 ml plasma [13, 14]. Since many of the patients had a saturation of the plasma iron binding capacity that was greater than 80 per cent, plasma from a normal human donor was labelled in vitro using ^{59}Fe citrate (The Radiochemical Centre, Amersham). The ^{59}Fe dose was diluted to 1 ml with pH 2 saline and added to the plasma over 5 minutes with constant mixing. The plasma was then incubated at 37 °C for 30 minutes, and brought to 90 per cent iron saturation with ferrous ammonium sulphate before injection. Bone marrow erythroid/myeloid (E:M) ratios were obtained from a 1000 cell count on bone marrow aspirate smears. Erythroid expansion was calculated from ferrokinetic or morphological data as a multiple of normal erythroid activity, the latter being taken as an EIT of 100 μmol/litre blood/24 hours or E:M ratio of 0.3.

Results

Absorption of radioiron

The mean value for the retention of the test dose of ^{59}Fe was 55 per cent (range 20 to 89 per cent) in 20 patients with β thalassaemia intermedia, and 31 per cent (range 5 to 71 per cent) in 7 patients with congenital sideroblastic anaemias (Fig. 1). Despite the increased iron stores present in many of these

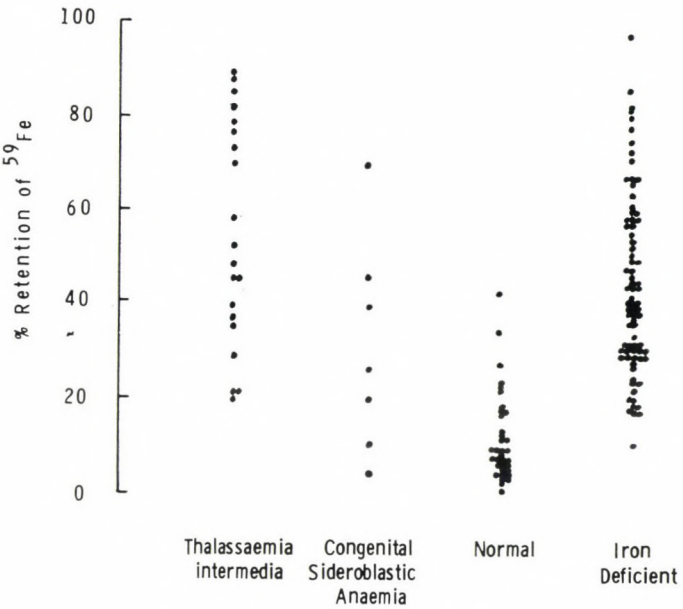


Fig. 1. Iron absorption in patients receiving a test dose of 5 mg oral iron ($^{59}FeSO_4$)

patients (see Fig. 2), iron absorption was closer to that of an iron deficient group (mean 45.5 per cent, n = 90) than that of iron replete normal controls (mean 14.7 per cent, n = 35).

Rate of iron loading

The plasma ferritin level was directly related to liver iron concentration in 10 patients in whom liver biopsies were obtained (data not shown). The amount of iron overload increased with age in both β thalassaemia intermedia and sideroblastic anaemia (Fig. 2). However, the rate of iron loading varied widely between patients and additional risk factors for iron loading are clearly involved.

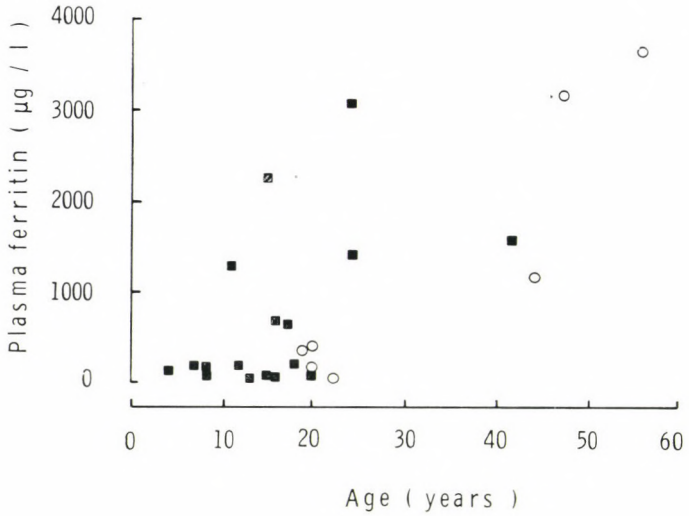


Fig. 2. Plasma ferritin levels as a function of patient age in β thalassaemia intermedia (■) or sideroblastic anaemia (○)

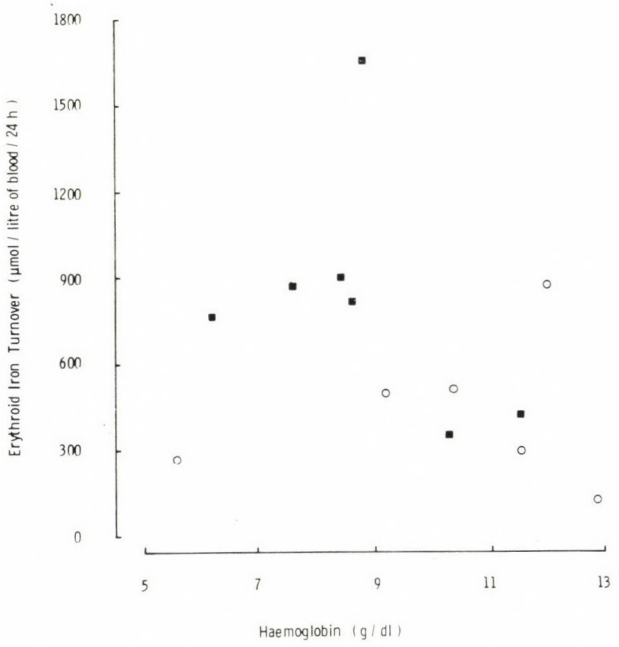


Fig. 3. Relationship between haemoglobin level and erythroid iron turnover in patients with β thalassaemia intermedia (■) or sideroblastic anaemia (○)

Effects of anaemia and erythroid expansion

The degree of anaemia and the extent of erythroid expansion were poorly correlated with each other (Fig. 3) and their effects were therefore examined separately. The haemoglobin deficit (taken as 14 g/dl minus the patient's circulating haemoglobin level) or the erythroid expansion (expressed as a multiple of

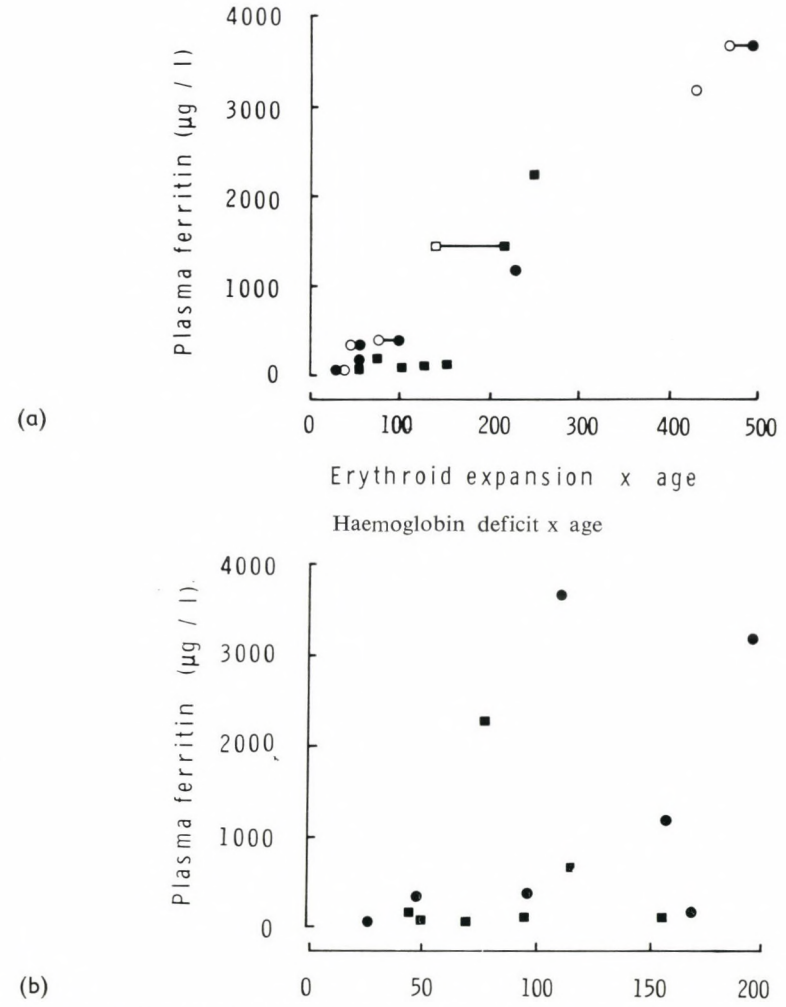


Fig. 4. Relationship between plasma ferritin and (a) degree of anaemia or (b) erythroid expansion, in patients with β thalassaemia intermedia (■) or sideroblastic anaemia (●). The degree of anaemia was determined as (14 minus the patient's circulating haemoglobin level in g/dl), and the erythroid expansion (times normal) from ferrokinetic studies (closed symbols) or marrow E : M ratios (open symbols). In 5 subjects paired values for the two measures of erythroid expansion are shown

normal erythroid activity) was multiplied by the patient's age (representing the duration of exposure to each potential risk factor). In the 14 patients in whom a comparison of these two measurements could be made the product of haemoglobin deficit and age showed a poor correlation with the level of iron loading (Fig. 4a), whereas the product of erythroid expansion and age was a much better guide (Fig. 4b). In addition, in those patients in whom erythroid expansion was assessed by both ferrokinetic and morphological means, a good correlation between the two measurements was obtained (Fig. 4b). This suggests that assessment of erythroid expansion from simple marrow examination may be as good as the more detailed ferrokinetic measurements in predicting the risk of iron loading.

Effect of iron chelation treatment

In four adult patients with β thalassaemia intermedia, control metabolic iron balance studies confirmed a positive iron balance (mean +2.9 mg/day, range +1.2 to 3.9 mg). Oral DF (1 g taken with meals) converted this to a small negative

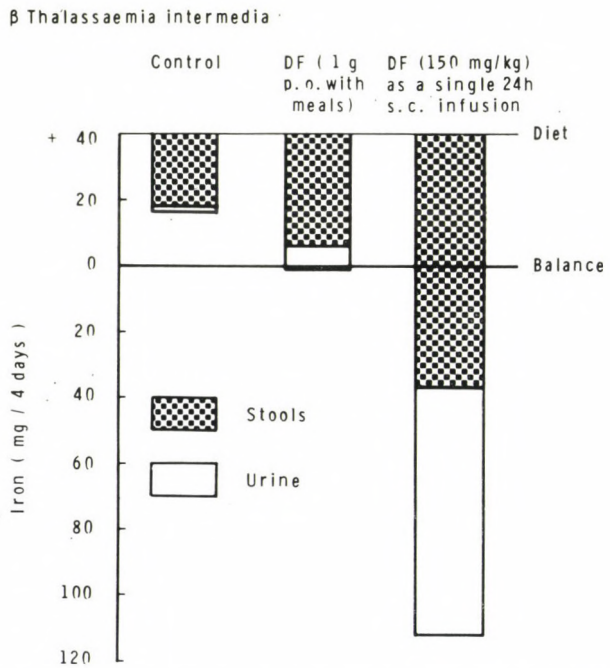


Fig. 5. Metabolic balance study in a patient with β thalassaemia intermedia. A constant diet (10 mg iron/day) was taken during three consecutive 4-day periods. During a control period the total iron intake (40 mg) was not matched by the total iron excretion in stools and urine, and a net iron gain (positive iron balance) resulted. The positive iron balance was abolished by oral DF (1 g in 10 ml water t.d.s.), and converted to substantial net iron loss by a single 24 h subcutaneous infusion of 8 g DF (150 mg/kg)

iron balance (mean −1.2 mg/day, range −0.2 to 2 mg). However, a single subcutaneous infusion of 150 mg DF/kg/24 hours produced a much more substantial net iron loss over a balance period of equivalent length (Fig. 5).

Discussion

These studies have shown that in non-transfused patients with sideroblastic anaemia or with β thalassaemia intermedia, the degree of anaemia is a poor guide to the amount of increased gastrointestinal iron absorption and the rate of iron loading. On the other hand, the magnitude of the erythroid expansion correlated much better with the risk of developing iron overload. This is consistent with the hypothesis [15] that excess food iron absorption is directly related to increased plasma iron turnover. The correlation with erythroid expansion held good despite the limitations inherent in a single measurement taken during the course of a disease which may evolve with time, and despite other obvious potential causes of variation (e.g. differences in the amount of available iron in the patients' usual diets, and differences in iron balance between men and women). It should be emphasised that the degree of anaemia is clearly not the only determinant of the magnitude of erythroid expansion. Other factors may include both the nature of the underlying disease and the degree of displacement of the haemoglobin oxygen-dissociation curve (e.g. with different amounts of circulating Hb F in β thalassaemia intermedia) [6]. Clearly, gross erythroid expansion may be present at a near normal haemoglobin level, and the possibility that otherwise asymptomatic patients may be at risk of iron overload will be missed if erythroid expansion is not assessed. In this study a simple determination of the erythroid/myeloid ratio from bone marrow smears appeared as good as ferrokinetic measurements of erythroid expansion in predicting the risk of iron loading. This is important, both because it avoids the use of radioisotopes and because the more detailed investigations are time consuming and are unlikely to be available in many parts of the world where β thalassaemia intermedia is common.

The price of neglecting the risk of iron loading in these patients is the development of tissue iron toxicity. Indeed several of our older patients have evidence of cardiac damage, hepatic cirrhosis, or endocrine failure [6, 9]. Reduction of iron absorption by dietary modification and/or the use of oral iron chelating agents to reduce the amount of available food iron [16] might offer these patients the hope of a life free from these complications of iron overload. Although our metabolic iron balance studies have indicated that the net dietary iron gain can be negated by the use of oral desferrioxamine (DF), subcutaneous infusions of the drug both reduce radioiron absorption [17] and give a much more marked negative iron balance. Further studies have shown that even in subjects with no evidence of excess iron stores, single infusions of 150 mg DF/kg/24 hours gave total (faeces plus urine) iron excretions that ranged from 41 to 92 mg (unpublished data). This raises the possibility that in patients with gross ineffective erythropoiesis who are shown to be at risk of iron loading, subcutaneous DF used only

occasionally (e.g. a single 24 hour infusion every 3 to 4 weeks) may be sufficient to prevent iron accumulation completely and thus potentially to avoid the complications of iron overload. Such an approach is likely to be more practical and acceptable to patients than the inefficient and very expensive use of prophylactic oral DF.

We are grateful to Dr. S. T. Callender and Dr. G. Warner, Nuffield Department of Clinical Medicine, Oxford, for allowing us to use their data on radioiron absorption in normal and iron deficient subjects, and to Miss Hesma Goodburn who provided excellent technical assistance. This work was supported by the Wellcome Trust, London, and a Wellcome Research Travel Grant made it possible for M. T. P. to present these data at the 19th Congress of the International Society of Haematology in Budapest, 1982.

References

1. Weatherall, D. J., Clegg, J. B.: *The Thalassaemia Syndromes* (3rd edition). Blackwell Scientific Publications, Oxford 1981.
2. Engle, M. A.: Cardiac involvement in Cooley's anemia. *Ann. N. Y. Acad. Sci. 119*, 694 (1964).
3. Heinrich, H. C., Gabbe, E. E., Oppitz, K. H., Whang, D. H., Bender-Gotze, Ch., Schafer, K. H., Schroter, W., Pfau, A. A.: Absorption of inorganic and food iron in children with heterozygous and homozygous β-thalassemia. *Z. Kinderheilk. 115*, 1 (1973).
4. Pippard, M. J., Callender, S. T., Warner, G. T., Weatherall, D. J.: Iron absorption and loading in β-thalassaemia intermedia. *Lancet ii*, 819 (1979).
5. Bannerman, R. M., Keusch, G., Kreimer-Birnbaum, M., Vance, V. K., Vaughan, S.: Thalassemia intermedia, with iron overload, cardiac failure, diabetes mellitus, hypopituitarism and porphyrinuria. *Am. J. Med. 42*, 476 (1967).
6. Pippard, M. J., Rajagopalan, B., Callender, S. T., Weatherall, D. J.: Iron loading, chronic anaemia, and erythroid hyperplasia as determinants of the clinical features of β-thalassaemia intermedia. In: *Advances in Red Blood Cell Biology*. D. J. Weatherall, G. Fiorelli., S. Gorini (eds). Raven Press, New York 1982, p. 103.
7. Soloman, L. R., Hillman, R. S., Finch, C. A.: Serum ferritin in refractory anemias. *Acta Haemat. 66*, 1 (1981).
8. Brain, M. C., Herdan, A.: Tissue iron stores in sideroblastic anaemia. *Brit. J. Haemat. 11*, 107 (1965).
9. Peto, T. E. A., Pippard, M. J., Weatherall, D. J.: Iron overload in mild sideroblastic anaemias. *Lancet i*, 375-378 (1983).
10. International Committee for Standardization in Haematology: Recommendations for measurement of serum iron in human blood. *Brit. J. Haemat. 38*, 291 (1978).
11. International Committee for Standardization in Haematology: The measurement of total and unsaturated iron-binding capacity in serum. *Brit. J. Haemat. 38*, 281 (1978).
12. Callender, S. T., Witts, L. J., Warner, G. T., Oliver, R.: The use of a simple whole-body counter for haematological investigations. *Brit. J. Haemat. 12*, 276 (1966).
13. Cook, J. D., Marsaglia, G., Eschbach, J. W., Funk, D. D., Finch, C. A.: Ferrokinetics: a biologic model for plasma iron exchange in man. *J. Clin. Invest. 49*, 197 (1970).
14. Cook, J. D., Finch, C. A.: Ferrokinetic measurements. In: *Iron* (Methods in Haematology, Vol. 1). J. D. Cook (ed.). Churchill Livingstone, Edinburgh 1980, p. 134.
15. Cavill, I., Worwood, M. and Jacobs, A.: Internal regulation of iron absorption. *Nature 256*, 328 (1975).
16. De Alarçon, P. A., Donovan, M., Forbes, G. B., Landaw, S., Stockman, J. A.: Iron absorption in the thalassemia syndromes and its inhibition by tea. *New Engl. J. Med. 300*, 5 (1979).
17. Pippard, M. J., Callender, S. T., Warner, G. T., Weatherall, D. J.: Iron absorption in iron-loading anaemias: Effect of subcutaneous desferrioxamine infusions. *Lancet ii*, 737 (1977).

Haematologia 17 (1), pp. 25–33 (1984)

Development and Evaluation of the Improved Iron Chelating Agents EHPG, HBED and their Dimethyl Esters

C. HERSHKO, R. W. GRADY,[1] G. LINK

Department of Medicine, Shaare Zedek Medical Center, Department of Nutrition, Hebrew University Hadassah Medical School, Jerusalem, 91 000, Israel; Division of Pediatric Hematology/Oncology,[1] Cornell University Medical College, New York, N.Y., USA

(Received 6 December 1982: accepted 10 January 1983)

The phenolic EDTA analogues ethylenediamine-N,N'-bis-(2-hydroxyphenylglycine) (EHPG), N,N'-bis(2-hydroxybenzyl)-ethylenediamine diacetic acid (HBED), and their respective dimethyl esters (dimethylEHPG and dimethylHBED) were studied in hypertransfused rats. Radioiron bound to these compounds was cleared mainly by the liver and excreted in the bile. After a single 40 mg i.m. injection, the percentage of radioiron removed from ^{59}Fe-ferritin-labelled hepatocytes and excreted in the bile was 4% in untreated controls, 24% for desferral, 42% for dimethylEHPG, 58% for EHPG, 63% for HBED, and 80% for dimethylHBED. DimethylHBED combines oral effectiveness with superior chelating ability, selective hepatocellular action, and low apparent toxicity. It may represent a significant advance in the development of new iron chelating drugs.

Keywords: EHPG, HBED, iron chelating agents.

Introduction

The poor oral absorption and high price of desferrioxamine (DF) underline the need for the development of improved chelating drugs for the management of transfusional iron overload. Of a large number of potential iron chelating drugs screened, four phenolic EDTA analogues were found to be particularly promising and were studied in detail. The objective of these studies was to define the source of iron chelated by these compounds in vivo, to compare their chelating efficiency with the standard chelating agent DF, and to assess their potential to mobilize iron when administered by the oral, as compared to the parenteral route.

Materials and Methods

Female Wistar rats of the Hadassah strain weighing 170 to 200 g were used throughout. All injections were performed through the tail veins. Animals were sacrificed under ether anesthesia by exsanguination through the abdominal aorta into heparinized syringes. Hypertransfusion was performed by two intravenous injections of 2 ml packed cells per 100 g body weight on days 4 and 1 prior to administration of radioiron labels. The mean hematocrit on the first day of study

was 60 per cent ± 1, the serum iron 459 $\pm$ μg/dl and the unsaturated iron-binding capacity less than 10 μg/dl.

A detailed description of the source, or procedure for the synthesis of the compounds employed in this study will appear in a manuscript presently in preparation.

Counting methods

The radioactivity of spleen, kidney, weighed portions of the liver, and 1 ml samples of blood were determined in an automatic well-type scintillation counter (Auto-Gamma Model 5360 scintillation spectrometer: Packard Instrument Co., Inc., Downer's Grove, Ill.) Whole body counts were performed in a small animal counter (Packard Model 446, Armac liquid scintillation detector). In order to measure the excretion of radioactivity following ^{59}Fe labeling, the animals were confined in solitary metabolic cages with stainless steel grid bottoms, and stool and urine were collected separately. In addition, radioiron excretion was also determined by whole body counts on the first and last days of the study, corrections being made for decay and differences in geometry. Recovery of radioactivity in the excreta as compared to the reduction in whole body radioactivity was over 90 per cent.

In preliminary studies employing ^{51}Cr-labeled erythrocytes, blood volume was found to be 6.4 ml/100 g body weight, and the proportion of circulating blood radioactivity trapped in the liver was 2.4 per cent. These factors were employed in subsequent studies to calculate net hepatic and total blood radioactivity. Residual tissue radioactivity was calculated from total body activity minus the combined radioactivity of liver, spleen, blood, and kidney.

Preparation of radioiron labels

Heat-damaged erythrocytes (DRBC). In vivo ^{59}Fe-labeled erythrocytes were prepared in rats by injecting 100 to 200 μCi of ^{59}Fe-citrate intravenously 5 days or more prior to harvesting the cells. Repeated injections of 50 to 100 μCi ^{59}Fe were given as required to maintain a specific radioactivity of 0.05 to 0.1 μCi of ^{59}Fe per mg of hemoglobin. Blood was removed in volumes of 0.5 to 1 ml, and after three washes in cool normal saline, cells were suspended in 5 vol of ACD formula B and incubated at 40°C for 15 min. After two more washings, the cells were resuspended in saline to a final concentration of 5 mg/ml hemoglobin and injected without delay in aliquots of 1 ml per animal.

Soluble ferritin. In vivo ^{59}Fe-labeled ferritin was prepared by injecting 100 to 200 μCi of ^{59}Fe-citrate into rats which had been given 12 mg of iron dextran the preceding week. The animals were sacrificed 24 h later, and purified radioactive ferritin was prepared by the method of Bjorklid & Helgeland [1.] Acrylamide gel electrophoresis of the ferritin preparation at pH 8.5 revealed a single protein band, and a single precipitin line was obtained on immunodiffusion against anti-

ferritin serum. The specific activity of ^{59}Fe-ferritin was 5 to 10 μCi/mg of iron. Shortly before injection, the purified ferritin concentrate was dissolved in sterile normal saline to a final concentration of 0.2 to 0.4 μCi/ml ^{59}Fe representing 40 μg of ferritin iron, and injected intravenously in aliquots of 1 ml per animal.

Transferrin. Freshly drawn rat plasma was incubated with $^{59}FeCl_3$ (specific activity 10 to 15 μCi/μg: Amersham Radiochemical Centre) which had been diluted in 0.005 N HCl and mixed with sufficient sterile 4% sodium citrate to ensure a molar ratio of citrate to iron in excess of 50 : 1. The final concentration of ^{59}Fe was 0.5 μCi/ml of plasma, and 1 ml aliquots were injected intravenously into all animals.

Initial processing of storage iron labels

This has been described in detail in previous communications [2, 3]. Briefly 89 per cent $\pm$ 1 of soluble ferritin and 81 per cent $\pm$ 2 of DRBC's were located to the liver and spleen within 1 h of intravenous injection. Following administration of DRBC's the cellular distribution of radioactivity in the liver determined by a quantitative autoradiographic method was 100 per cent in RE cells and 0 per cent in parenchymal cells. In contrast, both soluble ferritin and ^{59}Fe-transferrin were primarily located in parenchymal cells (97–100 per cent), with little labeling of RE cells (0–3 per cent). Thus, with one or the other source of radioiron, it was possible to selectively label either RE or parenchymal cells. The $t^{1/2}$ in plasma of intravenously injected soluble ferritin was 16 $\pm$ 1 min, and that of DRBC's was 2 $\pm$ 1 min. Within 1 day of injection, 72 per cent of ^{59}Fe-ferritin in the liver could be recovered in the ferritin fraction of the tissue homogenate, and the rest was soluble nonferritin iron. Hemoglobin in ^{59}Fe-DRBC was completely catabolized within 24 h of injection.

Ferritin iron was isolated from tissue homogenates by the method of Fulton and Ramsay [4] employing ammonium sulfate. For the chemical determination of ferritin iron, the ferritin fraction of tissue homogenates was incubated overnight in 3N HCl at 37°C and iron was determined colorimetrically. Total nonheme iron was determined by the method of Torrance and Bothwell [5]

Results

Table I describes the excretion and organ distribution of drugs incubated in vitro with tracer amounts of $^{59}FeCl_3$. Each compound was evaluated in 4 normal rats at a dose of 10 mg injected intramuscularly.

All four phenolic compounds showed an ability to promote the excretion of 60 to 80 per cent of the radioiron bound to them in vitro. In contrast with desferrioxamine, a substantial portion of excretion was found in the stool. Although these initial findings have shown that these iron chelators are able to compete with transferrin for the binding of ^{59}Fe, they gave no information on their ability to

Table 1

Excretion and Organ Distribution of the Radioiron Drug Complex

Compound	Urine	Stool	Blood	Liver	Residual
Ethylenediamine-N,N'-bis-(o-hydroxyphenylglycine)	0.1±0	4.8±0.1	27.1±3.6	12.4±1.7	54.6±6
(EHPG)	41.7±0.8	22.2±1.4	15.3±2.0	7.0±1.1	13.8±1.6
Ethylenediamine-N,N'-bis-(o-hydroxyphenylglycine)-dimethylester	35.9±2.2	28.9±1.7	12.9±1.3	5.2±0.6	17.1±2.5
N,N'-bis(2-hydroxybenzyl)-ethylene-diamine diacetic a. (HBED)	24.8±1.7	69.4±4.8	2.5±0.3	0.8±0.3	2.5±0.5
N,N'-bis(2-hydroxybenzyl-ethylene-diamine diacetic a. dimethyl ester	22.8±0.5	62.0±1.3	7.2±0.7	1.9±0.2	6.1±0.4
Desferrioxamine B	69.5±7.6	5.5±0.4	7.9±1.2	3.3±1.3	13.8±5.9

interact with iron storage in vivo. In order to study the ability of these drugs to promote the excretion of storage iron, iron stores were labeled with ^{59}Fe-ferritin or ^{59}Fe-DRBC.

Figure I shows the effect of iron chelating therapy on stores in hypertransfused rats labeled selectively by ^{59}Fe-DRBC. In untreated control rats about 60 per cent of the injected radioactivity was located to the liver and spleen, 7 per cent was in circulating RBC, and 20 per cent in residual tissues. Spontaneous ^{59}Fe excretion was 5 per cent and was mainly found in the stool. Each of the drugs tested was administered at a single intramuscular dose of 40 mg per animal given one hour after the radioiron probe. All measurements were made on day 6 of the study.

Desferrioxamine (DF) treatment resulted in a marked increase in both urinary and fecal excretion of radioactivity, and a simultaneous reduction in the radioactivity of spleen and liver. With the four phenolic ethylenediamine derivatives tested, there was some enhancement of urinary excretion, but the main portion of excreted radioactivity was found in the stool. This was 19 per cent for EHPG and 36–38 per cent for the other three compounds tested.

Drug interaction with parenchymal iron stores was studied in animals labeled with ^{59}Fe-ferritin (Fig. 2). In untreated control rats 88 per cent of the injected radioactivity was still located to the hepatic parenchyma at 6 days, and the spontaneous cumulative radioiron excretion was 4 per cent. Unlike ^{59}Fe-DRBC, in ^{59}Fe-ferritin labeled rats DF-induced radioiron excretion was limited almost entirely to the stool, and amounted to 4 per cent of the injected radioactivity. All four ethylenediamine derivatives tested produced a marked increase in fecal radioiron excretion. This amounted to 42 per cent of the injected radioactivity for

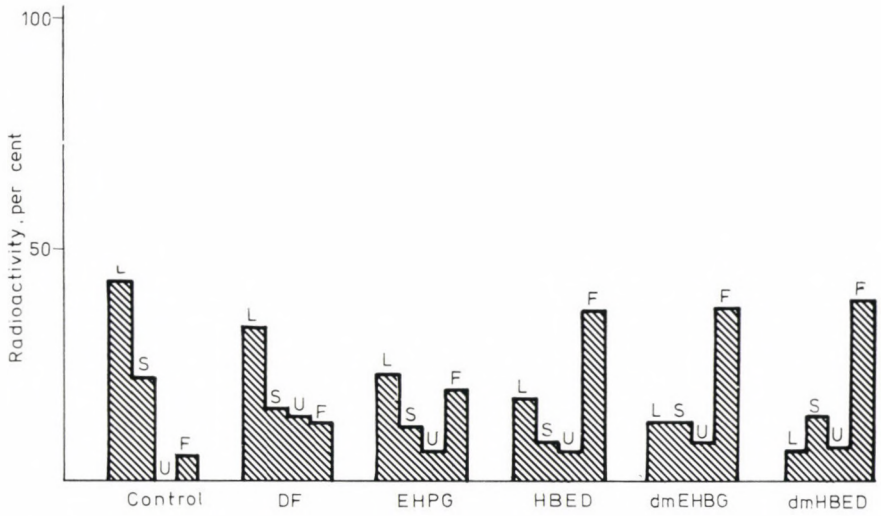


Fig. 1. Effect of iron chelating therapy on radioiron distribution in hypertransfused rats labeled by heat-denatured erythrocytes (^{59}Fe-DRBC). Each treatment represents a single i.m. injection of 40 mg drug per animal. DF = desferrioxamine; EHPG = ethylenediamine-N,N'-bis (o-hydroxyphenylglycine); HBED = N,N'-bis (2-hydroxybenzyl)ethylenediamine diacetic acid; and their dimethyl esters = dmEHPG and dmHBED. L = liver, S = spleen, U = urine, F = feces

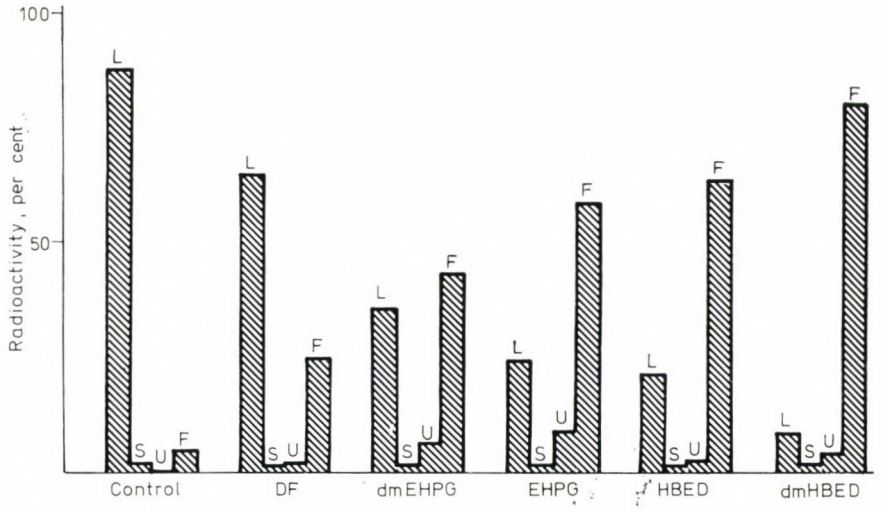


Fig. 2. Effect of iron chelating therapy on radioiron distribution in hypertransfused rats labeled with ^{59}Fe-ferritin. For explanation of abbreviations, see legend to Fig. 1

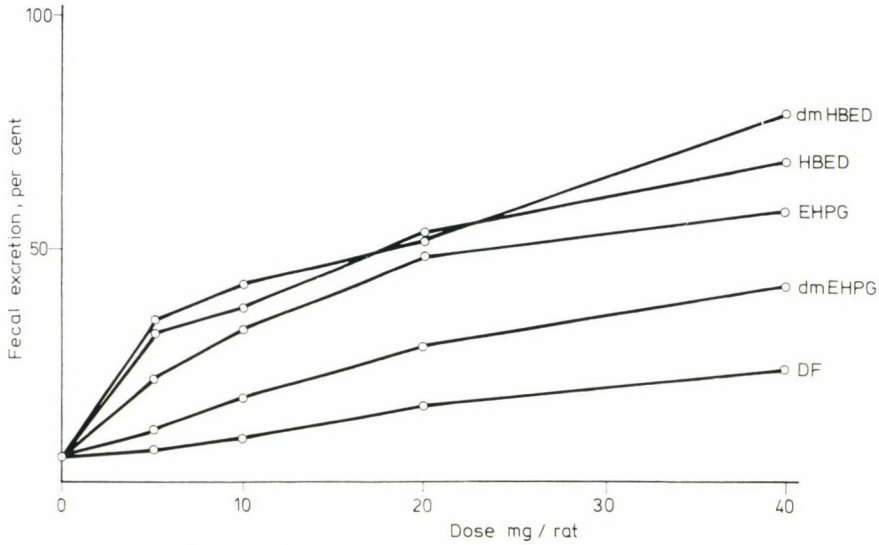


Fig. 3. Dose-response relationship in hypertransfused rats labeled by ^{59}Fe-ferritin. Per cent fecal excretion represents cumulative 6 day excretion after a single i.m. dose. For explanation of abbreviations see legend to Fig. 1

dimethyl EHPG and to as much as 80 per cent for dimethyl HBED. Residual hepatic radioactivity was 65 percent after DF, 35 per cent following dimethyl EHPG, and 8 per cent with dimethyl HBED.

The dose-response relationship for DF and the four phenolic drugs studied is shown in Figure 3. For comparison, spontaneous fecal excretion in untreated control animals is shown at dose zero. At all dose levels studied, the highest excretion was observed with dimethyl HBED, followed in descending order by HBED, EHPG, dimethyl EHPG, and finally DF. The most pronounced differences were observed at the lowest doses studied. For instance, net fecal excretion (treated minus control rats) at a single dose of 5 mg per animal was 1.9 per cent for DF as compared to 29.1 per cent for dimethyl HBED, a 15-fold difference in radioiron excretion. There was a linear dose-response relation with the less potent iron chelators such as DF and dimethyl EHPG. With the more powerful drugs such as EHPG, HBED, and dimethyl HBED there was also a steady increase in response with increasing dose, but with a relatively smaller effect at the higher dose range. This was most probably an artefact of the experimental model employed as at the highest dose levels hepatic radioiron reserves have been almost completely exhausted. Although most of the radioiron excretion was recovered in the stool, there was also some urinary excretion of radioactivity. This was roughly 11 per cent of the total excretion for EHPG and dimethyl EHPG, and about 3 per cent for HBED and dimethyl HBED.

The effect of oral as against intramuscular treatment was studied next. None of the orally administered drugs have shown any effect on urinary excretion of radioactivity. Similarly, fecal excretion following DF, EHPG, and HBED was negligible. However, the dimethyl esters of both EHPG and HBED showed significant enhancement of fecal excretion after oral administration. This amounted to 12.7 per cent of the total injected radioiron or 1/3 the effectiveness of intramuscular administration for dimethyl EHPG, and 52.0 per cent of total radioactivity or more than 2/3 the effectiveness of intramuscular injection for dimethyl HBED.

Finally, the effect of chelating therapy on hepatic iron stores was measured. About one half of the total hepatic nonheme iron stores was found in the ferritin iron fraction. Chelating therapy had a similar effect on both the total nonheme iron and ferritin iron stores, with the most striking reduction observed following dimethyl HBED. A single intramuscular injection of this drug resulted in a 72 per cent reduction in total hepatic nonheme iron and 74 per cent reduction in ferritin iron. This, however, was considerably less than the 91 per cent reduction in residual hepatic radioactivity in the same animals. Consequently, the specific activity of residual hepatic iron was much lower in HBED and dimethyl HBED-treated animals than in control, and DF-treated animals.

Discussion

In recent years, a number of studies have been devoted to the systematic screening of iron chelating compounds of potential usefulness [6–9]. Ideally, such compounds should be inexpensive, non-toxic, highly absorbable, and comparable in effectiveness to parenterally administered DF. Although useful information has been derived from in vitro systems employing cultured cells [6], animal models have been preferred by others because of their ability to yield information on intestinal absorption, toxicity, mechanism of iron chelation, and mode of excretion of the compounds studied. In the present study, we have employed a hypertransfused rat model in which distinct storage iron compartments could be labeled by selective radioiron probes. A number of potent iron chelating compounds have been identified with a preferential interaction with parenchymal iron stores. Some of these compounds are highly effective when given by the oral route, are apparently non-toxic, and may therefore fulfil all the expectations from an iron chelating drug of optimal quality.

Of the compounds showing encouraging preliminary results, the phenolic ethylenediamine derivatives EHPG [10], HBED [11] and their dimethyl esters seemed to be of particular interest.

The stability constants of the chelates formed by these aminopolycarboxylate ligands with ferric iron are very high [12]. Both EHPG and HBED were found to be very active in mouse and rat models of iron overload, although data are somewhat conflicting. Thus, EHPG was more active than HBED in the mouse screen despite the higher iron binding constants of the latter [8]. Esterification of

the active carboxyl group in both EHPG and HBED inhibited completely their activity in the in vitro Chang cell system [9], whereas in the in vivo rat model it has actually resulted in improved iron excretion by both oral and parenteral administration.

The experimental model employed in the present studies permits a distinction between parenchymal and RE iron chelation. In previous studies we have shown that it is possible to introduce radioiron specifically into hepatic parenchymal cells by the injection of ^{59}Fe-ferritin, and into RE cells by using ^{59}Fe-DRBC [2, 3, 14].

Both RE (^{59}Fe-DRBC), and parenchymal (^{59}Fe-ferritin) iron excretion was enhanced by all compounds tested. With DF, the standard reference compound, RE radioiron excretion was evenly distributed between urine and stool whereas parenchymal iron excretion was limited to the stool. These findings are in accordance with our previously reported observation [2], that DF-bound iron derived directly from RE cells in both rat and man is excreted by the kidneys whereas iron recycled from RE to parenchymal cells is chelated in situ and excreted in the bile. The phenolic EDTA analogues differed from DF in several respects. Firstly, and most importantly, they all were more effective. At the standard dose of 40 mg per rat, RE iron excretion was twice as much as with DF in 3 of the 4 compounds studied. Parenchymal iron excretion was even more impressive; 84 per cent of total body radioactivity was excreted after a single injection of dimethylHBED, the most active compound in the group, as compared to 25 per cent for DF.

The second difference from DF was the predominantly fecal excretion of iron with all compounds studied, irrespective of the source of radioiron. Lastly, the magnitude of radioiron excretion from parenchymal sources was much higher than that of RE radioiron for all of these compounds, in contrast to DF which showed an equal effect on both storage iron compartments. Thus, all of these chelating drugs were superior to DF both in the magnitude of response on a weight basis, and in their preferential interaction with parenchymal iron deposits. The improved chelating efficiency of the phenolic EDTA analogues was even more obvious in the study of dose-response relationships. At 5 mg per animal, the lowest dose studied, EHPG was 9 times, HBED 12 times, and dimethyl HBED 15 times more potent than DF.

Another important question was the ability of these drugs to induce iron excretion following oral administration. Neither DF, nor EHPG or HBED were able to promote radioiron excretion by oral treatment. However, the dimethyl esters of EHPG and HBED showed significant oral activity. DimethylEHPG retained 1/3, and dimethylHBED 2/3 of the effect of the same drug given by intramuscular injection. It is possible that an even better response could have been elicited if oral treatment was divided into multiple smaller doses instead of the single oral dose used in the present study.

These studies confirm and extend the recent reports of Pitt [8] and Grady and Jacobs [9] on the excellent in vivo chelating ability of dimethylHBED. In addition to a high oral absorption, it is 15 times as effective in promoting iron excre-

tion as DF, and its main target in the body is the hepatic parenchymal iron pool. Because of the predominantly biliary excretion of iron chelated by this drug, urinary iron measurements may be misleading, and fecal iron determinations will be an essential part of its clinical evaluation. If the apparent low toxicity of dimethyl-HBED in rodents is confirmed in higher species, and if its chelating efficacy in man is similar to that in experimental animals, this compound may represent a significant advance in the development of drugs for the clinical management of iron overload.

This study was supported by grant #2851 of the United States-Israel Binational Science Foundation.

References

1. Bjorklid, E., Helgeland, L.: Sex difference in the ferritin content of rat liver. *Biochem. Biophys. Acta 221*, 583 (1970).
2. Hershko, C.: Determinants of fecal and urinary iron excretion in desferrioxamine-treated rats. *Blood 51*, 415 (1978).
3. Hershko, C., Grady, R. W., Cerami, A.: Mechanism of iron chelation in the hypertransfused rat: definition of two alternative pathways of iron mobilization. *J. Lab. Clin. Med. 92*, 144 (1978).
4. Fulton, J. V., Ramsay, W. N. M.: The distribution of radioactive iron between ferritin and haemosiderin in rat tissues. *Biochem. J. 74*, 24 (1960).
5. Torrance, J. D., Bothwell, T. H.: A simple technique for measuring storage iron concentrations in formalinized liver samples. *S. Afr. J. Med. Sci. 33*, 9 (1968).
6. White, G. P., Jacobs, A., Grady, R. W., Cerami, A.: The use of Chang cells cultured in vitro to evaluate potential iron chelating drugs. *Brit. J. Haemat. 33*, 487 (1976).
7. Pitt, G. G., Cupta, G., Estes, W. E., Rosenkrantz, H., Metterville, J. J., Crumbiss, A. L., Palmer, R. A., Nordquest, K. W., Sprinkle Hardy, K. A., Whitcomb, D. R., Byers, B. R., Arceneaux, J. E. L., Gaines, C. G., Sciortino, C. V.: The selection and evaluation of new chelating agents for the treatment of iron overload. *J. Pharm. Exp. Ther. 208*, 12 (1979).
8. Pitt, C. G.: Structure and activity relationships of iron chelating drugs. In *Development of iron chelators for clinical use.* Martell, Anderson, Badman (eds). Elsevier-North-Holland, Amsterdam 1981. p. 105.
9. Grady, R. W., Jacobs, A.: The screening of potential iron chelating drugs. In *Development of iron chelators for clinical use.* Martell, Anderson, Badman (eds). Elsevier-North-Holland, Amsterdam 1981. p. 133.
10. Frost, A. E., Freedman, H. H., Westerback, S. J., Martell, A. E.: Chelating tendencies of N,N'-ethylenebis-2-(o-hydroxyphenyl)-glycine. *J. Amer. Chem. Soc. 80*, 530 (1958).
11. L'Eplattenier, F., Murase, I., Martell, A. E.: New multidentate ligands. VI. Chelating tendencies of N,N'-di(2-hydroxybenzoyl) ethylenediamine-N,N'-diacetic acid. *J. Amer. Chem. Soc. 89;* 837 (1967).
12. Martell, A. E.: The design and synthesis of chelating agents. In *Development of iron chelators for clinical use.* Martell, A. E., Anderson, W. F., Badman, D. G.: (eds). Elsevier-North Holland, Amsterdam 1981. p. 133.
13. Bothwell, T. H., Finch, C. A.: *Iron Metabolism.* Little, Brown and Co., Boston (1962).
14. Hershko, C., Cook, J. D., Finch, C. A.: Storage iron kinetics III. Study of desferrioxamine action by selective radioiron labels of RE and parenchymal cells. *J. Lab. Clin. Med. 81*, 876 (1973).

Haematologia 17 (1), pp. 35—55 (1984)

Globin Chain Precipitation, Deranged Iron Metabolism and Dyserythropoiesis in Some Thalassaemia Syndromes

S. N. WICKRAMASINGHE, MADELEINE HUGHES

Department of Haematology, St. Mary's Hospital Medical School, University of London, London W2 1 PG UK

(Received 10 December 1982: accepted 10 January 1983)

Electron microscope studies of the erythropoietic cells have revealed two morphologically distinct types of intracellular inclusion in homozygous β-thalassaemia and HbH disease, presumably reflecting different appearances of precipitated α- and β-chains, respectively. Inclusions resembling those seen in homozygous β-thalassaemia are also seen in HbE/β-thalassaemia and to a lesser extent in heterozygous β-thalassaemia. Dyserythropoietic changes including those indicative of deranged iron metabolism, are seen in some erythropoietic cells in all of the above-mentioned thalassaemia syndromes. Several of these changes are most prominent in homozygous β-thalassaemia and HbE/β-thalassaemia, less marked in heterozygous β-thalassaemia and least prominent in HbH disease. There are gross disturbances of proliferation and protein biosynthesis in erythroblasts in homozygous β-thalassaemia but not in HbH disease and this difference is reflected in a much greater degree of ineffective erythropoiesis in the former than in the latter. Results of studies into the relationship between the extent of α-chain precipitation in individual cells and both the disorder of proliferation and of protein biosynthesis are consistent with the view that these functional disturbances are related to the presence of precipitated α-chains and/or an expanded intracellular soluble α-chain pool. Some α-chains precipitate on centrioles and, presumably, thereby interfere with mitosis. The biochemical changes in the cell membrane of thalassaemic erythroblasts which result in their recognition and phagocytosis by macrophages remain to be determined.

Keywords: β-thalassaemia, erythropoiesis, HbH disease.

Introduction

It is now known that there are a variety of primary defects in the thalassaemia syndromes [1]. For example, while most but not all Asian patients with haemoglobin H (HbH) disease show a deletion of 3 of the 4 α-globin genes, some non-Asian patients show a combination of deleted and inactive genes. By contrast most cases of homozygous β-thalassaemia do not show a detectable deletion of the β-globin genes; in these cases the primary defect appears to be *(a)* a failure to produce β-mRNA due either to a defect of transcription or to an abnormality in the processing of a large molecular weight precursor of β-mRNA (known as β HnRNA), *(b)* the production of a functionally abnormal β-mRNA or *(c)* a translational defect (in the case of Ferrara β° thalassaemia). All these very different primary defects lead to a common secondary biochemical disturbance, namely a

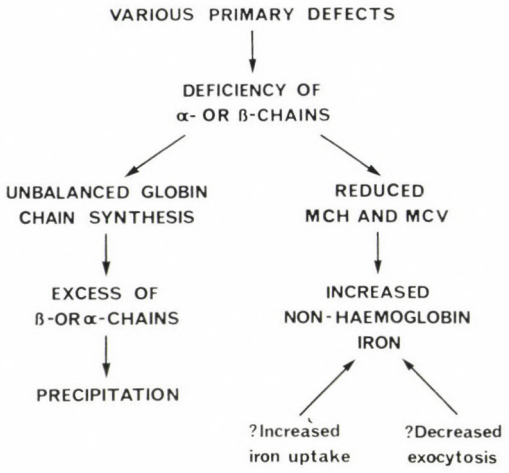


Fig. 1. Some biochemical disturbances affecting the erythroblasts in thalassaemia

depression of the rate of synthesis of one or more of the globin chains of the haemoglobin molecule. This secondary disturbance has two known consequences; an imbalance in globin chain synthesis and a reduction in the amount of haemoglobin produced per cell (Fig. 1). The unbalanced globin chain synthesis leads to an expansion of the intracellular soluble α- or β-chain pool and the precipitation of some of the excess globin chains within erythropoietic cells. The reduction in the quantity of haemoglobin produced per cell is associated with a low MCV and an increase in the non-haemoglobin iron within erythropoietic cells. It is not clear whether the increased non-haemoglobin iron merely represents the iron which could not be used for haemoglobin synthesis or whether there is an additional defect of iron uptake or exocytosis. The secondary disturbances summarized in Figure 1 are associated with an increased prevalence of dyserythropoietic changes as well as an increase in the extent of ineffective erythropoiesis.

In the present paper we describe ultrastructural evidence for the existence of the above-mentioned secondary disturbances in heterozygous and homozygous β-thalassaemia, HbE/β-thalassaemia and HbH disease. We also discuss the possible relationship between globin chain precipitation, iron overload of the erythron and ineffective erythropoiesis in these syndromes.

Globin Chain Precipitation

Several investigators have determined the degree of imbalance between α- and non-α-chain synthesis in the various thalassaemia syndromes by incubating blood reticulocytes with ^{3}H-leucine and then measuring the radioactivity in the α- and non-α-chains after a biochemical separation of these chains. In normal blood

reticulocytes, the ratio of the radioactivity in the α-chains to that in the β-chains (α : β ratio) is about 1, indicating that globin chain synthesis is more or less balanced. By contrast, this ratio or the α + β : γ ratio varies between 1.5 and 30.0 in homozygous β-thalassaemia [2–6], 1.5 and 2.5 in heterozygous β-thalassaemia [7–10] and 1.7 and 3.7 in HbE/β-thalassaemia [6, 11]. These high ratios indicate that there is an imbalance between α- and β-chain synthesis, with an excess of α-chains. By contrast, in HbH disease, the α : β ratio is 0.2–0.5 [2, 12–15], indicating the synthesis of an excess of β-chains relative to α-chains. Similar studies of bone marrow cells have indicated that there is also unbalanced globin chain synthesis in the erythroblasts in all of these thalassaemia syndromes [10, 14, 16–18].

There is now good evidence that inclusions consisting of globin chains are formed within the erythropoietic cells of patients with thalassaemia as a result of precipitation from an expanded free α- or β-chain pool. The initial evidence for globin chain precipitation came from studies of patients with homozygous β-thalassaemia by Fessas and his colleagues. These workers showed that if bone marrow cells from patients with homozygous β-thalassaemia were stained supravitally with methyl violet and then examined under the light microscope, a proportion of the erythroblasts contain violet-coloured inclusions [19]. In subsequent studies, they showed that such inclusions consist of haemoglobin-like material in which the protein consists predominantly, if not exclusively, of precipitated α-chains [20, 21]. Precipitation begins in the basophilic erythropoietic cells and both the percentage of erythroblasts containing precipitates and the average quantity of precipitate per affected cell increases in cell classes of increasing maturity [19, 22]. Ultrastructural studies of circulating erythroblasts [23] and of bone marrow erythropoietic cells [24] have confirmed the presence of intracellular inclusions (probably consisting of α-chains), in homozygous β-thalassaemia. In addition, they have shown that α-chain precipitates are present within the nuclei of some late erythroblasts [24, 25]. Recent ultrastructural studies have also revealed the presence of intracellular inclusions (probably consisting of β-chains) within the erythropoietic cells of cases of HbH disease [26, 27].

The inclusions present in the erythropoietic cells of patients with homozygous β-thalassaemia and HbH disease are ultrastructurally quite different, presumably reflecting different appearances of precipitated α- and β-chains, respectively. The α-chain precipitates appear as multiple small rounded masses of amorphous electron-dense material which tend to fuse with each other to form larger masses (Fig. 2). The β-chain precipitates appear to consist of bifurcating branches which radiate outwards from a central point (Fig. 3a) and it seems likely that the varying appearances of such precipitates under the electron microscope (Fig. 3b) result from varying positions of sectioning of this stellate structure. The percentage of marrow cell profiles containing α- and β-chain precipitates in homozygous β-thalassaemia and HbH disease, respectively, are given in Table 1.

Inclusions resembling those seen in homozygous β-thalassaemia are also seen in HbE/β-thalassaemia [29] and to a lesser extent in heterozygous β-thalassaemia [30]. It is evident from Table 1 that in HbE/β-thalassaemia 23 per cent of erythro-

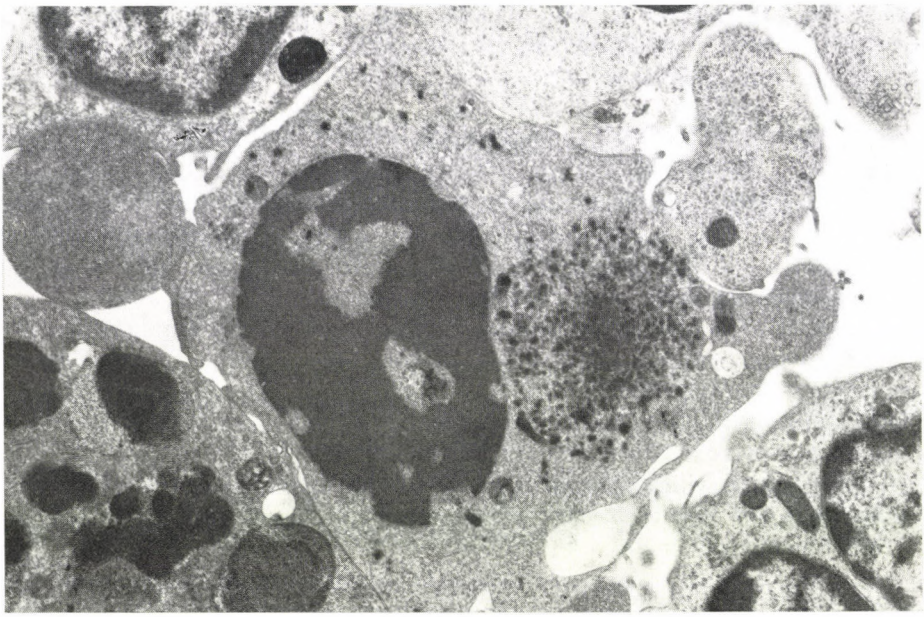


Fig. 2. Late erythroblast from the bone marrow of a patient with homozygous β-thalassaemia. The cytoplasm contains precipitated α-chains. × 10 900

Table 1

Prevalence of precipitates within the erythropoietic cells in the bone marrow of patients with thalassaemia syndromes

Diagnosis	No. of cases	% marrow cell profiles containing α- or β*-chain precipitates		Reference
		Erythroblasts	Non-nucleated profiles**	
Homozygous β-thalassaemia	4	29.8 21.6–40.1	40.8 25.7–58.1	29
HbE/β-thalassaemia	2	22.9 14.4, 27.6	49.9 37.0, 54.9	29
Heterozygous β-thalassaemia	6	1.3 0.2– 2.8	11.4 3.6–25.5	30
HbH disease	3	5.4 1.4– 8.7	13.0 6.1–19.4	28

* In the case of HbH disease only

** Containing ribosomes and mitochondria but not including part of a nucleus (i.e. profiles of reticulocytes and some erythroblasts)

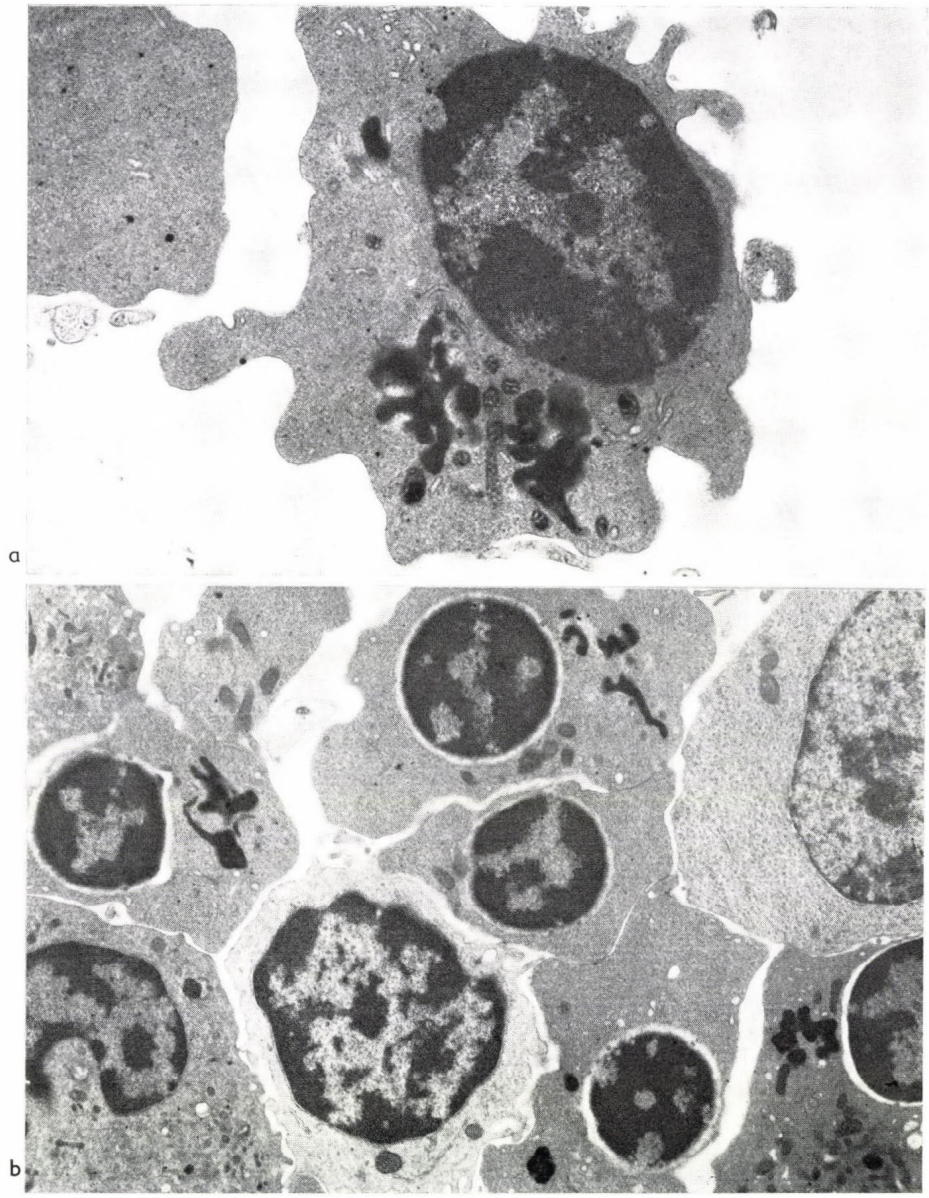


Fig. 3. Late erythroblasts from the bone marrow of two cases of HbH disease. (a) Stellate intracytoplasmic inclusion probably composed of precipitated β-chains. × 14 400. (b) Various other appearances of precipitated β-chains × 6700

blast profiles and 51 per cent of non-nucleated profiles contained α-chain precipitates and that these figures are similar to the corresponding values in homozygous β-thalassaemia. By contrast, in heterozygous β-thalassaemia only 1 per cent of erythroblast profiles and 10 per cent of marrow reticulocyte profiles contained precipitate. Furthermore, whereas precipitation commences early during erythropoiesis in homozygous β-thalassaemia and HbE/β-thalassaemia, it is found only in the most mature of the late erythroblasts in β-thalassaemia trait.

Ultrastructural Evidence of Deranged Iron Metabolism

The electron microscope reveals various forms of iron-containing material within normal erythropoietic cells. These consist of individual ferritin molecules scattered within the cytoplasm, intracytoplasmic aggregates of ferritin molecules (siderosomes or ferritin bodies), ferritin molecules lining rhopheocytotic vesicles, ferritin molecules within lysosomes and, in a very rare cell, iron-containing particles within mitochondria [31]. The siderosomes may or may not be limited by a membrane and the ferritin molecules within some of them are very closely packed with intercore distances less than 5 nm and should perhaps be described as haemosiderin rather than ferritin. There are about 500-1000 ferritin molecules in individual sections of normal proerythroblasts, and siderosomes are rarely seen in such sections. By contrast, in the late polychromatic erythroblasts there are fewer free ferritin molecules and a greater number of ferritin aggregates [31]. Ferritin molecules may also be found within autophagic vacuoles (Fig 4a). In a recent study of marrow from 3 normal volunteers we found that 8.3 per cent of erythroblast profiles contained siderosomes with diameters ranging between 80 and 290 nm (Fig. 4b). Iron-laden mitochondria were found in less than 0.2 per cent erythroblast profiles (Fig. 4c) and were somewhat more frequent in marrow reticulocytes (Fig. 4d).

Ultrastructural evidence of iron-overload in the erythropoietic cells of patients with homozygous β-thalassaemia was first reported by Bessis et al. [32] almost 25 years ago. More detailed descriptions were subsequently published by others [23, 24] but none of these studies have included quantitative data. The ultrastructural features that have been reported are: *(1)* increased amounts of dispersed ferritin molecules in the cytoplasmic matrix, *(2)* an increased number and size of ferritin aggregates within the cytoplasm (Figs 5a and 5b), *(3)* the presence of ferritin molecules within some globin-chain precipitates (Fig. 5c) and *(4)* an increased proportion of erythroblasts containing ferritin and other iron-containing material within their mitochondria. Studies from our laboratory have shown that all of these ultrastructural features of iron-overload are also found in HbE/β-thalassaemia [29], β-thalassaemia trait [30] and HbH disease [26, 27], except that the number of individual ferritin molecules per unit area of the cytoplasm of intermediate and late erythroblasts may not be increased in some of these syndromes [28]. The occurrence of ferritin molecules within some intra-erythro-

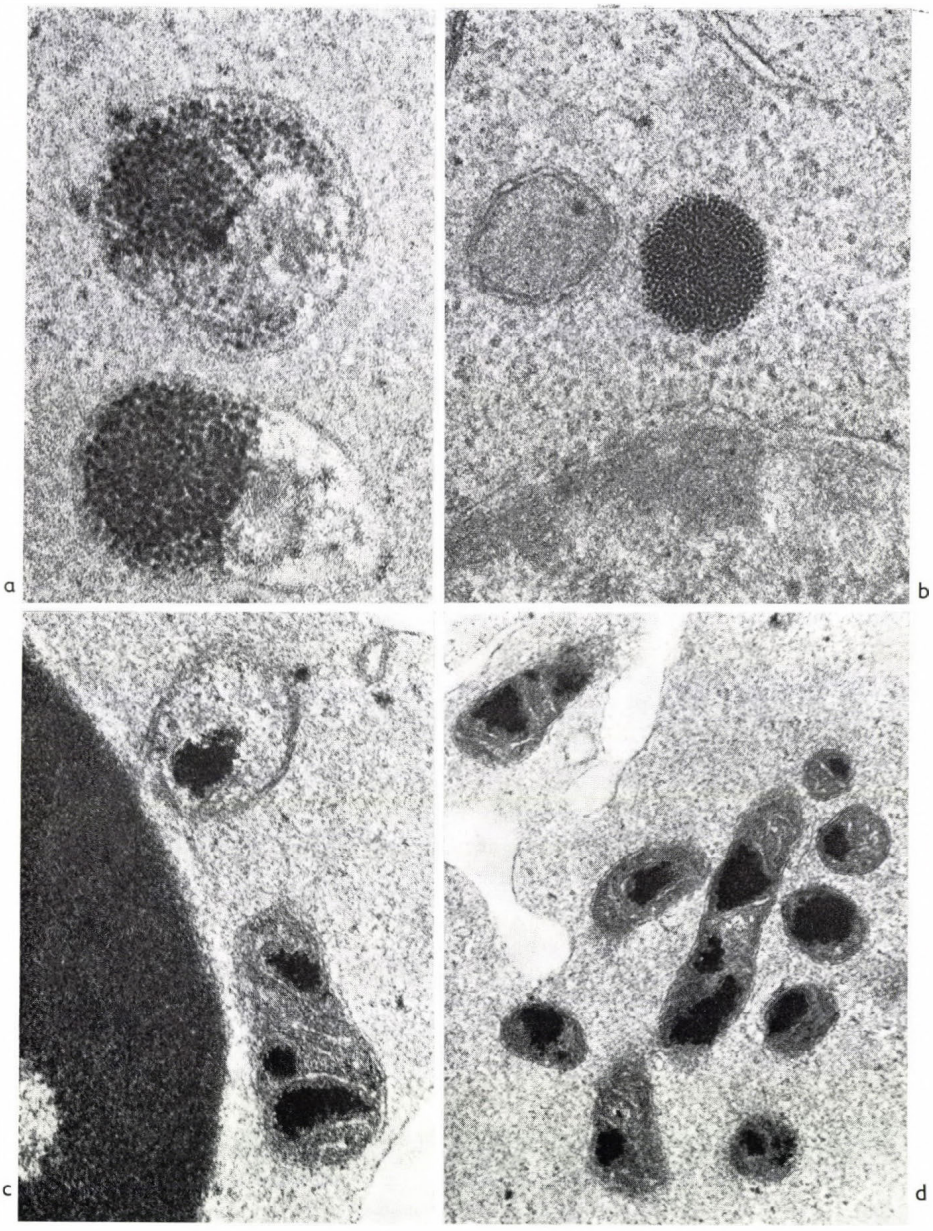


Fig. 4. Organelles of normal erythroblasts (a–c) and marrow reticulocytes (d) which contain or may contain 'visible' iron. (a) Two autophagic vacuoles containing ferritin. × 142 000. (b) Siderosome. × 79 000. (c) and (d) Iron-laden mitochondria (rare). (c) × 66 000; (d) × 38 600

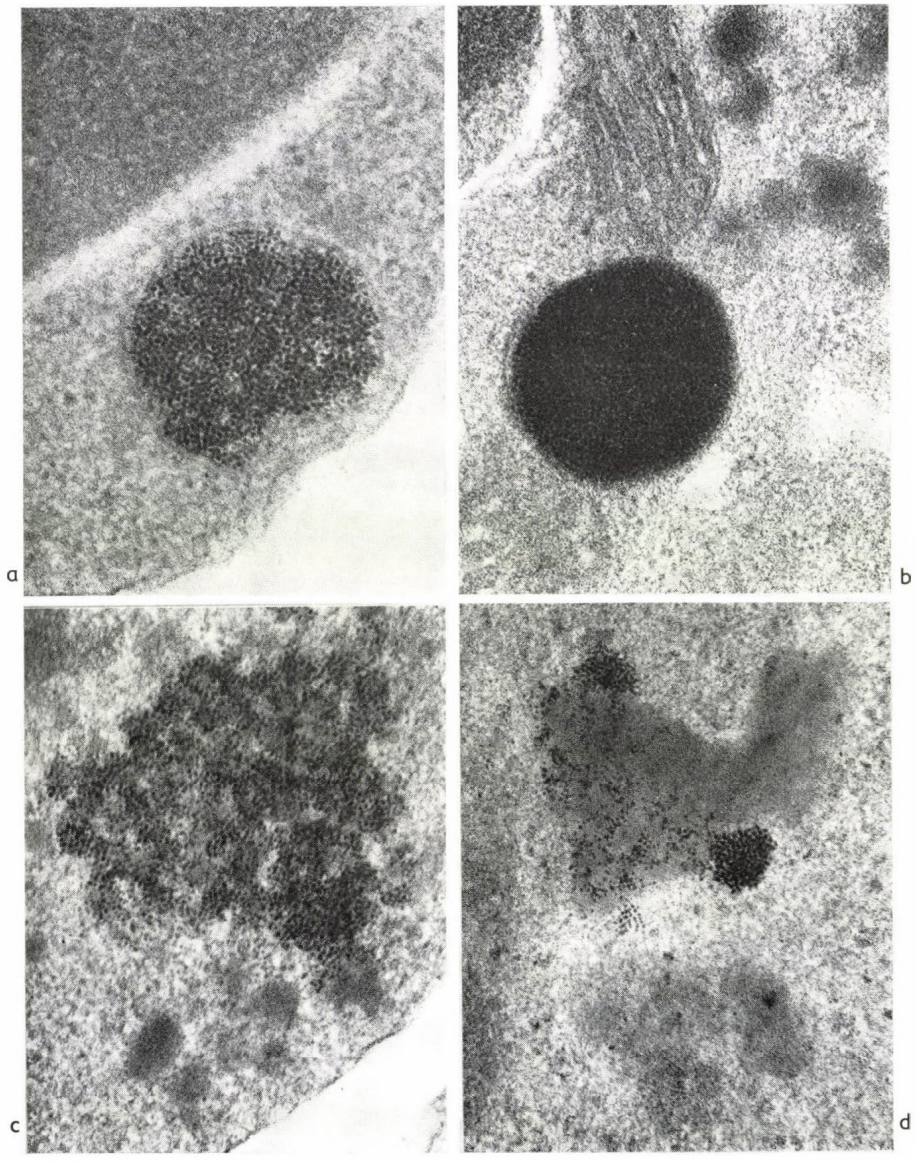


Fig. 5. Increased iron content of erythroblasts in thalassaemic bone marrow. (a) and (b) Abnormally large siderosomes (same magnification as normal siderosome in Fig. 4b). (c) Ferritin molecules within precipitated α-chains (homozygous β-thalassaemia) $\times$ 77 100. (d) Ferritin molecules within precipitated β-chains (HbH disease). $\times$ 54 400. Note that a few of the many small masses of precipitated α-chains (c) and one of the two large masses of precipitated β-chains (d) do not contain ferritin

blastic β-chain precipitates in HbH disease is illustrated in Figure 5d. The present authors have recently obtained some quantitative data on the proportion of erythroblast profiles containing siderosomes or iron-laden mitochondria and on the average diameter of siderosomes in various thalassaemia syndromes. These are given in Table 2.

Table 2

Some ultrastructural features encountered in consecutive erythroblast profiles in normal volunteers and various thalassaemia syndromes [28]. The sections were 90–150 nm thick

Ultrastructural feature	Normal (3 cases)*	Homozygous β-thalassaemia (2 cases)	HbE/β-thalassaemia (1 case)	Heterozygous β-thalassaemia (2 cases)	HbH disease (2 cases)
Erythroblasts with					
a) Siderosomes	8.3 (3.0–11.6)	25.5	16.7	5.1	22.3
b) Iron-laden mitochondria	0	4.8	19.4	3.8	1.8
c) Autophagic vacuoles	21.8 (5.0–36.8)	55.9	47.2	31.8	22.3
d) Autophagic vacuoles $\geq$290 nm in diameter	3.4 (3.0– 4.2)	32.4	20.8	15.3	10.7
e) Duplication of nuclear membrane >1 μm in length	0	0	2.8	1.9	0
f) Attachment to intercellular spindle bridge	0	1.4	0	3.8	2.7
g) Intercellular interactions	4.0 (0.8– 7.6)	11.7	2.8	14.7	4.5
Diameter of siderosomes (nm)					
Mean	185	192	204	200	235
Range	80— 291	73–447	98–400	100–366	100–446
Total number of cell profiles assessed	350	145	72	157	112

* Observed ranges given within brackets

Individual intracytoplasmic ferritin molecules are easily seen when unstained sections from material which has been post-fixed in osmium tetroxide are examined in the electron microscope. We found that the number of dispersed ferritin molecules per unit area of the cytoplasmic matrix of thin sections of intermediate and late erythroblasts from normal marrow varies markedly from 0–25 per 0.1 μm^2 (mean, 5.5; n = 45 cells); the sections were 90–150 nm thick and the areas of cytoplasm selected for study were free of rhopheocytotic vesicles and siderosomes.

In 2 cases each of homozygous β-thalassaemia and HbH disease the corresponding values for these parameters were, respectively, 4–57 per 0.1 μm^2 (mean, 18.0; n = 31 cells) and 1.8–37.0 per 0.1 μm^2 (mean, 16.6; n = 21 cells). Evidently some erythroblast profiles of cases of these two thalassaemia syndromes contain increased numbers of dispersed ferritin molecules in the cytoplasmic matrix. By contrast, no differences from normal were observed in the number of ferritin molecules per unit area of the cytoplasm in 2 cases of β-thalassaemia trait and 2 cases of HbE/β-thalassaemia studied.

Dyserythropoiesis

Minor ultrastructural 'peculiarities' are seen in some of the erythroblasts of normal individuals. Thus in a recent study of 350 consecutive erythroblast profiles in three marrow aspirates obtained from haematologically normal, healthy volunteers, the present authors found small autophagic vacuoles (Figs 4a, 6a and 6b) in 22 per cent of the profiles and slight to substantial degrees of myelinisation of the nuclear membrane (Fig. 7a) in 12 per cent. The contents of the autophagic vacuoles varied considerably and included ferritin molecules, myelin figures, amorphous material of varying electron density and 'empty' regions. The nuclear membrane was indistinct or appeared to be absent in small stretches in 2.9 per cent of the erythroblasts. About 2 per cent of the erythroblasts showed short (250–910 nm) stretches of 'duplication' of the nuclear membrane and frequently these 'duplicated' portions were continuous with endoplasmic reticulum. Occasional erythroblasts (1.7 per cent) showed intranuclear clefts which were 260–520 nm long (Fig. 7b). There were regions of interaction between some adjacent erythroblasts with ferritin molecules between the opposing cell membranes. The cell membranes in these regions were straight or convoluted. Occasionally, such interactions were present around finger-like protrusions of the cytoplasm of one erythroblast into another (Figs 7c and 7d). Inter-erythroblastic spindle bridges or karyorrhectic nuclei were not seen amongt the 350 cells studied consecutively, reflecting their low frequency in normal marrow. Although all of the above-mentioned ultrastructural features are sometimes referred to as dyserythropoietic it is likely that at least the autophagic vacuoles and the myelinisation of membranes are the morphological manifestations of degradative processes which play an important physiological role during normal erythropoiesis. Autophagic vacuoles are also seen in normal marrow reticulocytes. These resemble the autophagic vacuoles in normal erythroblasts except that they tend to be larger and more frequently contain mitochondria.

Some or all of the ultrastructural features mentioned in the preceding paragraph have been reported to be more prominent than in normal marrow in the four thalassaemia syndromes discussed in this paper [23–24, 26–30]. The dyserythropoietic changes other than those of iron overload in the erythropoietic cells, which have been reported in thalassaemia include abnormally large autophagic

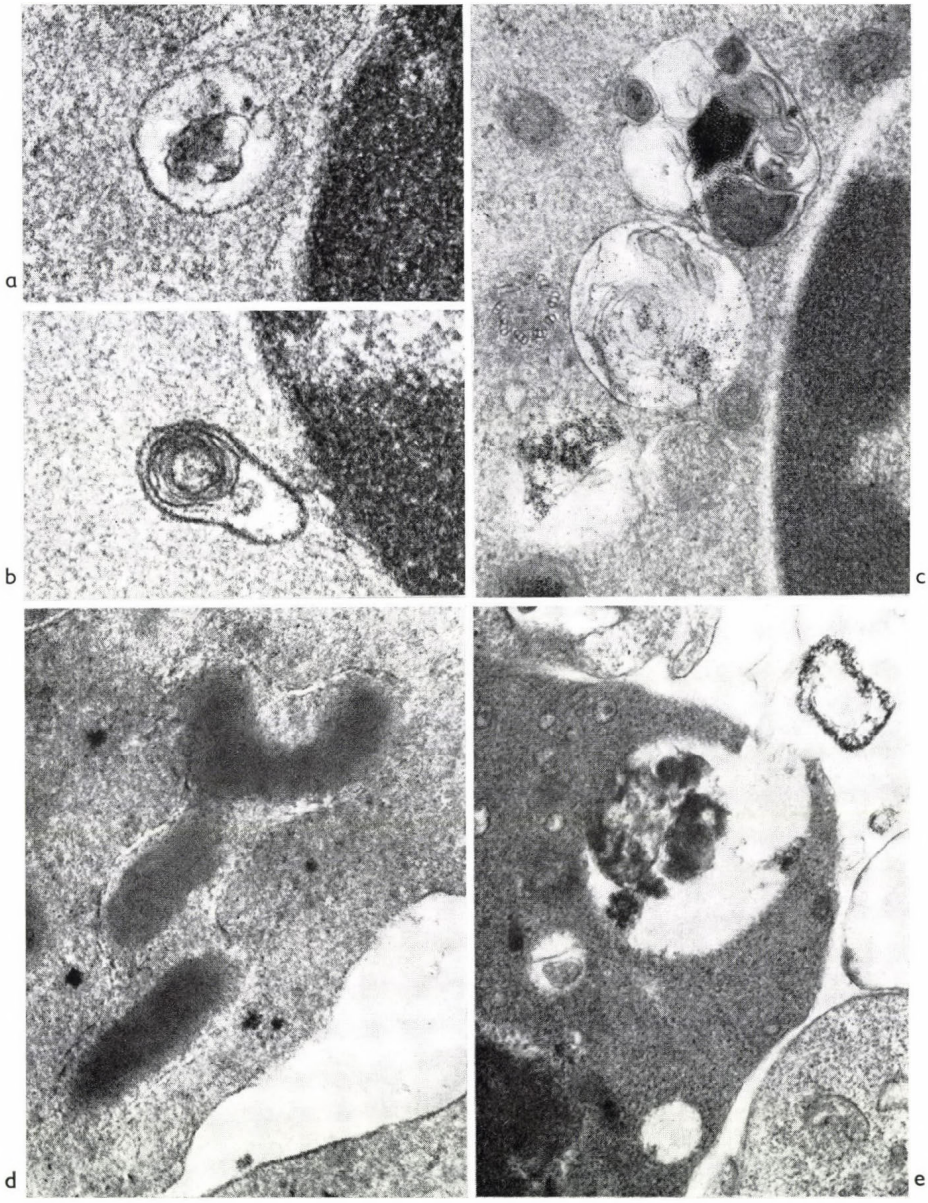


Fig. 6. Varying appearances of autophagic vacuoles in erythroblasts. (a) Small vacuole containing membranous material and electron-dense areas (normal marrow). × 74 800. (b) Small vacuole containing myelin figure (normal marrow). × 75 400. (c) Three large vacuoles containing varying amounts of membrane, ferritin molecules and electron-dense material (β-thalassaemia trait). × 40 200. (d) Large vacuole enclosing precipitated β-chains (HbH disease). × 47 000. (e) Autophagic vacuole in communication with the exterior at an area of attachment to the cell membrane (homozygous β-thalassaemia) × 25 300

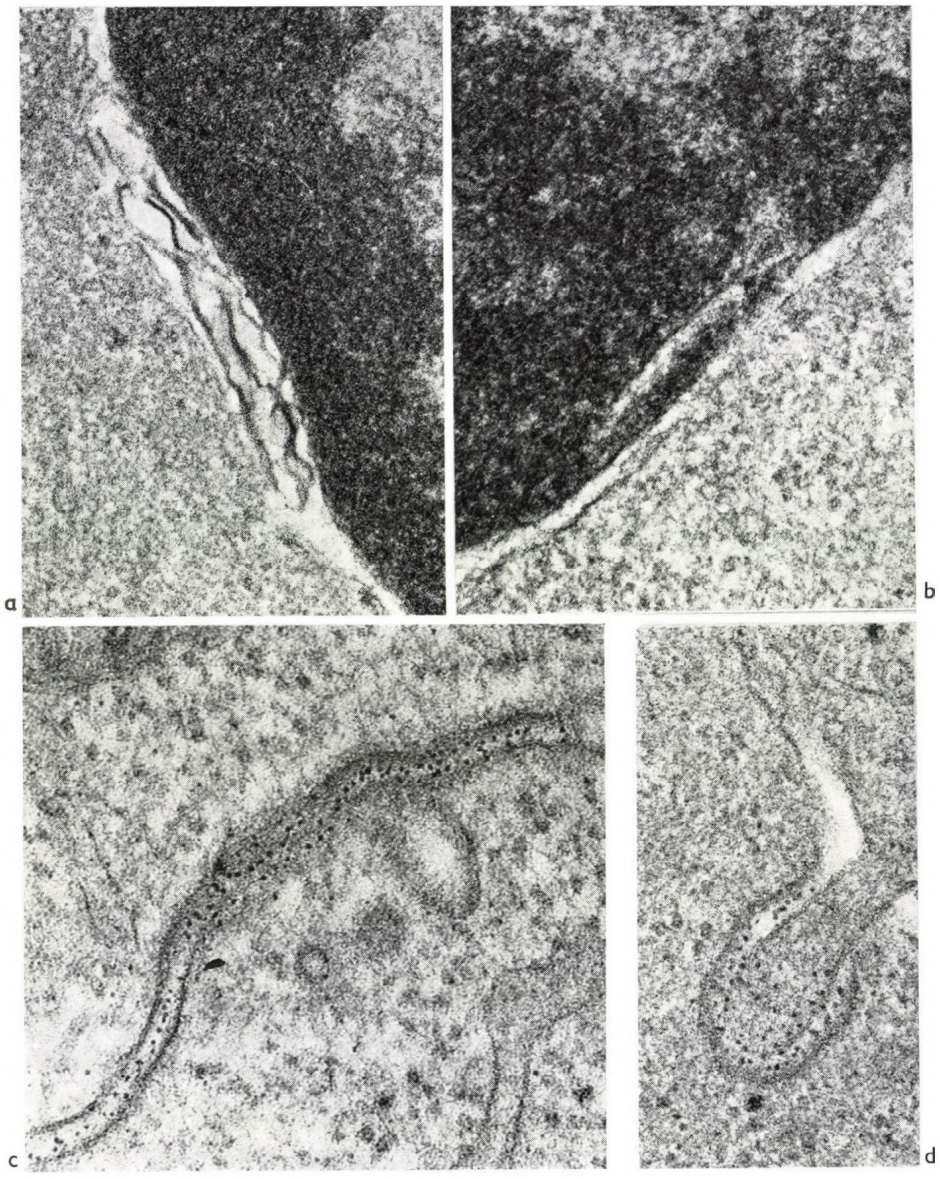


Fig. 7. Ultrastructural 'peculiarities' affecting some normal erythroblasts. (a) Myelinisation of a small part of the nuclear membrane. × 78 600. (b) Small intranuclear cleft. × 95 300. (c) Area of intercellular interaction with ferritin molecules between the opposing cell membranes. × 106 200. (d) Finger-like protrusion of the cytoplasm of one cell into another with intercellular ferritin at the area of interaction. × 106 200

vacuoles (Figs 6c–6e), abnormally long intranuclear clefts, an increased degree of myelinisation of parts of the nuclear membrane, duplication or loss of substantial parts of the nuclear membrane, the presence of cytoplasmic organelles within nuclear territory and an increased frequency of intererythroblastic spindle bridges. The autophagic vacuoles in the β-thalassaemia syndromes very occasionally contain α-chain precipitates and those in HbH disease sometimes contain β-chain precipitates (Fig. 6d). A few of these vacuoles are attached to the cell membrane or are in communication with the exterior as a result of membrane break-down at a site of attachment to the cell membrane [29, 33] (Fig. 6e). In HbH disease, some of the masses of precipitated β-chains are incompletely surrounded by a cytoplasmic membrane.

Some idea of the different degrees of dyserythropoiesis occurring in the β-thalassaemia syndromes and HbH disease can be gained from Table 2 which gives the results of a detailed analysis of 72–157 consecutive erythroblast profiles in each of these conditions. It is evident from Table 2 that, in general, the prevalence of dyserythropoietic changes was greatest in homozygous β-thalassaemia and HbE/β-thalassaemia and least in HbH disease.

Functional Disturbances

Erythroblast proliferation

The technique of combined Feulgen microspectrophotometry and ^{3}H-thymidine autoradiography has revealed major abnormalities in the cell cycle distribution of early polychromatic erythroblasts in homozygous β-thalassaemia [34, 35]. These abnormalities consist of *(1)* a marked pile-up of cells in the G_1 phase suggesting a substantial prolongation of the G_1 phase or a failure of some G_1 cells to enter the S phase or both, and *(2)* an excess of cells in the G_2 phase compared to a reduced number in the S phase, suggesting a failure of some G_2 cells to enter mitosis. A less marked accumulation of early polychromatic cells in the G_1 phase has also been reported in heterozygous β-thalassaemia [35]. By contrast, no abnormality has been found in the cell cycle distribution of the erythroblasts of two cases of HbH disease [27].

Impairment of protein biosynthesis

A severe depression or arrest of protein biosynthesis occurs in 16–45 per cent of the non-dividing late polychromatic erythroblasts of patients with homozygous β-thalassaemia [22]. Substantial numbers of erythroblasts showing a severe depression or arrest of protein synthesis were not found in 3 cases of HbH disease [26], but the available data are not quantitative and do not exclude a moderate degree of impairment of protein synthesis in some of the cells.

Ineffective Erythropoiesis

An important consequence of the various morphological and functional abnormalities affecting thalassaemic erythroblasts, is that an increased proportion of the erythropoietic cells does not mature into circulating erythrocytes but dies within the marrow. The degree of increase of ineffective erythropoiesis has been quantitated in some thalassaemia syndromes by determining ferrokinetic and erythrokinetic indices; it appears to be gross in homozygous β-thalassaemia [36, 37] and substantial but much less marked in heterozygous β-thalassemia [37–40] and probably also in HbH disease [41]. Ultrastructural studies have revealed the presence of erythroblasts at various stages of degradation within the bone marrow macrophages in both homozygous β-thalassaemia and HbE/β-thalassaemia [24, 29] (Fig. 8).

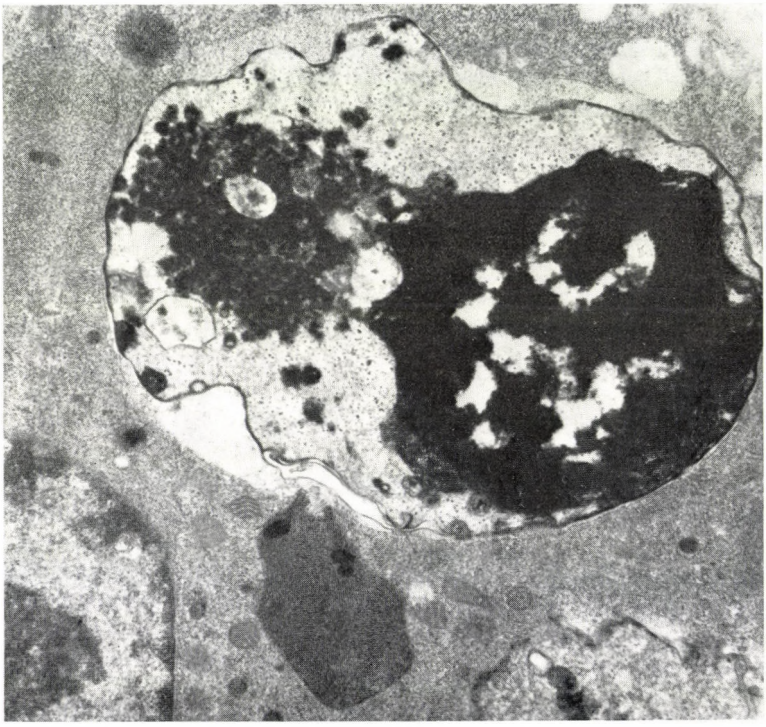


Fig. 8. Macrophage from the bone marrow of a case of homozygous β-thalassaemia showing a phagocytosed late erythroblast. The erythroblast has undergone some degradation but still contains intracytoplasmic α-chain precipitates. × 13 100

Relationship between Globin Chain Precipitation and Ineffective Erythropoiesis

The possibility that intracellular α-chain precipitates interfere with the entry of G_1 cells into S has been investigated in homozygous β-thalassaemia by correlating the extent of α-chain precipitation in individual cells with their capacity for DNA synthesis. Light microscope autoradiographic studies of marrow cells which had been incubated with ^{3}H-thymidine and subsequently stained supravitally with methyl violet showed that none of the early polychromatic erythroblasts containing moderate or large quantities of precipitated α-chains incorporated ^{3}H-thymidine and that 25 per cent of cells that did incorporate ^{3}H-thymidine contained small quantities of precipitate. However, the ^{3}H-thymidine labelling index of early polychromatic cells without any detectable precipitate was very low indicating that many cells without detectable precipitates were held up in G_1 [22]. One possible explanation for the latter observation is that the G_1 arrest may be related more to the expanded pool of soluble α-chains rather than to the precipitates formed from this pool. This view is supported by the observation that although the early polychromatic erythroblasts of heterozygotes for β-thalassaemia do not contain α-chain precipitates [30], they display a moderate accumulation in the G_1 phase [35]. Nevertheless, the tight relationship between the presence of moderate or large quantities of precipitate and the absence of ^{3}H-thymidine incorporation in homozygous β-thalassaemia is quite impressive and has been confirmed by electron microscope autoradiographic studies of ^{3}H-thymidine labelled marrow cells [42].

The relationship between the extent of α-chain precipitation and the depression of protein biosynthesis has been investigated by high-resolution autoradiographic studies of ^{3}H-leucine labelled marrow cells from patients with homozygous β-thalassaemia [42]. Such studies have indicated that the majority of the profiles containing moderate or large quantities of α-chain precipitates are unlabelled or weakly labelled. These data suggest, but do not prove, a causal relationship between the failure of protein synthesis and the presence of moderate to large quantities of α-chain precipitates or an associated expansion of the soluble intracellular α-chain pool.

That α-chain precipitates could have a damaging effect on erythroblast function is suggested from detailed ultrastructural studies of the erythroblasts of patients with the β-thalassaemia syndromes. For example, some cells show a loss and others a duplication of the portion of the nuclear membrane adjacent to a mass of precipitated α-chains [24, 25, 29, 30] (Figs 9a and 9b). Furthermore, recent studies [43] have shown that α-chain precipitates may form around centrioles (Fig. 9c). Clearly, the formation of substantial quantities of precipitate on and around the centrioles would be expected to interfere with the normal separation of the centrioles in early prophase, hinder the formation of the mitotic spindle and thus prevent the progress of cells through mitosis.

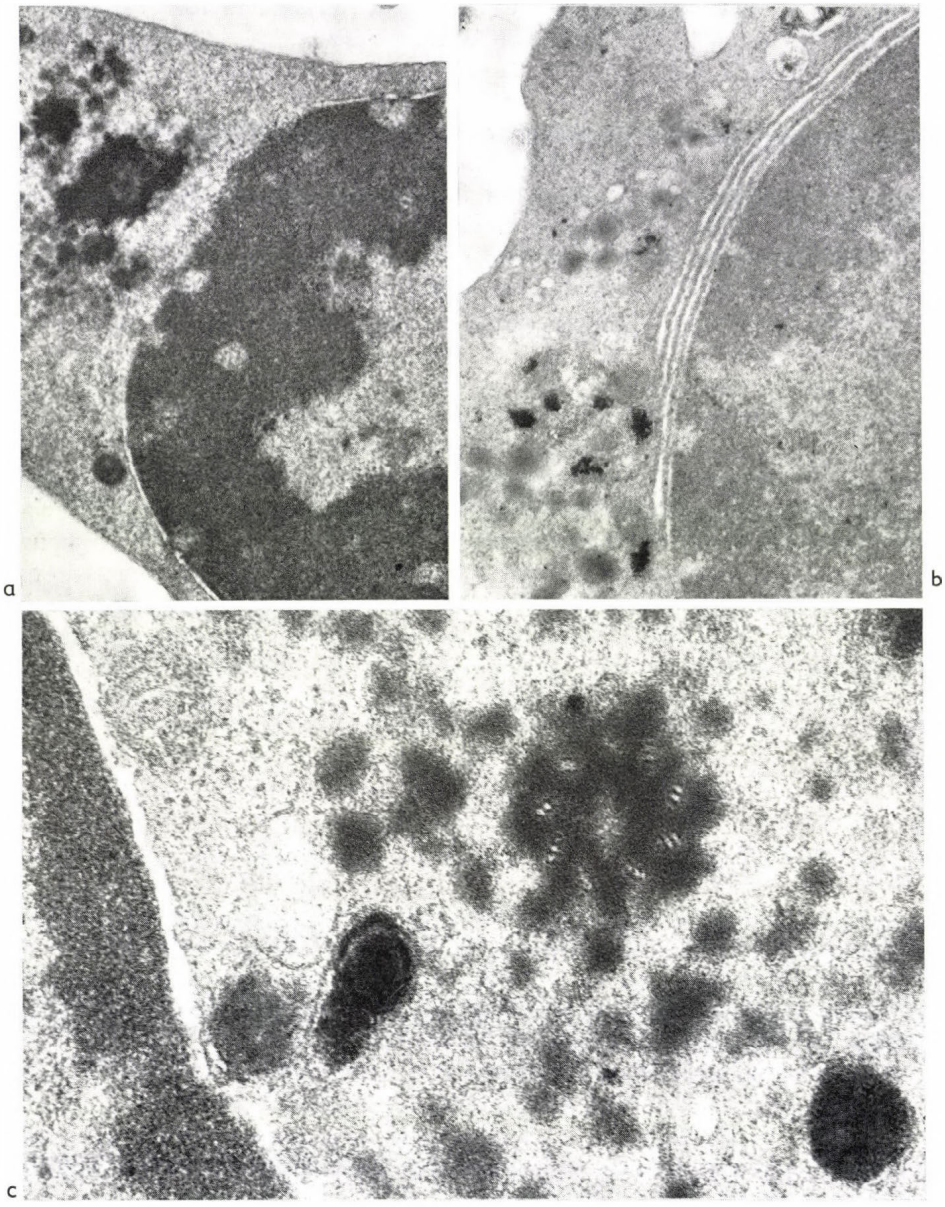


Fig. 9. Ultrastructural abnormalities in erythroblasts from β-thalassaemia syndromes. (a) Los of the nuclear membrane adjacent to precipitated α-chains (HbE/β-thalassaemia). × 25 400 (b) Reduplication of the nuclear membrane near masses of precipitated α-chains (HbE/β-thalassaemia). × 25 200. (c) Formation of α-chain precipitates on and around a centriole (homozygous β-thalassaemia) × 79 000

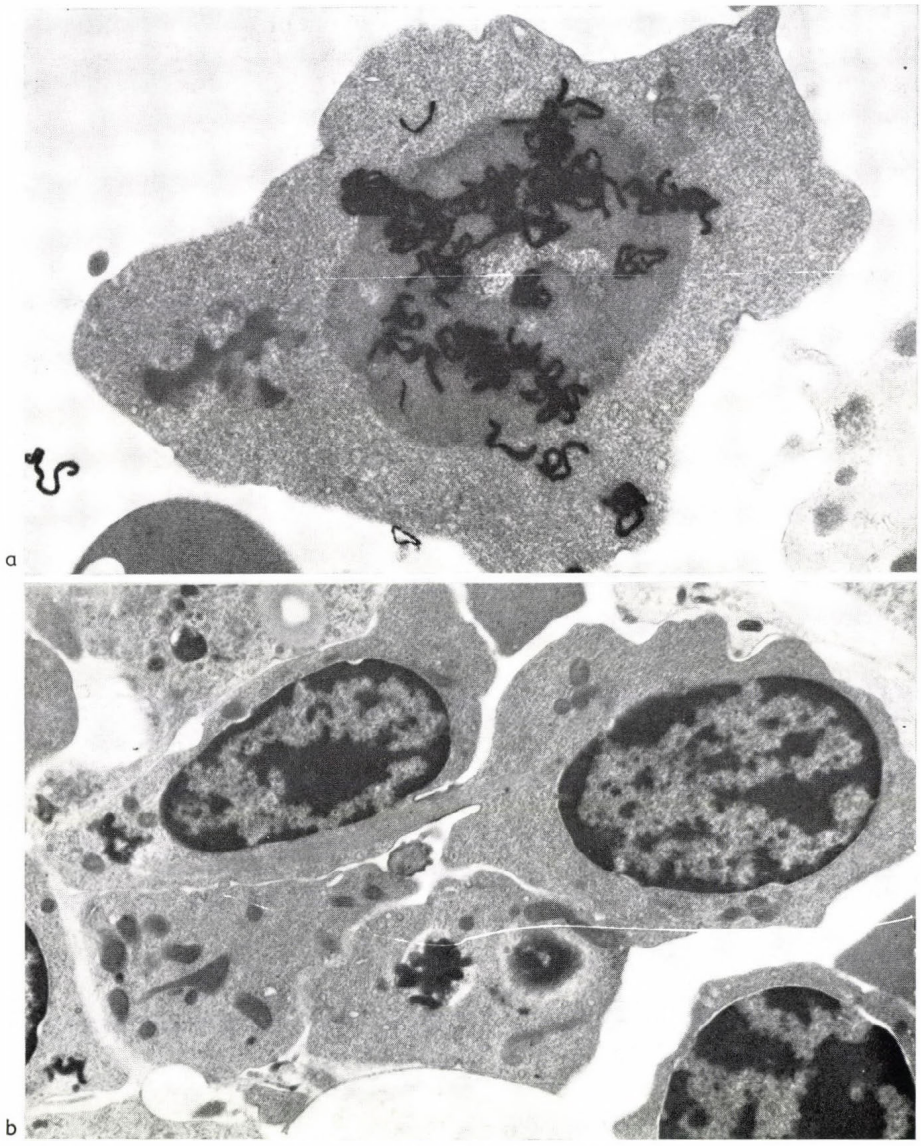


Fig. 10. DNA synthesis and mitosis in precipitate-containing erythroblasts from HbH disease (a) Electron microscope autoradiograph of an ^{3}H-thymidine labelled cell which contains precipitated β-chains [27]. × 17 900. (b) Intercellular spindle bridge between two erythroblasts, one of which contains precipitated β-chains. × 8 500

High resolution autoradiographic studies of ^{3}H-thymidine and ^{3}H-leucine labelled marrow cells have shown that in HbH disease, the presence of intracellular β-chain precipitates does not necessarily prevent DNA synthesis (Fig. 10a) or usually cause a gross impairment of protein biosynthesis [26, 27]. Furthermore, as β-chain precipitates have been occasionally found in erythroblasts in mitosis [27] and in erythroblasts which have completed mitosis but are still joined together by a spindle bridge (Fig. 10b), it seems that the presence of β-chain precipitates does not necessarily prevent mitosis.

Apparently, the presence in HbH disease of intracellular β-chain precipitates and/or an expanded soluble β-chain pool is associated with considerably less impairment of erythroblast function than the presence in homozygous β-thalassaemia of intracellular α-chain precipitates and/or an expanded soluble α-chain pool.

Relationship between Iron Overload of the Erythron and Ineffective Erythropoiesis

The extent, if any, to which the increased non-haem iron content within erythropoietic cells is responsible for the varying degrees of ineffective erythropoiesis in the different thalassaemia syndromes remains to be determined. This is a difficult problem to investigate at the single cell level for three main reasons. Firstly, it is impossible to quantitate the extent of iron-overloading in a cell without serially sectioning it, as a single ultrathin section only contains a very small fraction of the cell volume. Secondly, the increase in the ultrastructurally visible non-haem iron content in the thalassaemia syndromes is frequently associated with the presence of precipitated globin chains as well as other ultrastructural abnormalities, and previous studies have shown some correlations between the presence of α-chain precipitates on the one hand and the presence of dyserythropoietic changes, a pile-up of cells in G_1 and a failure of protein biosynthesis, on the other. Finally, and very importantly, it seems quite possible that any cellular damage which may result from the iron-overloading of erythroblasts may be mediated by a form of iron which is not visible in the electron microscope.

There is some evidence that at least in primary acquired sideroblastic anaemia, marked iron-loading of the mitochondria is associated with impaired cellular function. Thus, it has been shown that in this disorder, erythroblasts with heavily iron-laden mitochondria suffer from an impairment of protein biosynthesis, fail to synthesise DNA and tend to accumulate in the G_2 phase of the cell cycle [44, 45]. However, in homozygous β-thalassaemia, which is characterised by a gross degree of ineffective erythropoiesis, the proportion of erythroblasts with heavily iron-laden mitochondria is frequently relatively small and is considerably less than the proportion containing α-chain precipitates or displaying other dyserythropoietic changes (Tables 1 and 2). Therefore, at least in this thalassaemia syndrome, it seems unlikely that cellular dysfunction due to the iron-loading of mitochondria plays a major role in causing the ineffective erythropoiesis.

Rachmilewitz [46] has suggested that in the presence of an excess of ferritin and haemosiderin, superoxides which are generated during the auto-oxidation of globin chains [47], may cause peroxidation of membrane lipids. The importance of membrane peroxidation or of other biochemical consequences of iron overload in the pathogenesis of ineffective erythropoiesis in the various thalassaemia syndromes is, however, still uncertain.

Conclusion

The data discussed in this paper show that the presence in the β-thalassaemia syndromes of an excess of intracellular α-chains is much more damaging to erythrob lasts than is the presence in HbH disease of an excess of β-chains. The biochemical basis underlying this interesting difference remains to be explored. Recent studies have revealed that the treatment of DMSO-induced murine Friend leukaemia erythroblasts with inhibitors of protein synthesis [48] or with neuraminidase [49] leads to an enhancement of their interaction with syngeneic mouse peritoneal macrophages in vitro. The considerable increase of ineffective erythropoiesis in homozygous β-thalassaemia may, therefore, be a consequence of the gross impairment of protein biosynthesis which has been demonstrated in a substantial proportion of the erythroblasts in this disorder. It is possible that the impairment of protein biosynthesis causes secondary alterations and rearrangements of membrane macromolecules and that these changes in the cell membrane are recognised by the macrophages. It is not known whether the cell membranes of those thalassaemic erythroblasts which are phagocytosed by macrophages have a decreased sialic acid content when compared with those which mature successfully into circulating erythrocytes.

These studies were supported by the Medical Research Council.

References

1. Weatherall, D. J., Clegg, J. B.: *The Thalassaemia Syndromes*, 3rd ed. Blackwell Scientific Publications, Oxford (1981).
2. Weatherall, D. J., Clegg, J. B., Naughton, M. A.: Globin synthesis in thalassaemia: an in vitro study. *Nature 208*, 1061 (1965).
3. Bank, A., Marks, P. A.: Excess α chain synthesis relative to β chain synthesis in thalassaemia major and minor. *Nature 212*, 1198 (1966).
4. Bargellesi, A., Pontremoli, S., Conconi, F.: Absence of beta globin synthesis and excess alpha globin synthesis in homozygous β thalassaemia. *Eur. J. Biochem. 1*, 73 (1967).
5. Modell, C. B., Latter, A., Steadman, J. H., Huehns, E. R.: Haemoglobin synthesis in β-thalassaemia. *Brit. J. Haemat. 17*, 485 (1969).
6. Weatherall, D. J., Clegg, J. B., Na-Nakorn, S., Wasi, P.: The pattern of disordered haemoglobin synthesis in homozygous and heterozygous β-thalassaemia. *Brit. J. Haemat. 16*, 251 (1969).

7. Vigi, V., Volpato, S., Gaburro, D., Conconi, F., Bargellesi, A., Pontremoli, S.: The correlation between red cell survival and excess of α-globin synthesis in β-thalassaemia. *Brit. J. Haemat. 16*, 25 (1969).
8. Schwartz, E.: Heterozygous beta thalassaemia: balanced globin synthesis in bone marrow cells. *Science 167*, 1513 (1970).
9. Kan, Y. K., Nathan, D. G., Lodish, H. F.: Equal synthesis of α- and β-globin chains in erythroid precursors in heterozygous β-thalassemia. *J. Clin. Invest. 51*, 1906 (1972).
10. Chalevelakis, G., Clegg, J. B., Weatherall, D. J.: Imbalanced globin chain synthesis in heterozygous β-thalassaemic bone marrow. *Proc. nat. Acad. Sci. USA. 72*, 3853 (1975).
11. Assawamunkong, S.: Hemoglobin biosynthetic characterization of a classical and a mild β-thalassaemia in Thailand. MS Thesis, Chulalongkorn University (1969).
12. Clegg, J. B., Weatherall, D. J.: Haemoglobin synthesis in alpha-thalassaemia (haemoglobin H disease). *Nature 215*, 1241 (1967).
13. Kan, Y. W., Schwartz, E., Nathan, D. G.: Globin chain synthesis in alpha thalassemia syndromes. *J. Clin. Invest. 47*, 2515 (1968).
14. Wood, W. G., Stamatoyannopoulos, G.: Globin synthesis during erythroid cell maturation in α thalassemia. *Hemoglobin 1*, 135 (1976–1977).
15. Hunt, D. M., Higgs, D. R., Old, J. M., Clegg, J. B., Weatherall, D. J., Marsh, G. W.: Determination of α thalassaemia phenotypes by messenger RNA analysis. *Brit. J. Haemat. 45*, 53 (1980).
16. Braverman, A. S., Bank, A.: Changing rates of globin chain synthesis during erythroid cell maturation in thalassaemia. *J. Molec. Biol. 42*, 57 (1969).
17. Nathan, D. G., Benz, E. J.: Pathophysiology of the anaemia of thalassaemia. In: *Ciba Foundation Symposium 37 (new series) on Congenital Disorders of Erythropoiesis.* R. Porter, D. W. Fitzsimons (eds). Elsevier, Amsterdam (1976), p. 25.
18. Testa, U., Dubart, A., Hinard, N., Galacteros, F., Vainchenker, W., Rouyer-Fessard, P., Beuzard, Y., Rosa, J.: Beta°-thalassemia/HbE association. Hemoglobin synthesis in blood reticulocytes and bone marrow cells fractionated by density gradient and in blood erythroid colonies in culture. *Acta haemat.* (Basel) *64*, 42 (1980).
19. Fessas, P.: Inclusions of hemoglobin in erythroblasts and erythrocytes of thalassemia. *Blood 21*, 21 (1963).
20. Fessas, P., Luukopoulos, D., Thorell, B.: Absorption spectra of inclusion bodies in β-thalassemia. *Blood 25*, 105 (1965).
21. Fessas, P., Loukopoulos, D., Kaltsoya, A.: Peptide analysis of the inclusions of erythroid cells in β-thalassemia. *Biochim. biophys. Acta 124*, 430 (1966).
22. Wickramasinghe, S. N., Letsky, E., Moffatt, B.: Effect of α-chain precipitates on bone marrow function in homozygous β-thalassaemia. *Brit. J. Haemat. 25*, 123 (1973).
23. Polliack, A., Rachmilewitz, E. A.: Ultrastructural studies in β-thalassaemia major. *Brit. J. Haemat. 24*, 319 (1973).
24. Wickramasinghe, S. N., Bush, V.: Observations on the ultrastructure of erythropoietic cells and reticulum cells in the bone marrow of patients with homozygous β-thalassaemia. *Brit. J. Haemat. 30*, 395 (1975).
25. Polliack, A., Yataganas, X., Thorell, B., Rachmilewitz, E. A.: An electron microscopic study of the nuclear abnormalities in erythroblasts in beta-thalassaemia major. *Brit. J. Haemat. 26*, 201 (1974).
26. Wickramasinghe, S. N., Hughes, M., Hollan, S. R., Horanyi, M., Szelenyi, J.: Electron microscope and high resolution autoradiographic studies of the erythroblasts in haemoglobin H disease. *Brit. J. Haemat. 45*, 401 (1980).
27. Wickramasinghe, S. N., Hughes, M., Wasi, P., Fucharoen, S.: Morphology and kinetics of erythropoiesis in haemoglobin H disease. *Brit. J. Haemat. 49*, 185 (1981).
28. Wickramasinghe, S. N., Hughes, M.: Unpublished observations.
29. Wickramasinghe, S. N., Hughes, M., Wasi, P., Fucharoen, S., Modell, B.: Ineffective erythropoiesis in haemoglobin E/β-thalassaemia: an electron microscope study. *Brit. J. Haemat. 48*, 451 (1981).

30. Wickramasinghe, S. N., Hughes, M.: Ultrastructural studies of erythropoiesis in β-thalassaemia trait. *Brit. J. Haemat. 46*, 401 (1980).
31. Bessis, M. C., Breton-Gorius, J.: Iron metabolism in the bone marrow, as seen by electron microscopy: a critical review. *Blood 19*, 635 (1962).
32. Bessis, M., Alagille, D., Breton-Gorius, J.: Particularités des érythroblastes et des érythrocytes dans la maladie de Cooley. Étude au microscope électronique. *Rev. hémat. 13*, 538 (1958).
33. Wickramasinghe, S. N., Hughes, M., Higgs, D. R., Weatherall, D. J.: Ultrastructure of red cells containing haemoglobin H inclusions induced by redox dyes. *Clin. lab. Haemat. 3*, 51 (1981).
34. Wickramasinghe, S. N., McElwain, T. J., Cooper, E. H., Hardisty, R. M.: Proliferation of erythroblasts in beta-thalassaemia. *Brit. J. Haemat. 19*, 719 (1970).
35. Queisser, W., Betzler, M., Heimpel, H., Kleinhauer, E.: Erythropoietic cell proliferation in different clinical states of β-thalassaemia. *Acta haemat. (Basel), 45*, 303 (1971).
36. Sturgeon, P., Finch, C. A.: Erythrokinetics in Cooley's anemia. *Blood 12*, 64 (1957).
37. Malamos, B., Belcher, E. H., Gyftaki, E., Binopoulos, D.: Simultaneous radioactive tracer studies of erythropoiesis and red-cell destruction in thalassaemia. *Brit. J. Haemat. 7*, 411 (1961).
38. Pearson, H. A., McFarland, W., King, E. R.: Erythrokinetic studies in thalassemia trait. *J. Lab. Clin. Med. 56*, 866 (1960).
39. Gallo, E., Pich, P., Ricco, G., Saglio, G., Camaschella, C., Mazza, U.: The relationship between anemia, fecal stercobilinogen, erythrocyte survival and globin synthesis in heterozygotes for β-thalassemia. *Blood 46*, 693 (1975).
40. Cazzola, M., Alessandrino, P., Barosi, G., Morandi, S., Stefanelli, M.: Quantitative evaluation of the mechanisms of the anaemia in heterozygous β-thalassaemia. *Scand. J. Haemat. 23*, 107 (1979).
41. Pearson, H. A., McFarland, W.: Erythrokinetics in thalassemia. II. Studies in Lepore trait and hemoglobin H disease. *J. Lab. Clin. Med. 59*, 147 (1962).
42. Wickramasinghe, S. N.: The morphology and kinetics of erythropoiesis in homozygous β-thalassaemia. In: *Ciba Foundation Symposium 37 (new series) on Congenital Disorders of Erythropoiesis*. R. Porter, D. W. Fitzsimons (eds). Elsevier, Amsterdam (1976), p. 221.
43. Wickramasinghe, S. N., Hughes, M.: Precipitation of α-chains on the centrioles of erythroblasts in β-thalassaemia. *Brit. J. Haemat.* 52,681 (1982).
44. Wickramasinghe, S. N.: *Human Bone Marrow*. Blackwell Scientific Publications, Oxford (1975), pp. 339-342.
45. Wickramasinghe, S. N., Hughes, M.: Capacity of ringed sideroblasts to synthesize nucleic acids and protein in patients with primary acquired sideroblastic anaemia. *Brit. J. Haemat. 38*, 345 (1978).
46. Rachmilewitz, E. A.: The role of intracellular hemoglobin precipitation, low MCHC and iron overload on red blood cell membrane peroxidation in thalassemia. In: *Birth Defects: Original Article Series, XII*. Bergsma, D. Cerami, A. Paterson, C. M. Graziano J. H. (eds). Liss, New York 1976. p. 123.
47. Brunori, M., Falcioni, G., Fioretti, E., Giardina, B., Rotilio, J.: Formation of superoxide in the autoxidation of the isolated α and β chains of human hemoglobin and its involvement in hemichrome precipitation. *Eur. J. Biochem. 53*, 99 (1975).
48. Wiener, E., Wickramasinghe, S. N.: Impaired protein synthesis in erythroblasts enhances their phagocytosis by macrophages. *Brit. J. Haemat. 53*, 117 (1983).
49. Wiener, E., Wickramasinghe, S. N.: The interaction of DMSO-induced murine Friend leukaemia erythroblasts with cultured syngeneic mouse peritoneal macrophages: an *in vitro* model for the study of ineffective erythropoiesis. *Brit. J. exp. Path. 63*, 585 (1982).

Haematologia 17 (1), pp. 57–66 (1984)

Activation of Coagulation Factors on the Surface of Platelets*

P. N. WALSH

Thrombosis Research Center, Department of Medicine,
Temple University School of Medicine, 3400 N. Broad St., Philadelphia,
PA., 19130, USA

(Received 25 November 1982; accepted 14 January 1983)

Platelet physiology and coagulation biochemistry have traditionally been studied and conceptualized as separate and distinct entities. This brief summary of platelet-coagulant protein interactions presents an alternative viewpoint which emphasizes the effects of platelets in coagulation and the effects of coagulation proteins on platelet physiology. These interrelationships are so intimate and important that any consideration of the hemostasis mechanism that fails to take them into account is meaningless.
Keywords: Coagulation factors, platelets

Introduction

The participation of platelets in both primary and secondary hemostasis involves intimate and complex interrelationships with coagulation proteins [1–3]. Platelet aggregation is dependent upon the exposure of specific high affinity fibrinogen receptors in the plasma membrane after platelet stimulation [4, 5]. In addition, the generation of thrombin on the platelet surface via the intrinsic coagulation mechanism involves the sequestration and activation of coagulation proteins on the platelet surface [1–3]. This paper summarizes some of what is known about these interactions and provides a conceptual perspective for interpreting the following papers, all of which deal with various aspects of the interaction of platelets with coagulation proteins. More detailed and complete information on platelet-coagulant protein interactions can be found elsewhere [1–3].

Coagulation Proteins in Platelets

Platelets have been demonstrated to contain a number of molecules similar or identical to their counterparts in plasma (Table 1). For example, fibrinogen is present in the alpha granules of platelets and is released in soluble form when secretion is induced [6–16]. Total platelet fibrinogen comprises 4–10 per cent of total platelet protein and about 3–7 mg of intracellular fibrinogen are present per 10^{11} platelets [6–10]. There is some disagreement about whether platelet and plasma fibrinogen are identical or different molecules [17, 18]. Factor V is also present in alpha granules and is secreted in soluble form [19–29]. Recent estimates by Tracy et al. [30] using a radioimmune assay for factor V indicate that the amount

Table 1

Coagulant proteins in platelets

Protein	Amount present (per 10^{11} platelets)	Subcellular localization
Fibrinogen	3–7 mg	Alpha-granules
Factor V	250–770 μg	Alpha-granules
F VIII/R:Ag	10–28 units	Alpha-granules
High Mw Kininogen	18–170 μg	Alpha-granules
Factor XI	1.2–6.1 units	Plasma membrane
Factor XIII ('a' subunit)	50% of the total in blood	Cytosol

See text for references and details

of factor V in human platelets is between .25 and .77 mg per 10^{11} platelets which amounts to some 4600 to 14 000 molecules of factor V per platelet. Factor VIII related antigen devoid of the coagulant factor VIII molecule is also stored in and released from alpha granules [13, 16, 31–40] as is a molecule with immunological similarity to high molecular weight kininogen [41]. We have recently presented evidence that a molecule functionally and immunologically similar to plasma factor XI but distinct in molecular weight and subunit structure is present in the platelet membranes of normal donors and those with severe plasma factor XI deficiency who have no bleeding problems [41–43]. Finally, the a subunit of the cross-linking protein, factor XIII is present in the cytosol of platelets and comprises 50 per cent of the factor XIII activity found in the blood [44, 45]. In addition, there are a number of platelet specific proteins and coagulation inhibitor molecules present in platelets [3].

Interactions between Platelets and Coagulation Proteins

The sequence of events thought to occur when the coagulation mechanism is activated is complex and involves many interrelationships between the extrinsic and intrinsic mechanisms. Each of these reactions occurs on an activating surface. This surface is a lipoprotein component for the tissue factor-factor VII mechanism of factor X activation. However, for the intrinsic system there is evidence that the platelet can serve as the activating surface at many stages of the reaction sequence [1–3].

Contact activation

A number of studies have addressed the question of the possible role of platelets in the initiation of intrinsic coagulation [46–55]. Recent evidence from our laboratory indicates that platelets can promote the proteolytic activation of

factor XII to factor XIIa in the presence of the enzyme kallikrein and a cofactor protein, high molecular weight kininogen [52]. These interactions are shown in schematic form in Fig. 1 in which the platelet membrane in the unactivated state is represented by a smooth surface and after activation by ADP, collagen or thrombin as a rough activated surface. Additional studies from our laboratory indicate that platelets activated by thrombin or by collagen can bind the zymogen factor XI in the presence of high molecular weight kininogen [56]. This platelet-bound factor XI can then be activated proteolytically to factor XIa [52] in the presence either of factor XII a or of kallikrein (Fig. 2). Since kallikrein does not activate factor XI in the absence of platelets even in the presence of negatively charged surfaces, this proteolytic activation of factor XI by kallikrein in the absence of

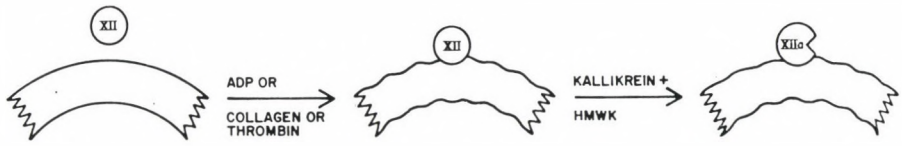


Fig. 1. Activation of Factor XII on the platelet surface. The crescent represents the platelet membrane in the unactivated (smooth) and activated (rough) states. Coagulation factor XII is designated by the Roman numeral in the zymogen form and in the activated form (indicated by the subscript 'a'. HMWK = High Mw Kininogen. See text for explanation

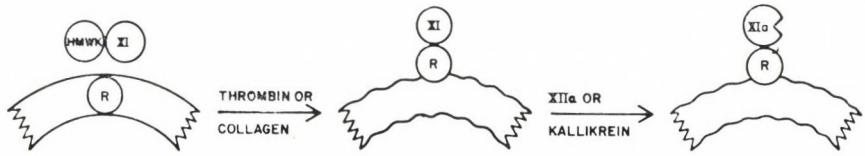


Fig. 2. Activation of Factor XI on the platelet surface. See Fig. 1 for abbreviations and text for explanation. R = hypothetical receptor

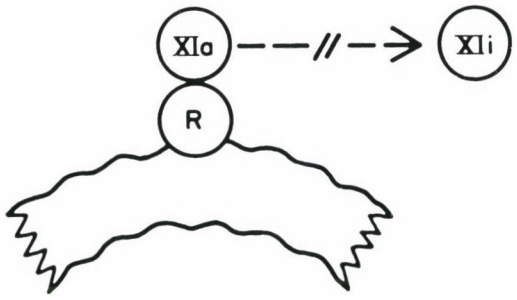


Fig. 3. Protection of Platelet Bound Factor XIa from inactivation by plasma proteinase inhibitors. See Figs 1 and 2 for abbreviations and text for explanation

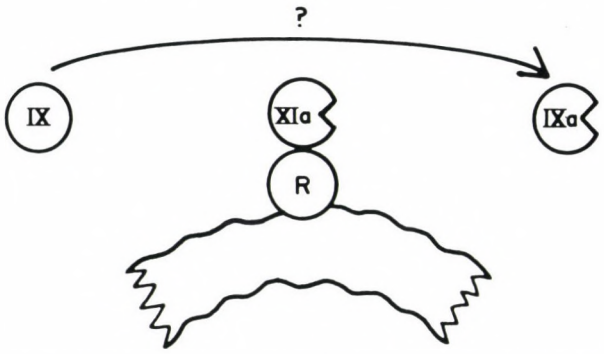


Fig. 4. Possible role of platelets in factor IX activation. See Figs 1 and 2 for abbreviations and text for explanation

added factor XII has been suggested as the basis for the absence of a bleeding syndrome in patients whith factor XII deficiency [52]. Additional evidence from our laboratory indicates that platelet associated factor XIa is protected from inactivation by plasma protease inhibitors [57, 58] such as antithrombin III and alpha-l-antitrypsin although detailed studies using purified proteins have not been published to confirm this hypothesis (Fig. 3). There is only indirect evidence [46–48, 52–54, 57, 58] that platelet associated factor XIa can activate factor IX and further work is required utilizing purified proteins to investigate the question whether platelets have a role in factor IX activation and, if so, by what mechanisms does this reaction occur (Fig. 4).

Factor X activation

Although a number of investigators have studied the contribution of phospholipids to the activation of factor X [59–61], very little work has been published on the role of platelets in this reaction. Evidence from several laboratories [58,

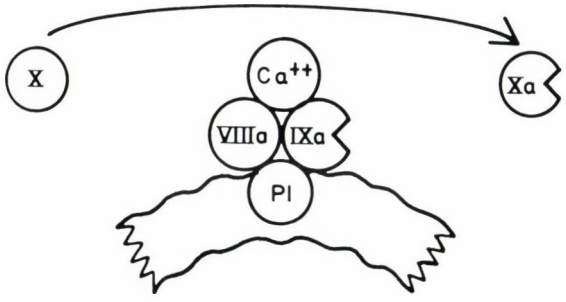


Fig. 5. Possible role of platelets in factor X activation. See Figs 1 and 2 for abbreviations and text for explanation

62, 63] including our own, is consistent with the view that an enzymatic complex is formed on the activated platelet surface consisting of the cofactor, factor VIII in the activated form, the enzyme factor IXa, calcium and a platelet cofactor which may be phospholipid in nature [58–73]. The nature of this complex and of the platelet membrane sites upon which it is assembled require further elucidation. A reasonable hypothesis to be tested is presented in Figure 5. After factor Xa is formed it is bound to high affinity sites in the platelet membrane in the presence of calcium ions [74–81]. The receptor for factor Xa would appear to be the activated form of factor V which itself binds to unidentified high affinity specific receptor sites in platelet membranes [80, 81]. One consequence of the formation of this complex is the protection of factor Xa from inactivation by plasma protease inhibitors such as antithrombin III [58, 74, 82]. These events are represented in schematic form in Figs 5 and 6.

Prothrombin activation

Another consequence of this assembly of proteins on the platelet surface is the formation of the prothrombinase complex (Fig. 7), which consists of an enzyme (factor Xa), a protein cofactor (factor Va), a platelet cofactor (platelet factor 3)

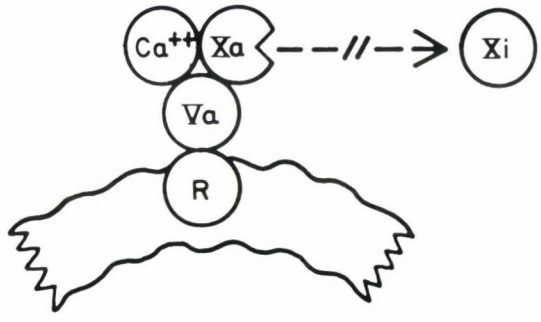


Fig. 6. Protection of Platelet Bound Factor Xa from Inactivation by antithrombin III. See Figs 1 and 2 for abbreviations and text for explanation

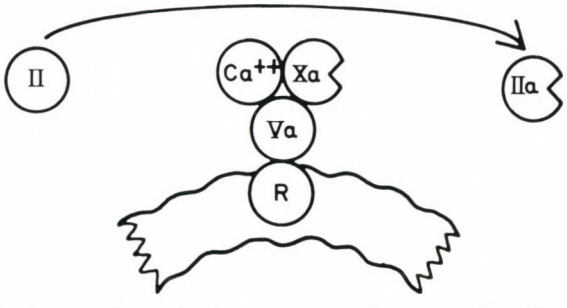


Fig. 7. Role of platelet membrane in the activation of prothrombin. See Figs 1 and 2 for abbreviations and text for explanation

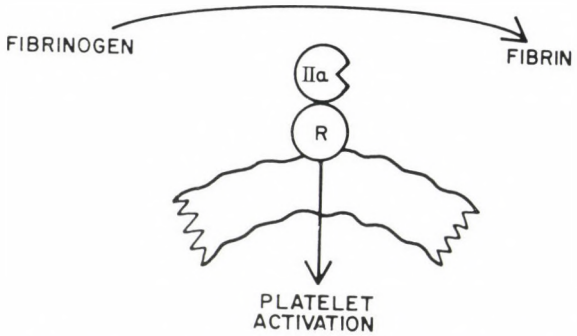


Fig. 8. Activation of platelets and conversion of fibrinogen to fibrin by platelet bound thrombin. See Figs 1 and 2 for abbreviations and text for explanation

and calcium ions [74–84]. Functionally the formation of this enzymatic complex is accompanied by a 300 000-fold acceleration in the rate of prothrombin activation compared with that which occurs with factor Xa alone [74, 75, 77, 79–81].

After thrombin is formed it is also bound to high affinity specific receptors in platelet membranes with two consequences: first, the conversion of fibrinogen to fibrin and, second, the activation of platelets by complex mechanisms which probably involve both stoichiometric (ligand-receptor) interactions and catalytic (proteolytic) effects of bound enzyme on platelet membrane sites (Fig. 8). The details of these interactions of platelets with thrombin have been reviewed elsewhere [85–86].

Interactions of Platelets with Fibrinogen and Fibrin

Finally the process of platelet aggregation requires the interaction of platelets with another coagulation protein, fibrinogen, which is bound specifically to high affinity receptors in platelet membranes exposed by ADP and postulated to

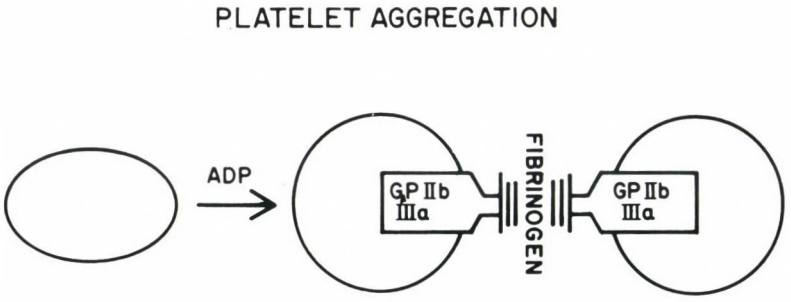


Fig. 9. Roles of fibrinogen and membrane glycoproteins in platelet aggregation. See Figs 1 and 2 for abbreviations and text for explanation

consist of glycoproteins 2B and 3A. These receptor mediated mechanisms of platelet aggregation have been reviewed in detail elsewhere [3] and are represented schematically in Figure 9.

This work was supported in part by DHES grants HL-14217, HL-25661 and by a grant from the Council for Tobacco Research (No. 1389). The authors are grateful to Ms. Patricia Pileggi for excellent secretarial assistance.

References

1. Walsh, P. N.: Platelet coagulant activities and hemostasis: An hypothesis. *Blood 43*, 597 (1974).
2. Majerus, P. S., Miletich, J. P.: Relationship between platelets and coagulation factors in hemostasis. *Ann. Rev. Med. 29*, 41 (1978).
3. Walsh, P. N.: Platelet-coagulant protein interactions. In: *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. R. W. Colman, J. Hirsh, V. J. Marder, E. S. Salzman (eds). Lippincott Co., Philadelphia 1982, p. 404.
4. Marguerie, G. A., Plow, E. F., Edgington, T. S.: Human platelets possess an inducible and saturable receptor for fibrinogen. *J. Biol. Chem. 254*, 5357 (1979).
5. Bennett, J. S., Vilaire, G.: Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J. Clin. Invest. 64*, 1393 (1979).
6. Nachman, R. L., Marcus, A. J., Zucker-Franklin, D.: Immunologic studies of proteins associated with subcellular fractions of normal human platelets. *J. Lab. Clin. Med.* 69, 651 (1967).
7. Levine, R. H., Freirich, E. J.: Effect of storage up to 48 hours on response to transfusion of platelet-rich plasma. *Transfusion 4*, 251 (1964).
8. Keenan, J. P.: Platelet fibrinogen. I. Quantitation using fibrinogen-sensitized tanned red cells. *Med. Lab. Techn. 29*, 71 (1972).
9. James, H. L., Bradford, H. R., Ganguly, P.: Quantitation of human platelet fibrinogen. *Circulation 50*, 284 (1974).
10. Lopaciuk, S., Solum, N. O.: Bovine platelet proteins. I. Soluble proteins studied by immunophoresis and polyacrylamide disc electrophoresis. *Thromb. Diath. Haemorrh. (Stuttg.) 21*, 409 (1969).
11. Day, H. J., Solum, N. O.: Fibrinogen associated with subcellular platelet particles. *Scand. J. Haemat. 10*, 136 (1973).
12. Lopaciuk, S., Lovette, K. M., McDonagh, J., Chuang, H. Y. K., McDonagh, R. P.: Subcellular distribution of fibrinogen and factor XIII in human blood platelets. *Thromb. Res. 8*, 453 (1976).
13. Kaplan, K. L., Broekman, J., Chernoff, A., Lesznik, G. R., Drillings, M.: Platelet -granule proteins: Studies on release and subcellular localization. *Blood 53*, 604 (1979).
14. Davey, M. G., Luscher, E. F.: Release reactions of human platelets induced by thrombin and other agents. *Biochim. Biophys. Acta 165*, 490 (1968).
15. Keenan, J. P., Solum, N. O.: Quantitative studies on the release of platelet fibrinogen by thrombin. *Brit. J. Haemat. 23*, 461 (1972).
16. Koutts, J., Walsh, P. N., Plow, E. F., Fenton, J., Bouma, B. N., Zimmerman, T. S.: Active release of human platelet factor VIII-related antigen by adenosin diphosphate, collagen, and thrombin. *J. Clin. Invest. 62*, 1255 (1978).
17. James, H. L., Ganguly, P., Jackson, C. W.: Characterization and origin of fibrinogen in blood platelets. A review with recent data. *Thromb. Haemost. 38*, 939 (1977).
18. Doolittle, R. F., Takagi, T., Cottrell, B. A.: Platelet and plasma fibrinogen are identical gene products. *Science 185*, 368 (1974).

19. Walsh, P. N., Mills, D. C. B., White, J. C.: Metabolism function of human platelets washed by albumin density gradient separation. *Brit. J. Haemat. 63*, 285 (1977).
20. Tangen, O., Lestrup, E. P., Berman, H. J.: Characterization of clotting factors associated with blood platelets after gel filtration. *Thromb. Diath. Haemorrh. (Stuttg.) 30*, 289 (1973).
21. Raccuglia, G.: Factor VIII (anti-hemophilic factor) and factor V (proaccelerin) activity of platelets. *Proc. Soc. Exp. Biol. (N. Y.) 104*, 309 (1960).
22. Breederveld, K., Giddings, J. C., ten Cate, J. W., Blood, A. L.: The localization of factor V within normal human platelets and a demonstration of a platelet-factor V antigen in congenital factor V deficiency. *Brit. J. Haemat. 29*, 405 (1975).
23. Osterud, B., Rapaport, S. I., Lavine, K. K.: Factor V activity of platelets: Evidence for an activated factor V molecule and for a platelet activator. *Blood 49*, 819 (1977).
24. Miletich, J. P., Majerus, D. W., Majerus, P. W.: Patients with congenital factor V deficiency have decreased factor Xa binding sites on their platelets. *J. Clin. Invest. 62*, 824 (1978).
25. Pifer, D. D., Colman, R. W., Chesney, C. M.: Subcellular localization and secretion of factor V by human platelets. *Thromb. Haemost. 38*, 126 (1977).
26. Chesney, C. M., Pifer, D. D., Colman, R. W.: Factor V coagulant activity of human platelets generated by collagen. *Circulation 58*, 209 (1978).
27. Ittyerah, T. R., Rawala, R., Colman, R. W.: Immunochemical studies of bovine platelet factor V. *Thromb. Haemost. 42*, 324 (1979).
28. Miletich, J. P., Jackson, C. M., Majerus, P. W.: Properties of the factor Xa binding site on human platelets. *J. Biol. Chem. 253*, 6908 (1978).
29. Kane, W. H., Lindhout, M. J., Jackson, C. W., Majerus, P. W.: Factor Va-dependent binding of factor Xa to human platelets. *J. Biol. Chem. 255*, 1170 (1980).
30. Tracy, P. B., Eide, L. L., Bowie, E. J. W., Mann, K. G.: Radioimmunassay of factor V in human plasma and platelets. *Blood 60*, 59 (1982).
31. Howard, M. A., Montgomery, D. C., Hardisty, R. M.: Factor VIII-related antigen in platelets. *Thromb. Res. 4*, 617 (1974).
32. Hoyer, L. W., de los Santo, R. P., Hoyer, J. R.: Anti-hemophilic factor antigen localization in endothelial cells by immunofluorescent microscopy. *J. Clin. Invest. 52*, 2737 (1973).
33. Piovella, F., Ascari, E., Sitar, G. M., Malamani, G. D., Cattaneo, G., Magliulo, E., Storti, E.: Immunofluorescent detection of factor VIII-related antigen in human platelets and megakaryocytes. *Haemostasis 3*, 288 (1974).
34. Nachman, R. L., Jaffe, E. A.: Subcellular platelet factor VIII antigen and von Willebrand factor. *J. Exp. Med. 141*, 1101 (1975).
35. Coller, B. S., Hirshman, R. J., Gralnick, H. R.: Studies of the factor VIII/von Willebrand's factor antigen of human platelets. *Thromb. Res. 6*, 469 (1975).
36. Ruggeri, Z. M., Mannucci, P. M., Bader, R., Barbui, T.: Factor VIII-related properties in platelets from patients with von Willebrand's disease. *J. Lab. Clin. Med. 91*, 132 (1978).
37. Shearn, S. A. M., Giddings, J. C., Peake, I. R., Blood, A. L.: A comparison of five different rabbit antisera to factor VIII and a demonstration of factor VIII-related antigen in normal and von Willebrand's disease platelets. *Thromb. Res. 5*, 585 (1974).
38. Bouma, B. N., Hordijk-Hos, J. M., de Graaf, S., Sixma, J. J., van Mourik, J. A.: Presence of factor VIII-related antigen in blood platelets of patients with von Willebrand's disease. *Nature 257*, 510 (1975).
39. Slot, J. W., Bouma, B. N., Montgomery, R., Zimmerman, T. S.: Platelet factor VIII-related antigen: Immunofluorescent localization. *Thromb. Res. 13*, 871 (1978).
40. Zucker, M. B., Broekman, M. J., Kaplan, K. L.: Factor VIII-related antigen in human blood platelets. Localization and release by thrombin and collagen. *J. Lab. Clin. Med. 94*, 675 (1979).

41. Schmaier, A. H., Tuszynski, G. P., Walsh, P. N., Colman, R. W.: Immunological identification of human platelet kininogen. *Clin. Res. 28*, 323A (1980).
42. Lipscomb, M. S., Walsh, P. N.: Human platelets and factor XI. Localization in platelet membranes of factor-XI-like activity and its functional distinction from plasma factor XI. *J. Clin. Invest. 63*, 1006 (1979).
43. Tuszynski, G. P., Bevacqua, S. J., Schmaier, A. H., Colman, R. W., Walsh, P. N.: Factor XI antigen in human platelets. *Blood 59*, 1148 (1982).
44. Luscher, E. F.: Ein fibrinstabilisierender Faktor aus Thrombocyten. *Schweiz. Med. Wschr. 87*, 1220 (1957).
45. McDonagh, J., Kiesselbach, T. H., Wagner, R. H.: Factor XIII and antiplasmin activity in human platelets. *Amer. J. Physiol. 216*, 508 (1969).
46. Schiffman, S., Rapaport, S. I., Chong, M. M. Y.: Platelets and initiation of intrinsic clotting. *Brit. J. Haemat. 24*, 633 (1973).
47. Schiffman, S., Rimon, A., Rapaport, S. I.: Factor XI and platelets: Evidence the platelets contain only minimal factor XI activity and antigen. *Brit. J. Haemat. 35*, 429 (1977).
48. Walsh, P. N.: The role of platelets in the contact phase of blood coagulation. *Brit. J. Haemat. 22*, 237 (1972).
49. Vicic, W. J., Ratnoff, O. D., Saito, H., Goldsmith, G. H.: Platelets and surface-mediated clotting activity. *Brit. J. Haemat. 43*, 91 (1976).
50. Vecchione, J., Zucker, M. B.: Procoagulant activity of platelets in recalcified plasma. *Brit. J. Haemat. 31*, 423 (1975).
51. Walsh, P. N., Lipscomb, M. S.: Comparison of the coagulant activities of platelets and phospholipids. *Brit. J. Haemat. 33*, 9 (1976).
52. Walsh, P. N., Griffin, J. H.: Contributions of human platelets to the proteolytic activation of blood coagulation factors XII and XI. *Blood 57*, 106 (1981).
53. Connellan, J. M., Bowden, D. S., Smith, I., Castaldi, P. A.: The use of antibodies to investigate the role of factor XI in platelets. *Thromb. Res. 17*, 225 (1980).
54. Firkin, B. G., Booth, P., Hendrix, L., Howard, M. A.: Demonstration of a platelet bypass mechanism in the clotting system using an acquired anticoagulant. *Amer. J. Haemat. 5*, 81 (1978).
55. Osterud, B., Harper, E., Rapaport, S. I., Lavine, K. K. J.: Evidence against collagen activation of platelet associated factor XI as a mechanism for initiating intrinsic clotting, *Scand. J. Haemat. 22*, 205 (1979).
56. Greengard, J. S., Walsh, P. N., Griffin, J. H.: Binding of factor XI to human platelets. *Blood 58*, 688 (1981).
57. Walsh, P. N.: The effects of collagen and kaolin on the intrinsic coagulant activity of platelets. Evidence for an alternative pathway in intrinsic coagulation not requiring factor XII. *Brit. J. Haemat. 22*, 393 (1972).
58. Walsh, P. N., Biggs, R.: The role of platelets in intrinsic factor Xa formation. *Brit. J. Haemat. 22*, 743 (1972).
59. Lundblad, R. L., Davie, E. W.: The activation of anti-hemophilic factor (Factor VIII) by activated Christmas factor (activated Factor IX). *Biochemistry 3*, 1720 (1964).
60. Lundblad, R. L., Davie, E. W.: The activation of Stuart factor (factor X) by activated anti-hemophilic factor (activated factor VIII). *Biochemistry 4*, 1123 (1965).
61. Schiffman, S., Rapaport, S. I., Chong, M. M. Y.: The mandatory role of lipid in the interaction of factors VIII and IX. *Proc. Soc. Exp. Biol. Med. 123*, 736 (1966).
62. Hultin, M. B.: Role of human factor VIII in factor X activation. *J. Clin. Invest. 69*, 950 (1982).
63. Walsh, P. N.: Different requirements of intrinsic factor Xa forming activity and platelet factor 3 activity and their relationship to platelet aggregation and release. *Brit. J. Haemat. 40*, 311 (1978).
64. Hougie, C., Denson, K. W. E., Biggs, R.: A study of the reaction product of factor VIII, and factor IX by gel filtration. *Thrombos. Diathes. Haemorrh. (Stuttg.) 18*, 211 (1967).

65. Hemker, H. C., Kahn, M. J. P.: Reaction sequence of blood coagulation. *Nature 215* 1201 (1967).
66. Barton, P. G.: Sequence theories of blood coagulation reevaluated with reference to lipid-protein interactions. *Nature 215*, 1508 (1967).
67. Barton, P. G., Jackson, C. M., Hanahan, D. J.: Relationship between factor V and activated factor X in the generation of prothrombinase. *Nature 214*, 923 (1967).
68. Hemker, H. C., Kahn, M. J. P., Devilee, P. P.: The absorption of coagulation factors into phospholipids. *Thrombos. Diathes. Haemorrh. (Stuttg.) 24*, 214 (1970).
69. Osterud, B., Rapaport, S. I.: Synthesis of intrinsic factor X activator. Inhibition of the function of formed activator by antibodies to factor VIII and factor IX. *Biochemistry 9*, 1854 (1970).
70. Chuang, T. F., Sargeant, R. B., Hougie, C.: The intristic activation of factor X in blood coagulation. *Biochim. Biophys. Acta 273*, 287 (1972).
71. Váradi, K., Hemker, H. C.: Kinetics of the formation of the Factor X activating enzyme of the blood coagulation system. *Thromb. Res. 8*, 303 (1976).
72. Hemker, C., Lindout, M. J., Vermeer, C.: Blood coagulation factors at phospholipid surfaces. *Ann. N. Y. Acad. Sci. 283*, 104 (1977).
73. Előd i, S., Váradi, K.: Optimization of conditions for the catalytic effect of the factor IXa–factor VIII complex. *Thromb. Res. 15*, 617 (1979).
74. Miletich, J. P., Jackson, C. M., Majerus, P. W.: Interaction of coagulation factor Xa with human platelets. *Proc. Nat. Acad. Sci. USA, 74*, 4033 (1977).
75. Dahlback, B., Stenflo, J.: Binding of bovine coagulation factor X, to platelets. *Biochemistry 17*, 4939 (1978).
76. Jevons, S., Barton, P. G.: Biochemistry of blood platelets. Interaction of activated factor X with platelets. *Biochemistry 10*, 428 (1971).
77. Majerus, P. W., Miletich, J. P., Kane, W. H., Hofmann, S. L., Stanford, N., Jackson, C. M.: The formation of thrombin on the platelet surface. In: *The Regulation of Coagulation*. K. G. Mann, F. B. Jr. Taylor (eds). Elsevier/North-Holland, New York 1980, p. 215.
78. Tollefsen, D. M., Jackson, C. M., Majerus, P. W.: Binding of the products of prothrombin activation to human platelets. *J. Clin. Invest. 56*, 241 (1975).
79. Tracy, P. B., Peterson, J. M., Nesheim, M. E., McDuffie, F. C., Mann, K. G.: Interaction of coagulation factor V and factor Va with platelets. *J. Biol. Chem. 254*, 10354 (1979).
80. Kane, W. M., Lindhout, M. J., Jackson, C. M., Majerus, P. W.: Factor Va-dependent binding of factor Xa to human platelets. *J. Biol. Chem. 255*, 1170 (1980).
81. Tracy, P. B., Nesheim, M. E., Mann, D. G.: Coordinate binding of factor Va and factor Xa to the unstimulated platelet. *J. Biol. Chem. 256*, 743 (1981).
82. Marciniak, E.: Factor-Xa inactivation by antithrombin III: Evidence for biological stabilization of factor X, by factor V-phospholipid complex. *Brit. J. Haemat. 24*, 391 (1973).
83. Bevers, E. M., Comfurius, P., Van Rijn, J. L. M. L., Hemker, H. C., Zwaal, R. F. A.: Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Europ. J. Biochem. 122*, 429 (1982).
84. Bevers, E. M., Comfurius, P., Zwaal, R. E. A.: The nature of the binding site for prothrombinase at the platelet surface as revealed by lipolytic enzymes. *Europ. J. Biochem. 122*, 81 (1982).
85. Majerus, P. W., Tollefsen, D. M., Shuman, M. A.: The interaction of platelets with thrombin. In: *Platelets in Biology and Pathology*. J. L. Gordon (ed.) North-Holland, Amsterdam 1976, p. 241.
86. Mills, D. C. B., Macfarlane, D. E.: Platelet receptors. In: *Platelets in Biology and Pathology*. J. L. Gordon (ed.) North-Holland, Amsterdam 1976, p. 160.

Haematologia 17 (1), pp. 67—76 (1984)

Factor V : a Platelet Cytoskeletal Associated Protein

G. P. TUSZYNSKI, P. N. WALSH

Thrombosis Research Center, Temple University School of Medicine,
3400 N. Broad St., Philadelphia, PA 19140, USA

(Received 15 December 1982; accepted 18 January 1983)

Platelet cytoskeletons prepared from thrombin activated platelets contain specifically associated Factor Va. We postulate that Factor Va associates with the cytoskeleton through receptors present on the platelet surface. The following evidence supports this hypothesis. (a) Prior secretion of Factor Va is necessary for the association of Factor Va with the cytoskeleton. (b) Reagents that inactivate Factor Va on the surface of the platelet such as EDTA and proteolytic enzymes also inactivate the Factor Va on the cytoskeleton. (c) The platelet Factor Xa binding sites (i.e. Factor Va) are quantitatively retained on the platelet cytoskeleton. (d) Platelet cytoskeletons prepared from platelets that are thought to be deficient in Factor Va binding sites contain 25 per cent of Factor Va activity of normal platelet cytoskeletons and platelet cytoskeletons prepared from platelets deficient in Factor Va contain no Factor Va activity but regain control levels of Factor Va only when Factor Va is added to the platelets prior to the preparation of cytoskeletons.

Keywords: Factor Va, Factor Xa binding site, platelet cytoskeleton.

Introduction

The cell cytoskeleton of most cells is a fibrillar network that supports the plasma membrane [1]. These structures were first isolated and described by Yu et al. [2] who discovered that in the red cell most of the cytoskeleton was insoluble in Triton-X100. Other cells were later examined by the Triton extraction procedure and found to contain cytoskeletons that differed in protein composition [3]. These differences were due to the class of fibrillar material present. For example, fibroblasts contain actin filaments as well as a class of fibrils known as intermediate filaments [4].

The organization and composition of the platelet cytoskeleton is not fully known. Resting platelets are thought to contain a poorly organized cytoskeleton that is primarily composed of microtubules [5]. The amount of actin and associated proteins that appear in the Triton insoluble shell depend on the extraction medium [6]. Activated platelets contain a highly organized Triton insoluble cytoskeleton that is composed of actin, myosin and fibrin [7].

We have found that gel filtered platelets upon activation by thrombin form a large Triton insoluble cytoskeleton. This cytoskeleton has associated with it Factor Va [8]. We hypothesize that this associated Factor Va is bound to the cyto-

skeleton through surface membrane receptors that are anchored to the cytoskeleton either directly or by transmembrane linkage proteins. The following communication presents data in support of this hypothesis.

Materials and Methods

Proteolytic enzyme inhibitors, buffers, Sepharose-2B, 2-deoxy-D-glucose, antimycin A, reagents for clotting assays, and chromatographic resins, were purchased from Sigma Chemical Co., St. Louis, MO. Gluconolactone was purchased from Calbiochem, La Jolla, CA. Reagents for sodium dodecyl sulfate gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. The chromogenic substrate H-D-phenylalanyl-L-pipecolyl-L-argininep-nitroanilide dihydrochloride (S-2238) was purchased from Ortho Diagnostics, Raritan, NJ. Highly purified human thrombin was a gift from Dr. J. W. Fenton II. Silicone oils were obtained from William F. Nye, Inc., New Bedford, MA.

Isolation of platelets

Platelet-rich plasma (PRP) was prepared from citrated whole blood (0.38 per cent citrate final concentration) by centrifuging the blood at $140 \times g$ for 15 min. The PRP was gel filtered through Sepharose-2B equilibrated in Hepes-buffered Tyrode Ca^{2+} free solution [9] containing 0.1 per cent bovine serum albumin (BSA).

Preparation of cytoskeletons

Cytoskeletons were prepared as described by Tuszynski et al. [8].

Protein assays were performed according to the procedure of Lowry et al. [10]. Factor V clotting assays and chromogenic assays were performed as described previously [8].

Inhibition of platelet release

The platelet release reaction was inhibited by incubating platelets with the metabolic inhibitors 2-deoxyglucose (30 mM), antimycin A (9 mM) and gluconolactone (40 mM) at 37 °C for 30 min as described previously [8].

Purification of factor X, prothrombin and factor V

Factor X, prothrombin and Factor V were purified as previously reported [8]. Both proteins were at least 95 per cent pure as judged by SDS-gel electrophoresis.

Binding of ^{125}I-labeled factor Xa to cytoskeletons and platelets

Binding studies of radiolabeled Factor Xa to whole platelets and cytoskeletons were carried out by centrifugation through a silicone oil barrier technique as described previously [8].

Antibody studies

Monoclonal antibody to Factor V was a generous gift of Dr. Helen Glueck. Ten μl of an activated purified Factor V solution or a cytoskeletal suspension, each containing 1 unit/ml Factor Va activity, was incubated in the presence and absence of 1 μl of antibody or control serum. Clotting times were measured as a function of time of incubation at 4 °C.

Results

Demonstration of the presence of factor Va on platelet cytoskeletons

The presence of Factor Va on platelet cytoskeletons was established by the use of two specific assays for Factor Va (Table 1). The first is a coagulant assay utilizing Factor V deficient plasma and the second is a chromogenic assay which measures the ability of cytoskeletons to potentiate the rate of prothrombin activation. In this assay, the amount of thrombin generated was measured by the amount of the thrombin specific substrate S-2238 (H-D-phenylalanyl-L-pipecolyl-

Table 1

Factor Va activity of cytoskeletons obtained from thrombin activated platelets

Assay	Factor Va Activity/10^8 platelets
Coagulant	0.326 ± 0.012[a]
Chromogenic	0.72 ± 0.202[b]

[a] Units, one unit is the amount of coagulant activity present in one ml of normal plasma
[b] Units of thrombin generated/ml/min

L-arginine-p-nitroanilide) to yield as one of the products, paranitroaniline, a yellow chromophore. In addition, both of these assays could be inhibited by a specific antibody to Factor V. The time dependent antibody inhibition of Factor Va activity is shown in Figure 1. Therefore, these experiments strongly indicate that cytoskeletons contain Factor Va.

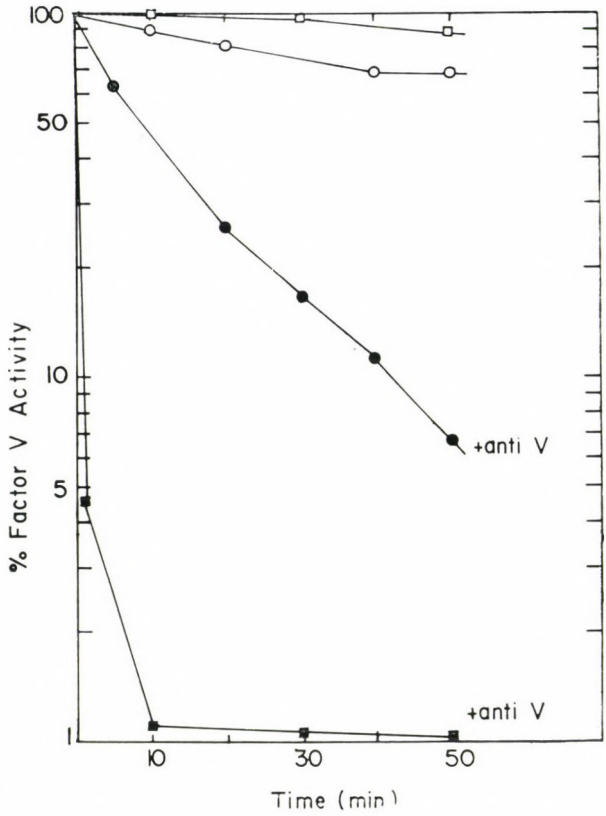


Fig. 1. Neutralization of cytoskeletal Factor Va and purified Factor Va with anti-Factor V antibody. 100 μl of a sonicated suspension of cytoskeletons containing 1 unit/ml Factor Va activity was assayed in the presence of 1 μl of anti-Factor V antiserum (●——●) or in the presence of 1 μl of control serum (○——○). 100 μl of a solution of purified Factor Va containing 1 unit/ml was assayed in the presence of 1 μl of anti-Factor V antiserum (■——■) or of 1 ml of control serum (□——□). Samples were incubated on ice and 10 μl aliquots were withdrawn and assayed for Factor Va coagulant activity at the times indicated. Figure reprinted with permission from reference [8]

Association of factor Va with the cytoskeleton by means of receptors present on the platelet surface

Several lines of evidence support the view that Factor Va is specifically associated to cytoskeletons presumably by platelet surface receptors. (a) Cytoskeletons prepared from platelets treated with agents that inhibit secretion such as deoxyglucose, antimycin A, and gluconolactone contained no Factor Va activity which implies that Factor V must first be released before it becomes associated with the cytoskeleton (Fig. 2). (b) Inactivation of platelet surface-bound but not

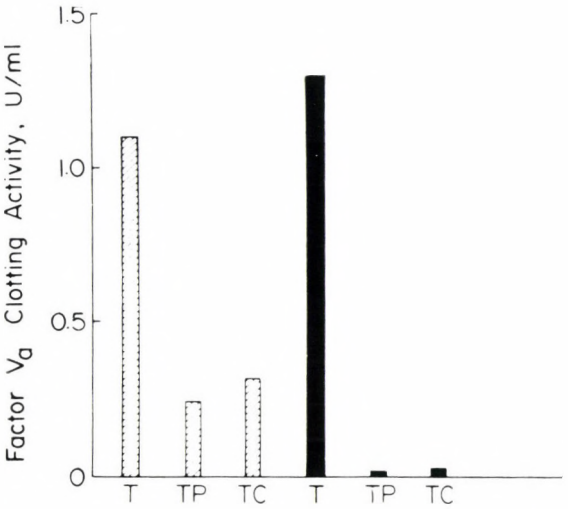


Fig. 2. Factor Va activity in thrombin activated control platelets and thrombin activated drug treated platelets. Both control and drug incubated platelets were treated with 1 unit/ml thrombin for 3 min and cytoskeletons prepared as described in reference [8.] Solid bars represent samples prepared from platelets preincubated for 30 min with 2-deoxyglucose (30 mM), antimysin A (9 mM) and gluconolactone (40 mM). Open bars represent corresponding controls. T = total platelet Factor Va activity, TP = total thrombin released platelet activity, TC = total cytoskeletal activity

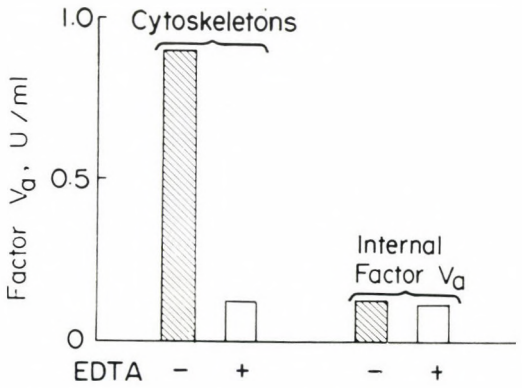


Fig. 3. Inhibition of Factor Va cytoskeletal activity by EDTA. One ml of 3×10^8 platelets per ml were either treated with 1 mM EDTA for 1 min at room temperature, washed three times and cytoskeletons prepared (open bars) or treated with buffer, washed three times and cytoskeletons prepared (hatched bars). Cytoskeletal Factor Va activity was measured with coagulant assay. Internal Factor Va activity was the activity released by Triton when cytoskeletons were made

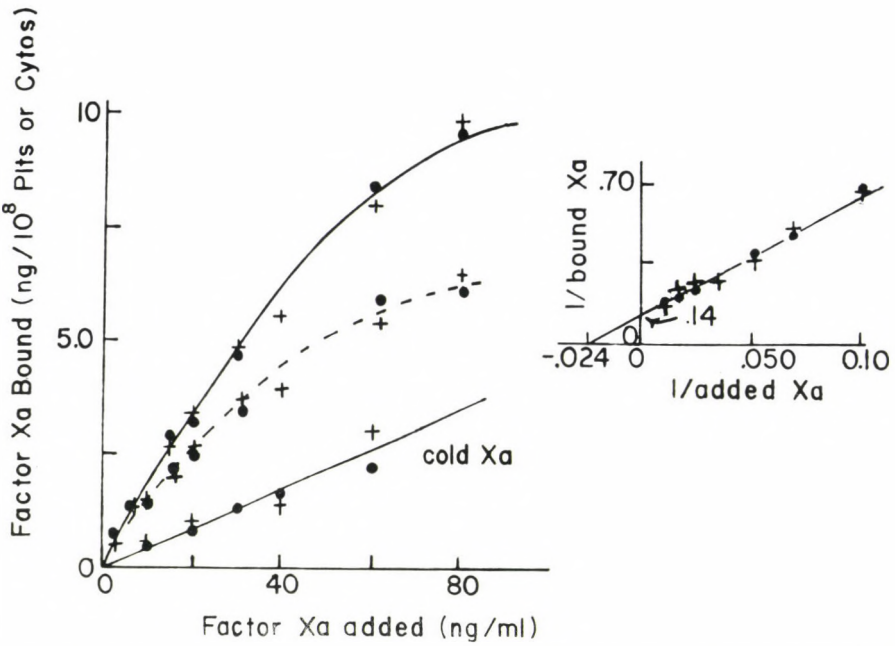


Fig. 4. Binding of ^{125}I-Factor Xa to thrombin activated platelets and determination of the amount bound to their corresponding cytoskeletons. Reaction mixtures contained 2.78×10^8 platelets per ml of Hepes buffered Tyrode solution pH 7.3 containing 1 mg/ml bovine serum albumin, 1 unit/ml thrombin, 2.5 mM $CaCl_2$ and radiolabeled Factor Xa, specific activity 400 cpm/ng. Bound ^{125}I-Factor Xa was the amount that pelleted through oil as described under Methods. Cytoskeletons were prepared by pelleting platelets in the presence of 0.5% Triton. Each point represents the average of two experiments. Each experiment was performed on different donor platelets and two determinations were performed for every point in each of the experiments. ●----●, ^{125}I-Factor Xa bound to platelets; +----+, ^{125}I-Factor Xa bound to cytoskeletons. Nonspecific binding was determined in the presence of 100-fold excess cold Factor Xa and is indicated in the figure by the straight line labeled cold Xa. The values corrected for non-specific binding were obtained by subtracting the total Factor Xa bound at 100-fold excess cold Factor Xa from the total amount bound. The dotted line represents the total Factor Xa bound and corrected for nonspecific binding. The inset shows the corrected data plotted as a double reciprocal plot giving the maximum binding sites as 7.14 ng Factor Xa per 10^8 platelets or cytoskeletons or 960 molecules per platelet or its cytoskeleton having a dissociation constant of 10.4×10^{-10} M for either platelets or cytoskeletons. Figure reprinted with permission from reference [8]

intracellular Factor Va with EDTA (Fig. 3) results in the disappearance of Factor-Va-associated cytoskeletal activity suggesting that the Factor Va which is bound to the cytoskeleton interacts with the platelet surface and is not simply trapped or coprecipitated from intracellular storage sites. (c) Cytoskeletons and whole platelets contain the same number of Factor Xa binding site which is postulated to

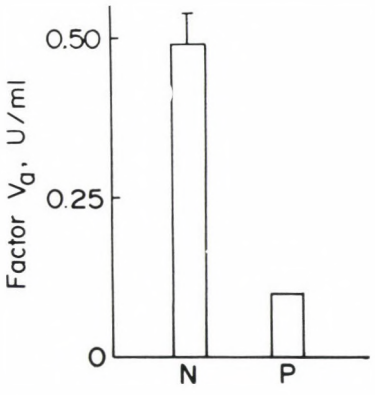


Fig. 5. Cytoskeletal activity from Factor V receptor deficient platelets. 2×10^8 cytoskeletons per ml obtained from controls were tested for Factor Va activity shown by the bar marked N. An equal number of cytoskeletons from a patient with deficient Factor Va was assayed for Factor V shown by the bar marked P. The control platelet activities were an average of ten determinations

be Factor Va [11] suggesting that the binding site for Factor Va arose on the cytoskeleton from a site on the surface of the cell (Fig. 4). We measure approximately 960 Factor Xa binding sites on whole platelets or their respective cytoskeletons which have a dissociation constant of 10.4×10^{-10} M. These values are in good agreement with the values obtained for whole platelets by others [11]. (d) Cytoskeletons prepared from a patient thought to be deficient in Factor Va binding sites [12] contained only 25 per cent of the Factor Va activity present on control cytoskeletons (Fig. 5). These results suggest that the Factor Va receptors present on the platelets of these patients are quantitatively retained on the platelet cytoskeleton. (e) Cytoskeletons prepared from a patient whose platelets contained 1.8 per cent of normal Factor Va levels contained less than 2.0 per cent of normal cytoskeletal Factor Va levels (Fig. 6). However, when exogenous Factor Va was added to these platelets and cytoskeletons were made, patient cytoskeletal Factor Va activities were restored to control values (Fig. 6). Addition of exogenous Factor Va to control platelets resulted in only a 30 per cent increase in the activity of cytoskeletal associated Factor Va, suggesting that cytoskeletal Factor Va receptor sites are normally saturated in controls. These results clearly establish that only exogenous Factor Va added to platelets can become associated with the platelet cytoskeleton.

In conclusion, the above experiments suggest that externalized Factor V becomes bound to platelets upon thrombin activation and becomes tightly associated to the platelet cytoskeleton. The association is postulated to occur through a transmembrane linking of the platelet Factor V receptor with the platelet cytoskeleton.

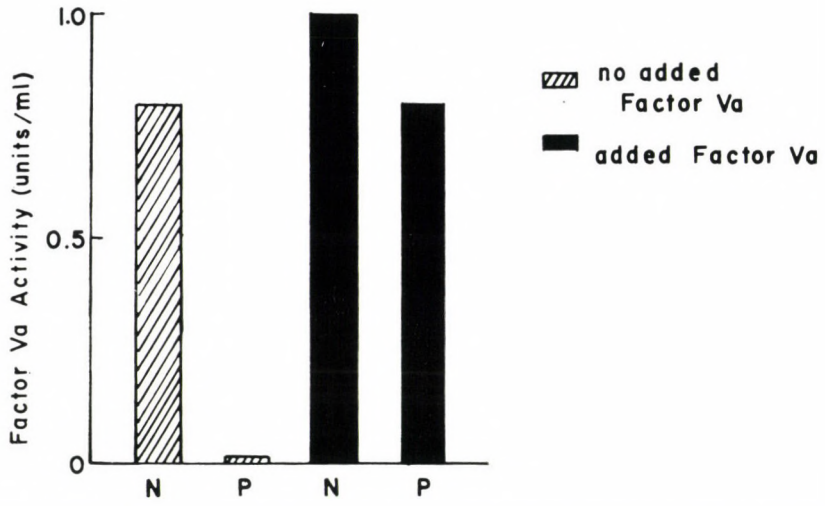


Fig. 6. Cytoskeletal Factor Va activities of control and Factor V deficient platelets. Factor Va levels of cytoskeletons prepared as described under Methods from control platelets (N) (2.3×10^8/ml) and from platelets of a Factor V deficient patient (P) (2.3×10^8/ml) are shown as hatched bars. Factor Va levels of cytoskeletons from patient (P) and control (N) platelets to which 2 units/ml of Factor Va were added prior to cytoskeleton preparation are shown by black bars. Factor Va coagulant activities were determined as described under Methods. Figure reprinted with permission from reference [8]

Discussion

We have shown that cytoskeletons contain Factor Va on the basis of the following evidence:

a) Cytoskeletons possess Factor-Va coagulant activity and cytoskeletons potentiate the Factor Xa catalysed activation of prothrombin (Table 1).

b) The Factor V coagulant activity and the prothrombin-activating ability of cytoskeletons were inhibited by anti-Factor V antiserum (Fig. 1).

c) The activity is inhibited by EDTA (Fig. 2), is sensitive to proteases and to heat and is not inactivated by DFP. These properties are shared with purified Factor Va [8].

d) The cytoskeletons and platelets bind radiolabeled Factor Xa in a manner consistent with the receptor being Factor Va (Fig. 3).

The association of Factor Va with the platelet cytoskeleton most likely occurs by means of receptors present to the platelet surface. The following results support this hypothesis:

a) When the platelet release reaction is inhibited (Fig. 2) and Factor Va is not secreted from platelets, no Factor Va becomes associated with the platelet cytoskeleton. Therefore, in order to become associated with the platelet cytoskeleton. Factor Va must be released from the platelet and bind to the cell surface.

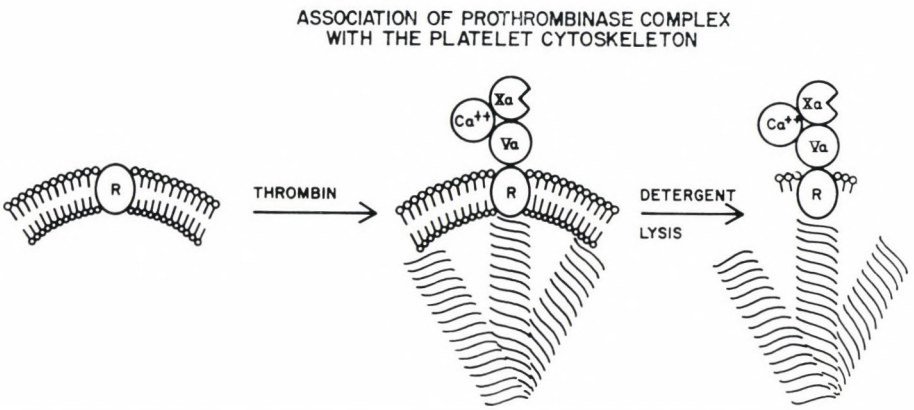


Fig. 7. Scheme for the association of Factor Va and the prothrombinase complex with the platelet cytoskeleton

b) If our contention that Factor Va must first bind to the cell surface before becoming associated with the cell cytoskeleton is correct, then inactivation of surface bound Factor Va by such reagents as EDTA or proteases should inactivate cytoskeletal Factor Va. This is indeed what was found (Fig. 3). Cells treated with EDTA contain no Factor Va on their cytoskeletons.

c) Radiolabeled Factor Xa added to thrombin activated platelets bound to the platelets in a saturable and reversible manner (Fig. 4) consistent with previous studies [11]. Remarkably, the platelet bound Factor Xa was quantitatively recovered with the platelet cytoskeleton (Fig. 4). These experiments indicate that Factor Va the platelet receptor for Factor Xa [11] is retained quantitatively on the cytoskeleton. The most likely explanation of this is that surface bound Factor Va becomes bound very tightly [11] to Factor Xa and this surface complex upon the activation of the platelets by thrombin becomes associated with the cytoskeleton by means of a transmembrane linkage protein.

d) Finally, by the use of Factor Va deficient platelets and platelets deficient in the receptors for Factor Va, we could show that cytoskeletons prepared from these platelets were either totally deficient in Factor Va (Fig. 6) or partially deficient in Factor Va (Fig. 5). Restoration of the cytoskeleton Factor Va activity could only be accomplished by addition of exogenous Factor Va to the platelets prior to preparation of the cytoskeleton (Fig. 6). The most likely explanation of these results is that platelet surface bound Factor Va associates with internal cytoskeletal elements as a consequence of platelet thrombin activation.

The experimental results described above can be summarized in the form of the scheme shown in Figure 7. This scheme depicts the generation of a large Triton insoluble cytoskeleton beneath the plasma membrane as a result of platelet thrombin activation. The cytoskeleton then becomes linked to transmembrane

receptors that bind Factor Va and other elements of the prothrombinase complex such as lipid [13]. Precedence for such transmembrane linkage associations of surface membrane proteins with the internal cell cytoskeleton in other systems is numerous [14, 15]. Therefore, it is likely that in the platelet system transmembrane linkages between surface receptors of Factor Va and the internal cytoskeleton may play an important role in stabilizing or anchoring the platelet surface prothrombinase complex in order to efficiently generate thrombin during platelet aggregation and clot formation.

This research was supported by Health and Human Services Grants HL 28149. HL 25455, and HL 25661 and Grant No. CTR 1389 from the Council for Tobacco Research, Inc. The authors are grateful to Ms. Patricia Pileggi for excellent secretarial assistance,

References

1. Wolosewick, J. J., Porter, K. R.: Microtrabecular lattice of the cytoplasmic ground substance. *J. Cell Biol. 82*, 114 (1979).
2. Steck, T. L.: The organization of proteins in the human red blood cell membrane. *J. Cell Biol. 62*, 1 (1974).
3. Brown, S., Levinson, W., Spudich, J. A.: Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. *J. Supramolec. Struct. 5*, 119 (1976).
4. Frank, E. D., Tuszynski, G. P., Warren, L.: Localization of vimentin and desmin in BHK21/C13 cells and in baby hamster kidney. *Exp. Cell Res. 139*, 235 (1982).
5. White, G. J.: Platelet microtubules and microfilaments. Effects of cytochalasin B on structure and function. In: *Platelet Aggregation*. J. Caen (ed.). Masson, Paris 1971, p. 15.
6. Lucas, R. C., Gallagher, M., Stracher, A.: Actin and actin binding protein in platelets. In: *Contractile Systems in Non-Muscle Tissues*. S. V. Perry R. S. Adelstein, A. R. Margath (eds). Elsevier North-Holland Biomedical Press, Amsterdam 1976, p. 133.
7. Tuszynski, G. P., Kornecki, E., Niewiarowski, S., Knight, L., Srivastava, S.: Platelet cytoskeletons contain receptors for fibrinogen. *J. Cell Biol. 95*, 3A (abstract) (1982).
8. Tuszynski, G. P., Walsh, P. N., Piperno, J. R., Koshy, A.: Association of coagulation factor V with the platelet cytoskeleton. *J. Biol. Chem. 257*, 4557 (1982).
9. Timmons, S., Hawiger, J.: Separation of human platelets from plasma proteins including factor VIII by a combined albumin gradient-gel filtration method using Hepes buffer. *Thromb. Res. 12*, 297 (1978).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193*, 265 (1951).
11. Kane, W. H., Majerus, P. W.: The interaction of human coagulation Factor Va with platelets. *J. Biol. Chem. 257*, 3963 (1982).
12. Miletich, J. P., Majerus, D. W., Majerus, P. W.: Patients with congenital factor V deficiency have decreased factor X, binding sites on their platelets. *J. Clin. Invest. 62*, 824 (1978).
13. Schick, P. K., Tuszynski, G. P. VanderVoort, P. W.: Human platelet cytoskeletons: specific content of glycolipids and phospholipids. *Blood 61*, 163 (1983).
14. Singer, I. I.: The fibronexus: A transmembrane association of fibronectin containing fibers and bundles of 5 nm microfilaments in hamster and human fibroblasts. *Cell 16*, 675 (1979).
15. Bennett, V., Stenbuck, P. J.: Human erythrocyte ankyrin. *J. Biol. Chem. 255*, 2540 (1980).

Haematologia 17 (1), pp. 77–79 (1984)

Biological Action and Clinical Significance of Antithrombin III

U. ABILDGAARD

Medical Department A, Aker Hospital, Oslo 5, Norway

(Received 5 August 1982; accepted 1 February 1983)

The story of antithrombin III (AT-III) dates from the turn of the century. It was felt that thrombin, having converted fibrinogen to fibrin in the hemostatic plug, must be inactivated. This early prophecy of a complex between one thrombin and one AT-III molecule was in fact confirmed some 70 years later. In 1965, Egeberg provided the biological proof that familial low AT-III activity is combined with a strong tendency to thrombosis [1]. Since that time, chemical and clinical research on AT-III have progressed hand in hand.

Purification of AT-III from plasma has made it possible to study the structure and function of the molecule (Table 1). Although AT-III inactivates 5 of the activated coagulation factors (or enzymes), most of our knowledge concerns inactivation of T thrombin and factor Xa. But it is probably this function at several levels of the coagulation pathway which explains that an apparently modest decrease in AT-III activity, to about half of that found in normal plasma, leads to thrombotic tendency.

Table 1

Basic research 1967–1979

Purification, characterization
Interaction with thrombin, Xa (IXa)
Interaction with heparin, heparan sulfate
Immunoassay
Crossed immunoelectrophoresis: abnormal AT
AT–thrombin complex: neoantigens
Metabolism
Assay with chromogenic substrates

Many centers have participated in the outline of AT-III structure and function. As often, immunology has provided valuable tools for such studies (Ingemar Björk, Uppsala.)

In clinical medicine, too, many centers have contributed to progress in the knowledge of the relations between AT-III and diseases. The simple chromogenic substrate has proved particularly valuable for a rapid and accurate assay of AT-III. Table 2 summarizes some main clinical research topics. We do not know much about the metabolism of AT-III in disease. There is also a need for a common international protocol for the study of the effects of AT-III concentrate.

Acquired AT-III deficiency may result from decreased synthesis, increased consumption, or loss into urine or tissues. The group in Amsterdam has particularly studied the acquired deficiencies.

Table 2

Clinical topics 1965–1979

Congenital deficiency
Subtypes
Acquired deficiency
Liver disease
Proteinuria
DIC, postoperative septicaemia
P-pills
Heparin
Metabolism
Treatment with AT concentrate

I do not know why hereditary AT-III deficiency seems to have been diagnosed in Hungary more often than elsewhere. It may be related to ethnic differences. But I believe that the long tradition in AT research in Hungary is important. One has to look for AT-III deficiency to find it. Gerendás [2] designed a practical assay and suggested an enzymatic character of the thrombin–AT-III reaction.

The eloquent studies of Machovich [3] on the interactions between thrombin, heparin and AT-III have been most stimulating. The first abnormal AT molecule, AT-III Budapest, was described by Sas and coworkers [4].

A functional abnormality of the AT-III molecule may involve a reaction with the enzymes thrombin or factor Xa, or, as shown by Nagy from Pécs also heparin binding [5]. Nagy has particularly studied AT in liver disease.

References

1. Egeberg, O.: Inherited antithrombin deficiency causing thrombophilia. *Thrombos. Diathes. Haemorrh. 13*, 516 (1965).
2. Gerendás, M.: Die Thrombinaktivierung als Enzymprozess. *Thrombos. Diathes. Haemorrh. 4*, 56 (1960).
3. Machovich, R., Blaskó, G., Pálos, L. A.: Action of heparin of thrombin-antithrombin reaction. *Biochim. Biophys. Acta 379*, 193 (1975).
4. Sas, G., Blaskó, G., Bánhegyi, D., Jákó, J., Pálos, L. A.: Abnormal antithrombin III (antithrombin III 'Budapest') as a cause of a familial thrombophilia. *Thrombos. Diathes. Haemorrh. 32*, 105 (1974).
5. Nagy, I., Losonczy, H., Szaksz, I., Temesi, G., Hergert, K.: An analysis of clinical and laboratory data in patients with congenital antithrombin III (AT III) deficiency. *Acta Med. Acad. Sci. Hung. 36*, 53 (1979).

Haematologia 17 (1), pp. 81–86 (1984)

Hereditary Antithrombin III Deficiency: Biochemical Aspects

G. SAS

First Department of Medicine, Postgraduate Medical School, Szabolcs u. 35, H-1389 Budapest, Hungary

(Received 27 September 1982; accepted 4 February 1983)

Since the description of the first thrombophilic family with congenital AT-III deficiency, increasing numbers of different types of the condition have become evident. Initially the anomaly seemed to be homogeneous and simple: the three main characteristics of AT-III (thrombin inactivating and heparin cofactor activity, antigen concentration) were decreased. This type of AT-III deficiency (type 1) was later divided into type 1a and 1b on the basis of the heparin affinity of the AT-III molecule. The first family with a different qualitative AT-III disorder (type 2) was described by our group in 1974. In the members of this family the AT-III antigen concentration was normal, but the molecule had no functional activity (AT-III Budapest). In the last few years some new variants of type 2 hereditary AT-III disorders have been observed; they are characterized by a loss of one or more functional properties of the AT-III molecule.

Keywords: antithrombin III, classification, hereditary deficiency

*

Although only 19 years have passed since description of the first hereditary antithrombin III deficient family [1], the heterogeneity of the anomaly has become obvious. The present review will summarize the development of recent knowledge on the hereditary antithrombin III deficiencies, paying particular attention to the biochemical features of the various types of the disorder.

The antithrombin III molecule has three main special features, namely the thrombin-inactivating capacity, the activated Factor X inactivating capacity and the heparin cofactor activity. When we characterize antithrombin, we have to take account of its antigenic property and we have to examine its electrophoretic characteristics as well.

We do not know the exact mechanism by which heparin enhances the inactivation of thrombin or Factor Xa, but we do know that heparin somehow binds to antithrombin. We can easily prove the existence of the heparin-antithrombin binding directly, either by chromatographic methods or by crossed immunoelectrophoresis. However, the functional change of the antithrombin molecule caused by heparin occurs not necessarily on the basis of this bond. The accelerating effect of heparin on thrombin inactivation occurs far below the threshold concentration at which we can demonstrate heparin-antithrombin binding. This fact must be born

in mind when we study the pathological antithrombins. Indeed, in the case of antithrombin 'Budapest' the pathological antithrombin molecule has some binding activity to heparin, but it has no heparin cofactor activity [2].

Since the publication by Egeberg [1] of the first antithrombin deficient family, many authors have described similar thrombophilic families [3, 4]. Initially, the anomaly seemed to be homogeneous and simple. The thrombin-inactivating, and the heparin cofactor activities were both decreased in parallel with the antigen concentration, generally to about 50 per cent of the normal value. This type of antithrombin deficiency seems to be the most frequent variant of the disease; it was called by us Type 1. In the affected members of the families investigations with labelled antithrombin proved a decreased synthesis of antithrombin.

Later, applying heparin-affinity chromatography and modified crossed immunoelectrophoresis (mixing heparin with agarose), two sub-groups could be differentiated within Type 1 [5]. In Type 1a, antithrombin displays normal heparin affinity, while in Type 1b heparin-affinity is decreased in varying degrees from normal to zero.

In addition to the quantitative abnormality there is a qualitative disorder of the antithrombin molecule.

The first inherited qualitative abnormality of the antithrombin molecule was described by us in 1974 [2]. In this consanguinous thrombophilic family, the quantity of antithrombin was nearly normal, but the thrombin and Factor Xa inactivation was considerably decreased. At the beginning we assumed either a homogeneous population of pathological antithrombin molecules with diminished functional activity, or a heterogeneous population, one normal and one functionally inactive. Further investigations with the modified crossed immunoelectrophoresis technique supported the latter assumption: in the heparinized agarose gel we found a normal and a pathological population of antithrombins. This latter pathological population was termed antithrombin 'Budapest'. Experiments with gel-filtration proved that the pathological antithrombin had a greater than normal molecular size, about 100 000 daltons. Although this pathological antithrombin did not display any thrombin or activated Factor X inactivating capacity, it showed a decreased but still detectable affinity to heparin.

The main characteristic of any qualitative antithrombin anomaly is the discrepancy between the functional activity and its antigen concentration. Generally, the functional activity is decreased, while the antigen concentration is normal, as in cases of patients with the antithrombin 'Budapest' anomaly [6].

A detailed characterization of another pathological antithrombin was carried out on the members of a thrombophilic Japanese family [7]. In the affected members of this family the slightly decreased functional antithrombin activity was accompanied by a more significant decrease in antigen concentration. Because this had the opposite characteristics of antithrombin III 'Budapest' the anomaly was described as a 'reverse' type of the Budapest disorder. However, for a more precise characterization of this pathological antithrombin further biochemical data were needed.

Two years ago a new type of hereditary qualitative antithrombin abnormality was revealed by investigations of the heparin cofactor activity of the antithrombin molecule. The first cases of this variant were described in Hungary by Nagy et al. [8]. The common features of this disorder were a normal thrombin and Factor Xa inactivating capacity of the plasma of the affected individuals, with a decreased heparin cofactor activity.

In this group of patients, the antigen concentration of antithrombin is normal but the modified crossed immunoelectrophoresis technique indicated an abnormal pattern. We had the opportunity to study the proposita with antithrombin 'Paris' reported by Wolf et al. [9]. In the course of a joint work with Dr. Wolf we compared the immunoelectrophoretic pattern of antithrombin Budapest and antithrombin Paris. The experiments showed clearly an abnormal population of antithrombin molecules in the plasma of the French patient, and this abnormal antithrombin displayed a total loss of heparin binding capacity (Fig. 1). Similar results were published by Tran et al. [10] in the case of a Swiss thrombophilic family on the basis of biochemical analysis of the pathological antithrombin.

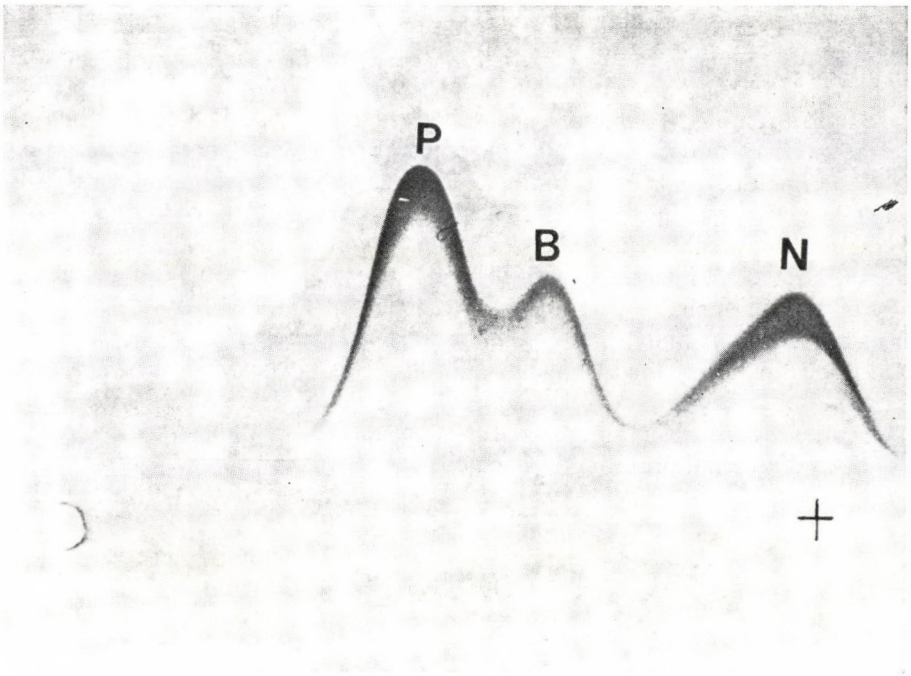


Fig. 1. Common antithrombin III pattern of the propositi with antithrombin III 'Budapest' and 'Paris' anomalies. A 50% mixture of the plasmas was prepared and crossed immunoelectrophoresis (heparin in agarose) was performed. Three main precipitation arches were obtained corresponding to the normal antithrombin III component of the propositi (N), antithrombin III 'Budapest' (B) and antithrombin III 'Paris' (P)

The susceptibility to thrombosis with this variant of antithrombin is, however, not quite clear. In these cases the inactivation of thrombin and Factor Xa is normal but heparin is incapable of enhancing their inactivation. According to present knowledge, heparin is either not present in normal human plasma or only in very small amounts. In other words, the inability of antithrombin to be influenced by heparin theoretically cannot predispose to thrombosis. In the case of antithrombin 'Paris', from 7 affected members of the family only a single one had repeated thromboses, and this could have occurred merely by chance.

In the case of the Ann Arbor family [11], the abnormality remained silent not causing thrombosis. Thus we have to conclude that this abnormal antithrombin either does not cause thrombosis or even this subgroup is heterogeneous. Further investigations are needed to clarify this point.

A new variant of hereditary antithrombin deficiency was described recently in Denmark by Sørensen et al. [12]. This antithrombin is characterized by a decreased thrombin-inactivating capacity and normal crossed immunoelectrophoretic pattern in the heparinized agarose gel. Further investigations in cooperation with Dr. Sørensen in our laboratory revealed that in the plasma of these patients the inactivation of Factor Xa is also normal.

Neither heparin affinity chromatography, nor gel filtration displayed any abnormality of antithrombin III 'Aalborg' as we call it after the town where this pathological antithrombin has been described [13].

Antithrombin III 'Aalborg' has raised some interesting questions about the biochemical features of antithrombin. It has been shown that thrombin, Factor IXa, and Factor Xa all cleave antithrombin at an arginin-serine bond which is the active site of the antithrombin molecule. Antithrombin III 'Aalborg' seems to contradict this assumption, because the abnormal thrombin inactivation and the normal Factor Xa inactivation could easily be explained by two different active sites on antithrombin, one responsible for thrombin, and the other for Factor Xa inactivation. Maintaining the original concept we may explain the antithrombin 'Aalborg' anomaly by the assumption that the active site at which cleavage occurs is normal but another part of the molecule is pathological and this is the part responsible for the specific binding of thrombin to antithrombin, while the binding of Factor Xa to antithrombin takes place normally.

FUNCTIONAL TESTS	Thrombin-inactivation FX_a-inactivation Heparin-cofactor activity (using natural or synthetic substrates)
IMMUNOCHEMICAL TESTS	Determination of AT-III. antigen concentration (Laurell's rocket or Mancinis method)
	Crossed immunoelectrophoresis (with and without heparin)

Fig. 2. Suggested methods for the investigation of patients with the suspicion of congenital antithrombin III deficiency

On the basis of the dissociated defects of thrombin and Factor Xa inactivation, as demonstrated in the case of antithrombin 'Aalborg', we may assume the existence of a localized Factor Xa inactivation defect of the molecule. An important practical consequence of the different inactivation mechanisms of thrombin and Factor Xa is that in a thrombophilic patient, in addition to the inactivation of thrombin we have to investigate the inactivation of Factor Xa as well.

Thus the following tests should be undertaken when investigating a thrombophilic patient with the suspicion of hereditary antithrombin deficiency (Fig. 2). Finally, Table 1 demonstrates our proposal for the classification of the hereditary antithrombin III deficiencies.

Table 1

Classification of the hereditary antithrombin III deficiencies

Type 1:	Characterized by a parallel decrease of functional activity and antigen concentration	
Subgroups:	*Type 1a:*	normal heparin affinity
	Type 1b:	abnormal heparin affinity
Type 2:	Characterized by disparity of antigen concentration and (one or more) functional activity(ies).	
Subgroups:	*Type 2a:*	normal antigen concentration, decreased thrombin inactivation, F Xa inactivation and heparin affinity (e.g. antithrombin III 'Budapest')
	Type 2b:	normal antigen concentration and heparin affinity, decreased thrombin inactivation (e.g. antithrombin III 'Aalborg')
	Type 2c:	normal antigen concentration, thrombin inactivation, F Xa inactivation, decreased heparin affinity (e.g. antithrombin III 'Paris')

References

1. Egeberg, O.: Inherited antithrombin deficiency causing thrombophilia. *Thrombos. Diathes Haemorrh. 13*, 516 (1965).
2. Sas, G., Blaskó, Gy., Bánhegyi, D., Jákó, J., Pálos, L. Á.: Abnormal antithrombin III (antithrombin III 'Budapest') as a cause of a familial thrombophilia. *Thrombos. Diathes. Haemorrh. 32*, 105 (1974).
3. Meer, J. van der, Stoepman-Van Dalen, E. A., Jansen, J. M. S.: Antithrombin III deficiency in a Dutch family. *J. Clin. Path. 26*, 532 (1974).
4. Marciniak, E., Farley, C. H., Simone, P. A. de: Familial thrombosis due to antithrombin III deficiency. *Blood 43*, 219 (1974).
5. Sas, G., Pető, I., Bánhegyi, D., Blaskó, Gy., Domján, Gy.: Heterogeneity of the 'classical' antithrombin III deficiency. *Thrombos. Haemostas. 43*, 133 (1980).
6. Sas, G., Pepper, D. S., Cash, J. D.: Further investigation on antithrombin III in the plasma of patients with the abnormality of 'antithrombin III Budapest'. *Thrombos. Diathes. Haemorrh. 33*, 564 (1975).
7. Matsuo, T., Ohki, Y., Kondo, S., Matsuo, O.: Familial antithrombin III deficiency in a Japanese family. *Thrombos. Res. 16*, 815 (1979).

8. Nagy, I., Losonczy, H., Szaksz, I., Temesi, C., Hergert, K.: An analysis of clinical and laboratory data in patients with congenital antithrombin III (AT III) deficiency. *Acta Med. Acad. Sci. Hung. 36*, 53 (1979).
9. Wolf, M., Boyer, C., Lavergne, J. M., Larrieu, M. J: A new familial variant of antithrombin III. 'Antithrombin III Paris'. *Brit. J. Haemat. 51*, 285 (1982).
10. Tran, T. H., Bondeli, C., Marbet, G. A., Duckert, F.: Reactivity of a hereditary abnormal antithrombin III fraction in the inhibition of thrombin and Factor Xa. *Thrombos. Haemostas. 44*, 92 (1980).
11. Penner, J. A., Hassouna, H., Hunter, M. J., Chockley, M. A.: A clinically silent antithrombin III defect in an Ann Arbor family. *Thrombos. Haemostas. 42*, 186 (1979).
12. Sørensen, P. J., Dyerberg, J., Stoffersen, E., Krogh Jensen, M.: Familial functional antithrombin III deficiency. *Scand. J. Haemat. 24*, 105 (1980).
13. Sørensen, P. J., Sas, G., Pető, I., Blaskó, Gy., Kremmer, T., Samu, A.: Distinction of two pathologic antithrombin III molecules: Antithrombin III 'Aalborg' and antithrombin III 'Budapest'. *Thrombos. Res. 26*, 211 (1982).

Haematologia 17 (1), pp. 87—91 (1984)

Antithrombin III Deficiency: Clinical Aspects

IBOLYA NAGY

First Department of Medicine, University Medical School,
Ifjúság útja 13, H-7624 Pécs, Hungary

(Received 19 November 1982; accepted 11 February 1983)

The clinical picture of antithrombin III (AT-III) deficiency is characterized by early manifestations of serious venous thromboembolism. The inheritance is autosomal dominant. The precipitating factors of the clinical signs are infections, trauma and pregnancy. For a correct diagnosis, application of various methods (immunological, amidolytic, electrophoresis) are needed.

There are several types of AT-III defect; they are characterized by a decrease in amount and function, a functional decrease, or a pathological heparin-binding. Adequate treatment of thromboembolic episodes caused by AT-III deficiency is a lifelong coumarin therapy. In pregnancy and after surgery heparin can only be given together with AT-III substitution. The decreased AT-III activity may change during treatment and this has a diagnostic importance.

Keywords: antithrombin III deficiency, clinical aspects, hereditary

*

The importance of AT-III decrease lies in the occurrence of serious thromboembolic episodes [1]. A characteristic feature is the early appearance of deep venous thrombosis usually between 10 and 20 years of age and sometimes even earlier. Sudden pulmonary embolism may also occur but arterial localization is infrequent; several cases have been observed, mainly in the carotid region. Men and women are equally affected. The inheritance is autosomal dominant, though abnormal AT-III may be inherited from both parents. The frequency of the abnormality is probably 1 : 10 000 [2]. The provoking or precipitating factors of the clinical symptoms are infections (often genitourinary), surgical operations, trauma and pregnancy. They usually seem to be mild in comparison to the serious clinical picture. Progression of the deep vein thrombosis is very rapid and may lead to death.

We have lost only one patient, a 14 years old girl operated upon for an ovarian cyst. Her AT-III decrease had not been discovered before. A serious deep vein thrombosis developed immediately after the operation. In spite of the treatment she died of repeated pulmonary embolisms. All her veins were practically obliterated.

The life expectancy of these patients depends to a large extent on the correct diagnosis and treatment.

The main diagnostic possibilities are shown in Table 1. In recent years several AT-III variants have been recognized [3], so one needs to apply various methods

Table 1

Methods used in the diagnosis of AT III deficiency

AT-III	activity
	thrombin inactivation
	Xa inhibition
	various amidolytic methods
AT-III	amount
	Behring M-partigen
	Laurell Rocket-electrophoresis
Crossed electrophoresis	
Other biochemical methods	

to reach the correct diagnosis. Determination of the amount of AT-III is not enough, one has to determine the activity and also the heparin-cofactor. The Gerendás-Rák method [4] measures the AT-III activity without and with heparin. The advantage of the method is the optimal thrombin and heparin concentration, so it is able to indicate an abnormal AT-III by the so-called paradoxical heparin effect. This means that in the presence of heparin the thrombin inactivation does not increase, it rather decreases. The method is useful in the diagnosis of any AT-III defect.

On the basis of present knowledge [5] AT-III abnormalities can be divided into three main types (Table 2).

Table 2

Types of hereditary AT-III defects

	AT-III		AT-III activity in the presence of heparin
	amount	function	
Type I	↓	↓	↓
Type II	normal	↓	↓
Type III	normal	normal	↓

Type I is a true decrease which is easy to recognize. The extremely low amount and activity of AT-III and the characteristic crossed electrophoretic pattern are of diagnostic value.

Type II is a functional decrease where the amount of AT III is normal but its activity is low. The diagnosis of this type may be difficult because during anticoagulant therapy the AT-III activity increases. Therefore, examination of the untreated family members is necessary.

Type III patients have a pathological AT-III activity only in the presence of heparin. The diagnosis of this type is most difficult. Beside the above mentioned

methods, heparin loading with therapeutical doses [6] and other biochemical examinations may be necessary.

These three types probably have different subtypes. The diagnosis depends to a great extent on the methods applied [7–9].

As seen in Figure 1, the immunologic and amidolytic methods are suitable only to detect type I, the 'true' decrease. For the diagnosis of other types, functional determinations in the presence of heparin seem to be better. The amount of heparin in the system is critical. For the treatment of AT-III defects, coumarin or other indirect anticoagulants should be applied (Table 3).

The question arises whether the patients need lifelong anticoagulant treatment. Our patients with a few exceptions are treated continuously but only those

Table 3

Treatment of hereditary AT-III defect

Coumarin:	lifelong treatment
Heparin:	in pregnancy at surgery but only with AT-III substitution!

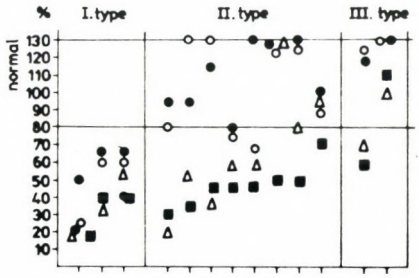


Fig. 1. Comparison of four methods in the detection of AT-III abnormality. ● - AT-III amount (Behring M-Partigen) [8]; ○ - AT-III activity with chromogenic substance S-2222 [8]; △ - AT-III function [4]; ■ - AT-III function in the presence of heparin [4]

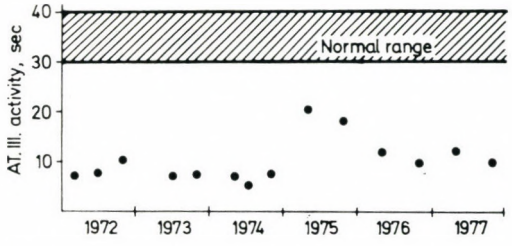


Fig. 2. AT-III activity in type I patient during coumarin treatment [4]

who suffer from clinically manifest thromboembolism. Symptom-free patients are not treated, only observed regularly.

Pregnancy poses a great problem in these patients. Very often it is the first provoking factor of thrombosis. These patients need therapy but coumarin-type anticoagulants, the best treatment otherwise, are contraindicated. At present low dose heparin and AT-III substitution seems to be the most useful.

In some cases AT-III activity has been observed to change during anticoagulant therapy. According to our experience in type I (Fig. 2) it remains always decreased, though the patients are clinically well and symptom-free. In types II and III the activity becomes normal during treatment (Fig. 3). The correct explanation of this observation is not clear, but it has an important significance in diagnostics [5].

As seen in Fig. 4, heparin alone has no effect on the AT-III activity of the patient. However, fresh plasma given together with heparin increases immediately the activity of AT-III. This is in good agreement with the facts, that heparin cannot effect as an anticoagulant without a normal AT-III level.

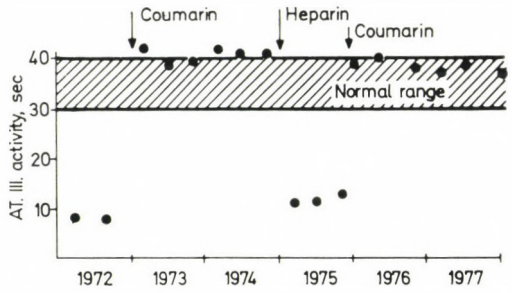


Fig. 3. AT-III activity in type II patient during coumarin and heparin treatment [4]

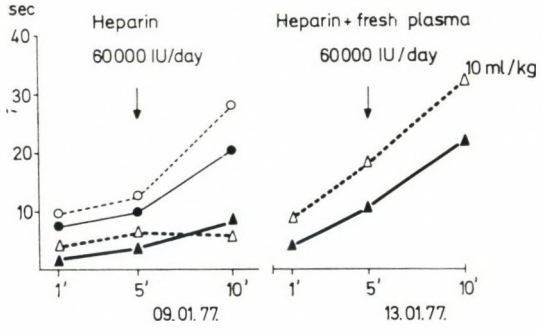


Fig. 4. AT activity after administration of heparin and fresh plasma. ○, ● - control samples taken from healthy blood donors; △, ▲ - blood samples of a patient with type I AT-III deficiency. The continuous line represents the basic AT-III activity, whereas the dotted one the activity measured in the presence of heparin [4]

Summarizing the clinical aspects of AT-III deficiency we can conclude as follows:

Hereditary AT-III defects can be divided into 3 types (Table 2).

Clinical picture is characterized by very early beginning of severe venous thrombosis.

Correct diagnosis of AT-III deficiency is based on applying various methods. The amidolytic methods are not always suitable. The same is true for the crossed immunoelectrophoresis which is not always characteristic to the type of the deficiency. In vivo heparin loading may also be necessary.

The treatment might be life saving, therefore it is important to examine untreated family members, too.

To get further informations about this very interesting and important protein abnormality we have some tasks in connection with these patients (Table 4).

Table 4

Future tasks

Longitudinal observation of patients
Repeated examinations, detailed biochemical analysis
To examine genetical or race markers (out of our 5 patients belonging to type I, 4 were gypsies)
Screening to determine the exact frequency of AT-III variants

References

1. Egeberg, O.: Inherited antithrombin III deficiency causing thrombophilia. *Thrombos. Diathes. Haemorrh. 13*, 516 (1965).
2. Abildgaard, U.: Antithrombin and related inhibitors of coagulation. In: *Recent Advances in Blood Coagulation*. L. Poller (ed.). Churchill Livingstone, Edinburgh 1981, p. 3.
3. Nagy, I., Losonczy, H.: Three types of hereditary antithrombin III deficiency. *Thrombos. Haemostas. 42*, 187 (1979).
4 Rák, K.: Thrombin inactivating ability (antithrombin III activity) of plasma and serum in liver cirrhosis. *Magy. Belorv. Arch. 22*, 3 (1969). (In Hungarian.)
5. Nagy, I., Losonczy, H., Szaksz, I., Temesi, C., Hergert, K.: An analysis of clinical and laboratory data in patients with congenital antithrombin III (AT III) deficiency. *Acta Med. Acad. Sci. Hung. 36*, 53 (1979).
6. Nagy, I., Losonczy, H.: The importance of the heparin-effect in the recognition of a decrease of antithrombin III. In: *Forschungsergebnisse der Transfusionsmedizin und Immunhaematologie*. H. Vinazzer (ed.). Medicus Verl. Berlin 1979.
7. Losonczy, H., Nagy, I.: Die Bedeutung der amidolytischen Methode bei der Typisierung der angeborenen Antithrombin III-Verminderung. *Folia Haemat. 109*, 184 (1982).
8. Nagy, I., Losonczy, H.: Vergleichende Untersuchungen zur Bestimmung der abnormen Antithrombin III-Aktivität. *Folia Haemat. 109*, 180 (1982).
9. Tran, T. H., Bounameaux, H., Bondeli, C., Honkanen, H., Marbet, G. A., Duckert, F.: Purification and partial characterization of a hereditary abnormal antithrombin III fraction of a patient with recurrent thrombophlebitis. *Thrombos. Haemostas. 44*, 87 (1980).

Haematologia 17 (1), pp. 93–100 (1984)

Plasma Fibronectin Concentration in Patients Admitted to Intensive Care Unit

H. O. ERIKSEN,[1] F. MOLKE-JENSEN,[1] INGE CLEMMENSEN[2]

[1]Department of Anaesthesiology and [2]Department of Clinical Chemistry, University Hospital, 2650 Hvidovre, Denmark

(Received 25 October 1982; accepted 17 January 1983)

The plasma fibronectin level was determined by electroimmune assay in patients under intensive care. The decrease in plasma fibronectin concentration was most pronounced in patients with sepsis and DIC. Survival of these patients was related to the extent of decrease of the plasma fibronectin concentration. Burn injury and the associated surgical treatment were followed by a decrease in fibronectin concentration which then normalized in 24–48 h after the burn injury. It is concluded that serial determinations of plasma fibronectin are essential for studying the relation of a low plasma fibronectin with the clinical condition.

Keywords: cold insoluble globulin, disseminated intravascular coagulation (DIC) syndrome, intensive care unit patients, plasma fibronectin, sepsis

*

Plasma fibronectins are a family of glycoproteins which are present in soluble form in different body fluids (plasma, cerebrospinal fluid and urine) [1, 2] and in insoluble form in many tissues [3, 4]. Fibronectin is synthesized by a variety of different cells: fibroblasts, macrophages, astroglia cells and endothelial cells [5–7]. Fibronectin interacts with many biological substances, like fibrin(ogen), heparin, collagen and bacterial cell walls of *Staphylococcus aureus* [8–10]. These interactions might explain some of the functions of plasma fibronectin.

Circulating fibronectin has been suggested to be of importance as an opsonin for the reticuloendothelial system [11]. A decrease in reticuloendothelial activity during extensive surgery, burns and trauma associated with sepsis has been observed [12]. A decreased plasma fibronectin concentration has been found to associate with depressed reticuloendothelial function in animal experiments [12, 13].

A decrease of plasma fibronectin concentration may be caused both by consumption as a result of RES function and by deposition in tissue injury. This may result in a decrease of reticuloendothelial function and an increased tendency to develop the disseminated intravascular coagulation syndrome (DIC).

The purpose of the present study was to investigate the plasma fibronectin level of patients admitted to intensive care units and to study its relation to major complications.

Materials and Methods

Patients

Plasma fibronectin concentration was studied in

(1) 374 healthy persons, 202 males and 172 females, ranging in age from 0 to 85 years [14];

(2) 109 patients admitted to the intensive care unit for mechanical ventilation. Of these patients *(i)* 20 had sepsis confirmed by positive blood culture, and *(ii)* 10 patients had disseminated intravascular coagulation (DIC). The diagnosis of DIC was based on clinical signs and laboratory results including decreased platelet counts and coagulation factors II–VII–X, presence of fibrin monomers, and increased fibrin degradation products (FDP); *(iii)* 49 patients who had major complications different from sepsis and DIC. The major complications were renal, heart or respiratory failure, or abdominal infection; *(iv)* 30 patients who had no major complication while in the intensive care unit;

(3) 16 patients sustaining second and third degree burns ranging 21 to 61 per cent of the body surface area (BSA). Their age ranged from 18 to 62 years (average 40 years).

The plasma fibronectin concentration of patients in the intensive care unit was estimated daily, if possible, until death or discharge.

Venous blood. The blood sample was drawn into tubes containing 0.3 ml dried dipotassium-EDTA 62 mmol $\cdot$ l^{-1}.

Plasma. Plasma was prepared by centrifugation at 1500 *g* for 10 min at room temperature. It was stored at -20 °C until use and thawed at 37 °C. It is of importance to thaw the plasma at 37 °C, to avoid fibrinogen precipitation. Precipitation of fibrinogen in plasma causes a decrease in fibronectin concentration as fibronectin binds to precipitated fibrinogen.

Buffer for electrophoresis was Tris-barbiturate buffer made up of Tris, 73 mmol $\cdot$ l^{-1}; diethylbarbituric acid, 25 mol $\cdot$ l^{-1}; calcium lactate, 0.3 mmol $\cdot$ l^{-1}; NaN_3, 2 mmol $\cdot$ l^{-1}, pH 8.6, I = 0.07 mmol $\cdot$ l^{-1}.

Agarose (Indubiose A 45) was from L'Industrie Biologique, Genevilliers, France.

Plasma standard. Pooled plasma from healthy adult donors served as the plasma fibronectin standard. The concentration of fibronectin in this plasma was determined using purified fibronectin as standard.

Plasma fibronectin concentration was determined by quantitative electroimmunoassay as described by Laurell [15]. The standard deviation was 28.2 mg $\cdot$ l^{-1} (0.06 μmol $\cdot$ l^{-1}), the degree of freedom (d.f.) = 45.

Purified human fibronectin was prepared from outdated blood bank plasma by gelatin-Sepharose [16]. The mass concentration was determined by using a specific absorbance coefficient of 12.8 l $\cdot$ mol^{-1} $\cdot$ cm^{-1} [1]. The purified fibronectin gave a single band on SDS-polyacrylamide electrophoresis under non-reducing

conditions. Crossed immunoelectrophoresis of the purified fibronectin showed one precipitate using anti-human serum in the second dimension.

Immunoglobulins against human fibronectin were produced and purified as described earlier [16, 17]. To inactivate plasmin present in the immunoglobulin preparation, pancreatic trypsin inhibitor (Trasylol®) was added to a final concentration of 5×10^{-6} mol $\cdot$ l^{-1} [18].

Statistical methods

Statistical significance of the plasma fibronectin concentration in the intensive care unit patients was evaluated by the Mann-Whitney non-parametric method (unpaired samples).

Results

Plasma fibronectin concentration in patients admitted to the respiratory intensive care unit. In view of the high individual variations of the plasma fibronectin concentration, the maximal depletion was measured. The plasma fibronectin concentration in healthy persons was used as the basic value.

The mean value of the maximal depletion of plasma fibronectin in patients with sepsis was 202.5 $\pm$ 20.1 mg $\cdot$ l^{-1} ($\pm$ 1 S.D.) and that in patients with DIC was 197 $\pm$ 31.5 mg $\cdot$ l^{-1} ($\pm$ 1 S.D.). Patients with major complications other than sepsis and DIC or without major complications had a maximum depletion of 140.2 $\pm$ 13.6 mg $\cdot$ l^{-1} ($\pm$ 1 S.D.) and 92.4 $\pm$ 18.9 mg $\cdot$ l^{-1} ($\pm$ 1 S.D.) respectively (Table 1).

The maximum depletion of plasma fibronectin was significantly higher in the patients with sepsis, DIC or major complications compared to the group of patients without major complications ($p < 0.05$). This was also true for the patients

Table 1

Patients admitted to respiratory intensive care unit

Group of patients	No. of patients	Maximum depletion of plasma fibronectin concentration (mean $\pm$1 S.D.) mg $\cdot$ l^{-1}
Sepsis	20	202.5 $\pm$ 20.1
DIC	10	197 $\pm$ 31.5
Major complications other than sepsis/DIC	49	140.2 $\pm$ 13.6
No major complication	30	92.4 $\pm$ 18.9

with sepsis in relation to those with major complications ($p < 0.05$). No significant difference in depletion was, however, observed between patients with DIC and those with sepsis or major complications ($p > 0.05$).

In the group of patients with DIC or sepsis ($n = 30$) the depletion of plasma fibronectin was significantly greater for the non-survivors than for those who survived ($p < 0.05$). No significant difference was observed between non-survivors and survivors in the groups with and without major complications ($p > 0.05$) (Table 2).

Plasma fibronection concentration in burn injury. Behaviour of the plasma fibronectin level after burn injury in 10 patients without any such complication as severe skin infection, sepsis, renal or cardiovascular failure etc., is demonstrated by the following case (Fig. 1).

A 27 years old male suffered a 35 per cent total body surface area burn of which 11 per cent was of third degree. On the day of admission the plasma fibronectin concentration was 300 mg $\cdot$ l^{-1}; it increased on the following days to reach the normal level within 48 h. A rebound effect (hyperopsonaemia) with 650 mg $\cdot$ l^{-1} was observed on day 14; during these two weeks no complication was observed.

At the time of surgery on day 17 three determinations of plasma fibronectin were done. One just before the beginning of surgery, one 2 h later and one at the end of the operation.

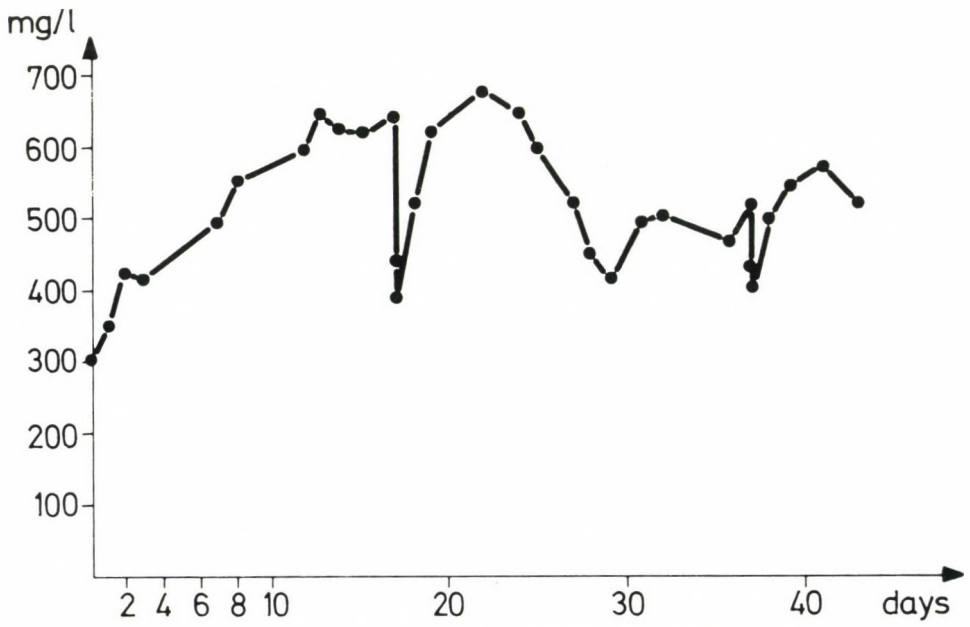


Fig. 1. Daily variation of plasma fibronectin concentration in burn injury without complication

Following surgical debridement of 11 per cent of the body surface area, a 38 per cent decrease was observed in the plasma fibronection concentration. The second operation took place on day 37 when debridement of 1.5 per cent of the body surface was performed; this time a 24 per cent decrease occurred in the plasma fibronectin concentration. After both operations a rebound effect was observed; this occurred after day 5 and day 4, respectively.

On day 42 the patient was discharged in a good clinical condition.

Patients with burn injury and major complications like sepsis, severe skin infections, renal or cardiovascular failure, showed certain common tendencies in the daily variation of the plasma fibronectin level (6 patients). This was illustrated by the following case (Fig. 2).

A 40 years old man suffered a burn extending to 37 per cent of the body surface, with 22 per cent of third degree. On the first postburn day the plasma fibronectin level was 100 mg $\cdot$ l^{-1}; on day 2–4 it was 375 mg $\cdot$ l^{-1}. At the same time the patient developed a heart failure and electrolyte imbalance. On day 5, surgical debridement of 22 per cent of the body surface was performed and this was followed by a 53 per cent decrease of the plasma fibronectin concentration and then by a rebound effect (hyperopsonaemia), reaching the maximum, 525 mg $\cdot$ l^{-1}, 3 days later. After day 10 the patient developed renal failure with a severe skin and lung infection and on days 21 and 27 positive blood cultures were obtained. In this period the plasma fibronectin concentration was between 250 mg $\cdot$ l^{-1} and 340 mg $\cdot$ l^{-1}.

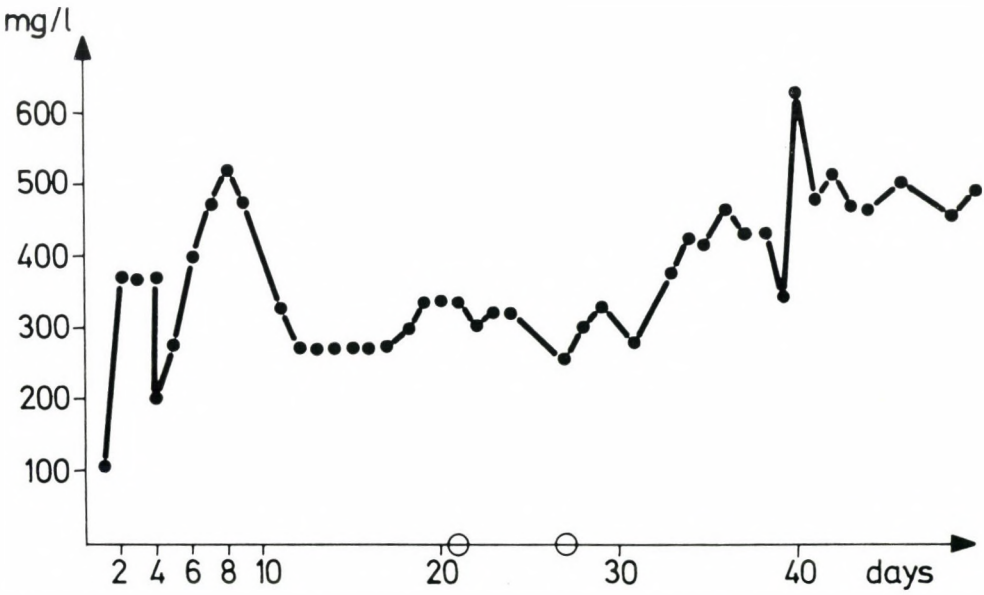


Fig. 2. Daily variation of plasma fibronectin concentration in burn injury associated with infection. The open circles on the abscissa indicate the days of positive blood culture

From day 31 after the burn, the patient's condition improved and this was followed by an increase of the plasma fibronectin level (Fig. 2). On day 50 the patient was discharged from the intensive care unit.

Discussion

In animal experiments, tissue injury is associated with a decreased opsonic activity of the plasma in the following 24 h [13]. The factor responsible for this has been identified as fibronectin [12, 13]. Its functional activity was determined in rat liver slices by measuring the uptake by Kupffer cells of lipid particles coated with labelled gelatin [19]. Fibronectin is supposed to act as an opsonin for these particles and heparin seems to be essential for the uptake [12, 19]. This bioassay, however, may not determine the true opsonic activity of fibronectin since it has been shown that only the first step, attachment of the particles to the phagocytic cell, depends on the presence of fibronectin [20].

In healthy persons the plasma fibronectin level has been found to depend on sex and age [14]. A high individual range with no daily variations has been observed [14]. Because of this, it is necessary to study the variations of the level by time interval in patients in whom plasma fibronectin consumption was suspected. Single determinations are difficult to evaluate in clinical studies.

Plasma fibronectin, whether measured by bioassay or an immunological assay, was found to decrease following major surgery, sepsis and in severely ill patients with DIC [12, 21, 22].

In the present study, the mean value of the maximum depletion of fibronectin was determined in different groups of patients (Table 1). As found also by others [22, 23], the decrease was most pronounced in patients with sepsis or DIC (Table 1). This might be explained by a consumption of fibronectin when fibrin is found in the microcirculation owing to DIC and when circulation fibrin complexes are removed

Table 2

Maximum depletion of plasma fibronection concentration (mean value ± 1 S.D.)

Group of patients	Survivors	Non-survivors
	mg · l^{-1}	
DIC/sepsis	172.8 ± 21.4 (n = 18)	242.4 ± 22.7 (n = 12)
Major complication other than DIC/sepsis	133.4 ± 14.3 (n = 42)	135.9 ± 32.5 (n = 7)
No major complication	76.4 ± 27.6 (n = 15)	108.4 ± 26.2 (n = 15)

from the blood by the reticuloendothelial system [12]. Whether in the group of patients with sepsis the decrease was a result of the sepsis, or the decrease preceded the sepsis as suggested by some authors [23], remains to be determined. Besides, the degree of the decrease seems to be of importance for survival (Table 2), as suggested by other authors [22].

The low plasma fibronectin level found immediately after the burn normalized in 24–48 h and at two weeks a tendency to rebound was observed. A decrease and a restoration of the level by 24 h after the injury was recorded also after physical trauma induced in rats and in a clinical study of patients with burn injury [23, 25]. The decreased plasma fibronectin concentration following burns and surgical treatment was in agreement with other findings [23, 24]. The magnitude of fibronectin depletion measured during surgery was correlated to the extent of debridement ($r = 0.82$). The decrease in plasma fibronectin observed after debridement might be due to the high affinity of fibronectin for exposed collagen or also to a consumption during the clearance of cell debris [12].

The importance of the decreased plasma fibronectin level in patients in a severe condition is difficult to evaluate, since the decrease is never observed by itself but always associated with a blood loss from the circulation and by consumption of other blood components, e.g. fibrinogen, originating from the complement system and the immunoglobulins.

It might be of clinical value to restore the plasma fibronectin concentration with cryoprecipitate .... which contains much fibronectin .... in patients with a high consumption of plasma fibronectin caused by sepsis or DIC.

*

This study has been supported by grants from the Danish Rheumatism Association. The authors wish to thank Mrs. Ryberg for technical assistance and Mrs. Vicki Nielsen for assistance in the preparation of the manuscript.

References

1. Mosesson, M. W., Umfleet, R. A.: The cold insoluble globulin of human plasma. I. Purification, primary characterization, and relationship to fibrinogen and other cold insoluble fraction components. *J. Biol. Chem. 245*, 5728 (1970).
2. Kuusela, P., Vaheri, A., Palo, J., Ruoslathi, E.: Demonstration of fibronectin in human cerebrospinal fluid. *J. Lab. Clin. Med. 92*, 595 (1978).
3. Stenman, S., Vaheri, A.: Distribution of the major connective tissue protein, fibronectin, in normal tissue. *J. Exp. Med. 147*, 1054 (1978).
4. Linder, E., Vaheri, A., Ruoslathi, E., Wartiovaara, J.: Distribution of fibroblast surface antigen in the developing chick embryo. *J. Exp. Med. 142*, 41 (1975).
5. Johansson, S., Rubin, K., Høøk, M., Ahlgren, T., Seljelid, R.: In vitro biosynthesis of cold insoluble globulin (fibronectin) by mouse peritoneal macrophages. *FEBS Lett. 105*, 313 (1979).
6. Vaheri, A., Ruoslathi, E.: A common cell-type specific, surface antigen in cultured human glial cells and fibroblasts loss in malignant cells. *J. Exp. Med. 143*, 64 (1976).
7. Chen, L. B., Maitland, N., Gallimore, P. H., McDougall, J. K.: Detection of large external transformation-sensitive protein on some epithelial cells. *Exp. Cell. Res. 106*, 1, 39 (1977).

8. Mosher, D. F.: Cross-linking of cold insoluble globulin by fibrin stabilizing factor. *J. Biol. Chem. 250*, 6614 (1975).
9. Rouslathi, E., Engvall, E.: Immunochemical and collagen-binding properties of fibronectin. *Ann. N.Y. Acad. Sci. 312*, 178 (1978).
10. Espersen, F., Clemmensen, I.: Clumping of *Staphylococcus aureus* by human fibronectin. *Acta Path. Microbiol. Scand. Sect. B. 89*, 317 (1981).
11. Saba, T. H., Blumenstock, F. A., Weber, P., Kaplan, J. E.: Physiologic role of cold insoluble globulin in systemic host defense. Implication of its characterization as the opsonic α_2-surface binding glycoprotein. *Ann. N.Y. Acad. Sci. 42*, 312 (1978).
12. Saba, T. M.: Reticuloendothelial systemic host defense after surgery and traumatic shock. *Circulat. Shock 2*, 91 (1975).
13. Kaplan, J. E., Saba, T. M.: Humoral deficiency and reticuloendothelial depression after traumatic shock. *Amer. J. Physiol. 230*, 7 (1976).
14. Eriksen, H. O., Clemmensen, I., Hansen, M. S., Ibsen, K. K.: Plasma fibronectin concentration in normal subjects. *Scand. J. Clin. Lab. Invest. 42*, 291 (1982).
15. Laurell, C. B.: Electroimmuno assay. *Scand. J. Clin. Lab. Invest. 29*, (Suppl. 124) 21 (1972).
16. Clemmensen, I., Andersen, R. B.: Different molecular forms of fibronectin in rheumatoid synovial fluid. *Arthr. and Rheum. 25*, 25 (1982).
17. Grinwell, F.: The serum dependence of baby hamster kidney cell attachment to a substratum. *Exp. Cell Res. 97*, 265 (1976).
18. Petersen, L. C., Clemmensen I.: Kinetics of plasmin inhibitor in the presence of synthetic substrate. *Biochem. J. 199*, 121 (1981).
19. Saba, T. M., Filkins, J. P.. Di Luzio. N. R.: Properties of the opsonic system regulating in vitro hepatic phagocytosis. *J. Reticuloendoth. Soc. 3*, 398 (1966).
20. Molnar, J., Gudewicz, P., Ming-Zong, L., Credo, B., Siefring, G., Lorance, L.: Role of plasma fibronectin in phagocytosis of gelatin coated colloids. *Fed. Proc. 38*, 303 (1979).
21. Scovill, W. A., Saba, T. M., Blumenstock, F. A., Bernard, H., Powers, S.: Opsonic α_2 surface binding glycoprotein therapy during sepsis. *Ann Surg. 188*, 521 (1978).
22. Mosher, D. F., Williams, E. M.: Fibronectin concentration is decreased in plasma of severely ill patients with disseminated intravascular coagulation. *J. Lab. Clin. Med. 91*, 729 (1978).
23. Scovill, W. A., Saba, T. M., Kaplan, I. C., Bernard, H., Powers, S.: Deficits in reticuloendothelial humoral control mechanism in patients after trauma. *J. Trauma 16*, 898 (1976).
24. Lanser, M. E., Saba, T. M., Scovill, W. A.: Opsonic glycoprotein (plasma fibronectin) levels after burn injury. *Ann. Surg. 192*, 776 (1980).
25. Di Luzio, N. R., Lindsey, E. S.: Surgery induced alterations in plasma recognition factor activity in normal renal donors and renal recipients. *Proc. Soc. Exp. Biol. Med. 143*, 715 (1973).

Haematologia 17 (1), pp. 101–106 (1984)

Significance of Plasma Fibronectin

INGE CLEMMENSEN

Department of Clinical Chemistry, Hvidovre Hospital, University of Copenhagen, 2650 Hvidovre, Denmark

(Received 25 October 1982; accepted 16 February 1983)

Fibronectin and fibrinogen are unique proteins, since they both are present in a soluble form in plasma and in an insoluble form in connective tissue. The insoluble fibronectin is always present in connective tissue, while the presence of fibrin is temporary. The two proteins may be associated under various conditions. Fibronectin is incorporated into polymerizing fibrin, e.g. in the tissue repair process and during thrombus formation. Fibronectin is also associated with other biological structures, such as collagen of all types, sulphated proteoglycans, blood platelets and the cell walls of *Staphylococcus aureus*. These properties result in a depletion of plasma fibronectin in certain clinical situations, such as disseminated intravascular coagulation (DIC), traumatic shock and severe burns. The decreased plasma fibronectin concentration might result in an increased fibrin polymer concentration in the circulation, thus accentuating the microvascular thromboses and the coagulation factor consumption in DIC. The practical clinical implication is that a low plasma concentration of fibronectin might define a group of patients with a high risk of developing DIC.

Keywords: fibrin, fibronectin, tissue repair

*

The high molecular weight glycoprotein fibronectin is present in extracellular fluids (plasma, cerebrospinal, amniotic and allantoic fluid), and in insoluble form in interstitial connective tissue. Prior to the suggestion of the name fibronectin, the protein has been designated by a variety of terms including: cold insoluble globulin (CIG), cell surface protein (CSP), large external transformation sensitive protein (LETS), soluble fibroblast antigen (SF-antigen), cell adhesion factor (CAF), α_2-opsonic glycoprotein, antigelatin factor. All of these terms are now included in the name fibronectin which indicate the properties of the protein to bind to fibrous proteins like collagen and fibrin (for reviews see Refs [1, 2]).

A variety of cells has been found to synthesize fibronectin: fibroblasts [3], astroglia [4], and endothelial cells [5].

The molecule has a M_r of 450 000 and is built up by two, probably identical, peptide chains held together by one disulphide bridge in the C-terminal part of the molecule [6]. Domains for the binding of many biological substances have been described in the molecule: fibrin, heparin, collagen of all types, sulphated proteoglycans and hyaluronic acid. The concentration of fibronectin in plasma varies between 0.3–0.6 g/l: it is lowest at birth and increases with age with a high degree

of individual variation [7]. The amount of fibronectin in serum is lower than in plasma as a result of binding of fibronectin to fibrin [8]. The fraction of fibronectin incorporated into fibrin clot during coagulation is variable (21–48 per cent of the plasma fibronectin). Several plasma components and physical factors may influence the amount of fibronectin which will bind to fibrin, e.g. the plasma fibrinogen concentration, the factor XIII (fibrin cross-linking factor) concentration and the temperature [8]. Quantitative estimation of fibronectin is therefore meaningful only for the study of plasma and not of serum. Fibronectin is a unique protein since it is present in soluble form in plasma and other body fluids and in insoluble form in connective tissue. This property is, at least partly, shared by fibrinogen (fibrinogen is only temporarily present in the tissue as fibrin). Both these proteins belongs to the plasma proteins with protective functions such as the immunoglobulins and the complement system, the proteinase inhibitors and the coagulation factors. Their functions are directed towards the limitation and repair of various types of injury.

Plasma fibronectin has 3 main functional roles: in tissue injury and repair, opsonin properties: antithrombotic activity.

Tissue Injury and Repair

Any damage to the normal endothelial lining cells of the vessels will start a chain of reaction; its best known result is the formation of the haemostatic blood clot (Fig. 1). The reaction of the protective systems in the blood are qualitatively the same whether the injury has thermal, bacteriological or immunological causes or is the consequence of, for example, arteriosclerotic changes in the arteries. Thus fibronectin and fibrinogen are well-known participants in the formation of the abcess membrane protecting the organism from invasion by microorganism or other foreign materials and are not solely associated with haemostatic functions [9]. As a result of vascular injury subendothelial structures with new binding sites partially caused by a different net surface charge, will be exposed to the blood. This has several effects: blood platelets and various coagulation factors will become activated and generate the enzyme thrombin from prothrombin. This in turn will convert fibrinogen to fibrin monomer, which polymerizes spontaneously to form the insoluble fibrin net (Fig. 1).

During the conversion of fibrinogen to fibrin the soluble plasma fibronectin will be incorporated into the fibrin meshwork (Fig. 2).

The exposure to collagen and glycosaminoglycans may also cause the plasma fibronectin adhering to these structures to be transformed into the matrix form of fibronectin.

Fibrin and fibronectin will be linked to each other by covalent bonds. Thrombin in the presence of calcium ions activates the coagulation factor XIII. This transglutaminase is responsible for stabilizing the fibrin net by the formation of covalent bonds, called cross links [10], and for forming covalent bonds between fibronectin and the fibrin monomers [11, 12]. Fibronectin has also an affinity to-

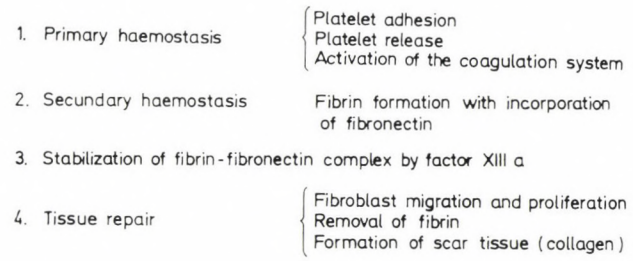


Fig. 1. Tissue injury and repair

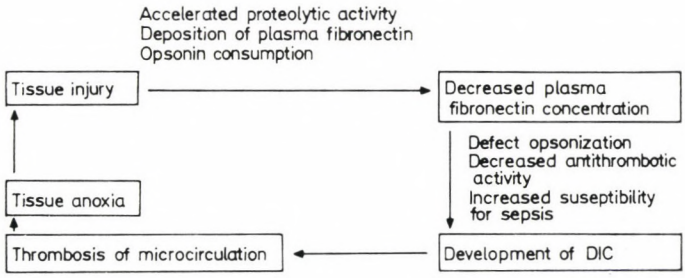


Fig. 2. Incorporation of soluble plasma fibronectin into the fibrin meshwork during the conversion of fibrinogen to fibrin

wards the unaltered fibrinogen molecule but only at low temperature. The affinity increases as fibrinogen is converted to fibrin, but is still temperature dependent [13]. The primary function of this fibrin-fibronectin net is to minimize the leakage of blood constituents from the damaged vessel.

The different factors involved in the first 3 steps in Figure 1 are all derived from plasma. The formation of the fibrin-fibronectin net is important for the following steps of the tissue repair process (Fig. 1). This is illustrated by a report by Remuzzi and coworkers [14]. They described a patient with Glanzmann's thrombasthenia associated with a fibronectin with a defective fibrin binding site. This defect resulted in an abnormal tissue repair process as demonstrated by a severely impaired scar tissue formation after a skin biopsy. Fibronectin cross-linked to fibrin is an important fibroblast attachment site and a substrate for fibroblast migration [15]. The importance of the cross-linking is also demonstrated in patients deficient in factor XIII. These patients show a defective wound healing [16]. The presence of fibronectin in granulation tissue and wound healing has recently been described [17–19].

The attachment of other cells to the fibrin-fibronectin net may also be of importance for normal wound healing and the formation of inflammatory reactions. Blood platelets adhere to fibrin and collagen, but the actual attachment may

at least partly be mediated through fibronectin bound to the platelet surface. The presence of platelets in the repair tissue is important since platelets secrete a potent growth factor for the fibroblasts.

Opsonin Properties

The protective role of fibronectin is not limited to its participation in the haemostatic reactions and the demarcation of local infections. It is probably directly involved in the clearance of bacteria through its opsonic activity in plasma.

Saba and coworkers demonstrated in a long series of experiments that plasma fibronectin enhances the removal of a gelatinized test particle by the reticuloendothelial system [20–23]. The process was accelerated by heparin. But as gelatin, which is a denatured collagen, specifically binds to fibronectin [24], this test system seems to be unsuitable for investigations of fibronectin activity as an unspecific opsonin.

It is however known that fibronectin binds to the cell wall of *Staphylococcus aureus* [25]. Both the binding site in the fibronectin molecule and the receptor protein in the staphylococcal wall have been isolated [26, 27]. Despite these interactions between *Staphylococcus aureus* and fibronectin, the opsonic activity is seemingly minor. This was recently demonstrated by Mosher and coworkers, who found that fibronectin mediated the attachment of *Staphylococcus aureus* to human neutrophils, but that it did not promote neutrophil phagocytosis of the bacteria [28]. As the bacteria were concentrated on the surfaces of neutrophils, the number of colony-forming units fell and the chemiluminescence increased [28].

The attachment to, but not phagocytosis of, gelatinized particles by human monocytes has also been described [29]. The role of fibronectin in sepsis seems complex and has not yet been fully elucidated. The role of fibronectin as an opsonin might be limited to the removal from the circulation of collagen and fibrin degradation products. Bang and coworkers have recently demonstrated the indigestibility of fibrinogen and fibrin degradation products by macrophages [30]. The attachment of fibrin(ogen) degradation products might be mediated by fibronectin as fibronectin binds to monocytes [29].

Antithrombotic Activity

Fibronectin might be of importance in the regulation of fibrin deposition and removal. Its influence on fibrin assembly and cross-linking is still unclear. It is however incorporated into soluble fibrin complexes produced by thrombin action on plasma and into the high molecular weight fibrin derivatives produced by the action of plasmin on fibrin, occurring in clinical conditions associated with disseminated intravascular coagulation [31]. Mosesson and coworkers have shown that fibronectin increases the surface binding between monocytes and particles or surfaces of collagen [29]. It is tempting to speculate that fibronectin acts as an opsonin in the

clearance of fibrin and collagen breakdown products [30]. It has been demonstrated by Kaplan and Snedeker that fibronectin increased the solubility of fibrin monomers in vitro [32]. Fibrin monomers result from the thrombin cleavage of fibrinogen and these monomers aggregate spontaneously under physiological conditions to form polymers. This would suggest that fibronectin is capable of influencing the clinical manifestations of some types of disseminated intravascular coagulation. The available results suggest a potential antithrombotic role for plasma fibronectin.

In a clinical situation with high catabolic rates such as major tissue injury with deposition of fibronectin in the injured tissue and with a high activity of the reticuloendothelial system, the consumption of fibronectin might exceed its synthesis and result in a low plasma concentration of fibronectin (Fig. 2) [33]. The low plasma fibronectin concentration might predispose to sepsis, resulting in a further consumption of fibronectin. This situation might also predispose to a deposition of soluble fibrin polymers in the microcirculation, leading to anoxic tissue damage and in turn cause further activation of the enzymes and cellular systems involved in inflammation. In such situations a vicious circle develops (Fig. 2) and survival depends on the ability to break it through. One way to overcome this might be a transfusion of cryoprecipitate in severely ill patients.

References

1. Yamada, K. B., Olden, K.: Fibronectins-adhesive glycoproteins of cell surface and blood. *Nature 275*, 179 (1978).
2. Mosher, D. F.: Fibronectin. In: *Progress in Hemostasis and Thrombosis*. Grune and Stratton, New York 1980, vol. 5, p. 111.
3. Wartiovaara, J. Linder, E., Ruoslahti, E., Vaheri, A.: Distribution of fibroblast surface antigen. *J. Exp. Med. 140*, 1522 (1974).
4. Vaheri, A., Ruoslahti, E., Westermark, B., Ponten, J.: A common cell-type specific surface antigen in cultured human glial cells and fibroblasts: Loss in malignant cells. *J. Exp. Med. 143*, 64 (1976).
5. Jaffe, E. A., Mosher, D. F.: Synthesis of fibronectin by cultured human endothelial cells. *J. Exp. Med. 147*, 1779 (1978).
6. Mosesson, M. W., Amrani, D. L.: The structure and biologic activities of plasma fibronectin. *J. Amer. Soc. Hemat. 56*, 145 (1980).
7. Eriksen, H. O., Clemmensen, I., Hansen, M. S., Ibsen, K. K.: Plasma fibronectin concentration in normal subjects. *Scand. J. Lab. Invest. 42*, 291 (1982).
8. Mosesson, M. W., Umfleet, R. A.: Cold-insoluble globulin of human plasma, *J. Biol. Chem. 245*, 5728 (1970).
9. Opie, E. L.: The transformation of sero-fibrinous into purulent pleurisy. *J. Exp. Med. 9*, 414 (1907).
10. Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey J., Oner, N., Brunner-Lorand, J.: Amine specificity and transpeptidation: inhibition of fibrin cross-linking. *Biochemistry 7*, 1214 (1968).
11. Mosher, D. F.: Cross-linking of cold-insoluble globulin by fibrin-stabilizing factor. *J. Biol. Chem. 250*, 6614 (1975).
12. Keski-Oja, J., Mosher, D. F., Vaheri, A.: Cross-linking of a major fibroblast-surface-associated glycoprotein (fibronectin) catalyzed by blood coagulation factor XIII. *Cell 9*, 25 (1976).

13. Ruoslahti E., Vaheri, A.: Interaction of soluble fibroblast surface antigen with fibrinogen and fibrin. *J. Exp. Med. 141*, 497 (1975).
14. Remuzzi, G., Marchesi, E., De Gaetan, G., Donati, M. B.: Abnormal tissue repair in Glanzmanns thrombasthenia. *Lancet 1*, 274 (1977).
15. Grinell, F.: Fibroblast receptor for cell-substrate adhesion: Studies on the interaction of baby hamster kidney cells with latex beads coated by cold insoluble globulin (plasma fibronectin). *J. Cell Biol. 86*, 104 (1980).
16. Ducker, F., Jung, E. Shmerling, D. H.: A hitherto undescribed congenital haemorrhagic diathesis probably due to fibrin stabilizing factor deficiency. *Thrombos. Diathes. Haemorrh. 5*, 179 (1960).
17. Kurkinen, M., Vaheri, A., Roberts, P. J., Stenman, S.: Sequential appearance of fibronectin and collagen in experimental granulation tissue. *Lab. Invest. 43*, 47 (1980).
18. Fursikawa, L. S. Foster, C. S., Harrist, T. J., Lanigan, J. M., Colvin, R. B.: Fibronectin in healing rabbit corneal wounds. *Lab. Invest. 45*, 120 (1981).
19. Hølund, B., Clemmensen, I., Junker, P., Lyon, H.: Fibronectin in experimental granulation tissue. *Acta Path. Microbiol. Scand. 90*, 159 (1982).
20. Saba, Th. M.: Reticulo-endothelial systemic host defence after surgery and traumatic shock. *Circulat. Shock 2*, 91 (1975).
21. Kaplan, J. E., Saba, T. M.: Humoral deficiency and reticuloendothelial depression after traumatic shock. *Amer. J. Path. 230*, 7 (1976).
22. Blummenstock, F. A., Weber, P., Saba, T. M.: Isolation of biochemical characterization of α_2-opsonic glycoprotein from rat serum. *J. Biol. Chem. 252*, 7156 (1977).
23. Blummenstock F. A., Saba, T. M., Weber, P., Laffin, R.: Biochemical and immunological characterization of human opsonic α_2SB glycoprotein: its identity with cold-insoluble globulin. *J. Biol. Chem. 253*, 4287 (1978).
24. Engvall, E., Rouslahti, E., Miller, E. J.: Affinity of fibronectin to collagen of different genetic types and to fibrinogen. *J. Exp. Med. 147*, 1584 (1978).
25. Kuusela, P.: Fibronectin binds to *Staphylococcus aureus. Nature 276*, 718 (1978).
26. Mosher, C., Practor, R. A.: Binding and factor $XIII_a$ mediated cross-linking of a 27-kilodalton fragment of fibronectin to *Staphylococcus aureus. Science 209*, 927 (1980).
27. Espersen, F., Clemmensen I.: Isolation of a fibronectin-binding protein from *Staphylococcus aureus. Infect. Immun. 37*, 526 (1982).
28. Proctor, R. A., Prendergast, E., Mosher, D. F.: Fibronectin mediates attachment of *Staphylococcus aureus* to human neurophils. *J. Amer. Soc. Hemat. 59*, 681 (1982).
29. Bevilacqua, M. P., Amrani, D., Mosesson, M. W., Bianco, C.: Receptors for cold-insoluble globulin (plasma fibronectin) on human monocytes. *J. Exp. Med. 153*, 42 (1981).
30. Bang, N. U., Chang, M. L., Mather, L. E., Burck, P. J., Von Frank, R. M., Zimmermann, R. E., Marks, C. A., Boxer, L. J.: Monocyte macrophage-mediated catabolism of fibrinogen and fibrin. *Ann. N.Y. Acad. Sci. 370*, 568 (1981).
31. Stahakis, N. E., Mosesson, M. W., Chen, A. B.: Cryoprecipitation of fibrin-fibrinogen complexes induced by cold-insoluble globulin of plasma. *Blood 51*, 1211 (1978).
32. Kaplan, J. E., Snedeker, P. W.: Maintenance of fibrin solubility by plasma fibronectin. *J. Lab. Clin. Med. 96*, 1054 (1980).
33. Mosher, D. F., Williams, E. M.: Fibronectin concentration is decreased in plasma of severely ill patients with disseminated intravascular coagulation. *J. Lab. Clin. Med. 91*, 729 (1978).

Haematologia 17 (1), pp. 107–113 (1984)

Effect of Erythropoietin on Acetylation and Phosphorylation of Bone Marrow Histones

A. D. PAVLOV, E. N. PASHUKOV

Department of Pathological Physiology and Central Research Laboratory,
Ryazan Medical Institute,
390012 Ryazan, Junnatov 8/1, kv. 34

(Received 14 December 1982; accepted 10 January 1983)

Polyacrylamide gel electrophoresis was used to reveal in the rabbit bone marrow cells 5 main fractions of histone proteins which are present in other eukaryotic cells. The degree of acetylation and phosphorylation increased after erythropoietin injection or exposure to hypoxia. Phosphorylation was enhanced in every fraction. Stimulation of acetylation was noted in histones rich in arginine. Erythropoietin had no effect on the synthesis of marrow histones in the early period after a hypoxic stimulus.

Keywords: bone marrow, erythropoietin, histones.

Introduction

Erythropoietin plays a central role in the regulation of erythropoiesis. Like any other hormone, erythropoietin stimulates the synthesis of RNA in target cells [2, 10, 14], and this is the molecular basis of differentiation of these cells into proerythroblasts. It is likely that factors controlling the matrix activity of DNA should be taken into account in the mechanisms of erythropoietin action. Histones give rise to non-specific repression of DNA, and certain structural modifications of these nuclear proteins during acetylation and phosphorylation are obviously one of the mechanisms of depression [6].

In the present study an attempt was made to elucidate whether acetylation and phosphorylation of the rabbit bone marrow histones do or do not change during the activation of RNA synthesis under the influence of erythropoietin.

Material and Methods

Experiments were carried out on 45 rabbits, weighing 2.0 to 3.5 kg each, and on 56 female F_1 (CBAxC_{57}B1) mice weighing 16 to 20 g. Structural modifications of histones were observed in the bone marrow of rabbits under conditions of increased production of erythropoietin (hypoxic hypoxia) and after hormone administration. Hypoxic hypoxia was induced by exposure in a hypobaric chamber to a simulated altitude of 6000 m (46 529 Pa). The preparation of urinary erythro-

poietin Pool H-I-TalSL (USA) with a specific activity of 31.8 IU/mg of protein was dissolved in normal saline prior to use and injected intravenously to intact rabbits in a single dose of 40 IU/kg.

Synthesis, acetylation and phosphorylation of histones were studied by using appropriate radioactive compounds: ^{14}C glycine (2046 MBq/mM), ^{14}C acetate (444 MBq/mM) and $Na_2H^{32}PO_4$; they were injected intravenously with 11 MBq/kg 2 h before sacrifice. Contamination of nucleic acids was estimated by measuring the incorporation of ^{3}H thymidine (792 MBq/mM) and ^{3}H uracil (1073 MBq/mM) which were injected intravenously with 19 MBq/kg. All the procedures were performed at 4 °C.

The nuclei from bone marrow were isolated according to the method of Chauveau et al. [8], and the purity of the preparations was controlled by light-microscopy of suspensions of nuclei stained with methyl green pironine. Isolation of histones from nuclei was made through the step of deoxyribonucleoprotein [1], then histones were fractionated according to Oliver et al. [15]. To determine the radioactivity of the obtained fractions each sample was weighed with a precision of 0.5 ± 0.02 mg then dissolved in water and after precipitation by an equal volume of cold 50 per cent trichloroacetic acid was filtered through a millipore membrane filter 'Hufs.' Radioactivity of the precipitates was estimated in a gas-flow counter and expressed in CPM/mg of protein. ^{3}H activity was measured by the liquid scintillation counter SL-4221 (Komef, France). The DNA content was determined by the diphenylamine method of Dische in Burton's modification [7], the RNA content by the orcin method [5]. The purity of the histone fractions was estimated during polyacrylamide gel electrophoresis by the method of Panyim and Chalkley [17], quantitative analysis of the protein bands was done by the method of Johns [13].

Bioassay of erythropoietic activity in plasma of hypoxic rabbits was performed by the method of Cotes [9] with slight modifications [3]. Mice were subjected to intermittent hypoxia (40 kPa) 18 h daily for 3 weeks. Three days after the hypoxic exposures had been stopped the test plasma samples were given s.c. in a dosage of 0.5 ml for 2 days. Mice injected with isotonic saline or normal plasma were used as controls. Twenty-four h following the last plasma sample injection, 37 kBq ^{59}Fe ascorbate was injected i.p. in 0.5 ml of saline and 24 h later 0.5 ml of blood was obtained by aortic puncture for measurement of iron uptake in a well-type scintillation counter and microhaematocrit. Data for mice with a haematocrit less than 60 per cent were discarded. Results were expressed in per cent of the injected radioiron in the calculated blood wolume, which was assumed to amount to 6 per cent of body weight. Estimation of erythropoietin units (EPO) was done from the ^{59}Fe uptake by reference to a standard curve prepared using the 2nd International Reference Preparation, 1971.

Results

Five main fractions of histones were found during gel electrophoresis of unfractionated histones of rabbit bone marrow: H1, H2A, H2B, H3 and H4 (Fig. 1). The purity of histone fractions was: H1 = 93 per cent; H2A + H4 (separation of these fractions was not performed because of the small amount of protein) = 83 per cent; H2B = 90 per cent; and H3 = 87 per cent. Fraction

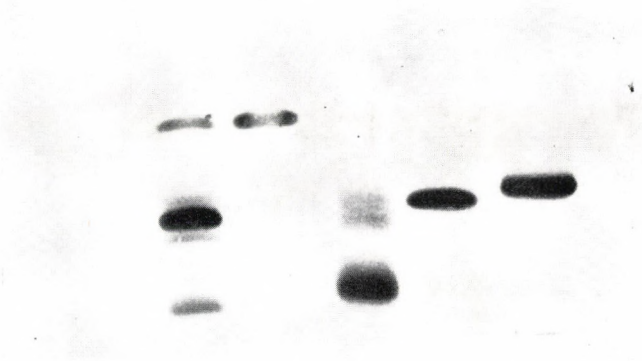


Fig. 1. Electrophoresis of rabbit bone marrow histones in polyacrylamide gel. TH — total, non-fractionated histones; H1, H2A+H4, H2B, H3—fractions

H2A + H4 contained 15 per cent of H2B fraction as an admixture, and fraction H3 contained 10 per cent of H4 fraction. Further purification of these fractions was not undertaken because of the possibility of splitting off the radioactive phosphorus incorporated into histones during the dialysis against ethanol [16].

The admixture of nucleic acids and some of their degradation products in histones was estimated by biochemical and radiometric methods. We found no colour reaction with diphenylamine or orcin and no radioactivity in histones after exposure to ^{3}H-thymidine or ^{3}H-uracyl. Thus, there was no admixture of nucleic acids in bone marrow histones.

The plasma titre of erythropoietin increased in rabbits after 4 h exposure to hypoxia (2.91 ± 0.15 per cent of ^{59}Fe uptake, or 0.12 I.R.P.) as compared with the control (0.77 ± 0.11 per cent of ^{59}Fe uptake).

The phosphorylation of histones of the rabbit bone marrow was not uniform; the highest specific activity was observed in fraction H1 (Table 1). After injection of erythropoietin and exposure to hypoxia the degree of phosphorylation increased in all histone fractions as compared to the control (Table 1).

The acetylation of rabbit bone marrow histones was more uniform than their phosphorylation (Table 2). An increase in acetylation was found only after the administration of erythropoietin: the well-defined increase in acetylation took

place in fraction H3 (Table 2). The rate of histone synthesis in the rabbit bone marrow did not change significantly under the influence of increased erythropoietin production (Table 3).

Table 1

Influence of exogenous erythropoietin and hypoxic hypoxia on ^{32}P incorporation into histones of rabbit bone marrow. Figures give the specific activities (mean ± S. E.) in CPM/mg of protein

Fraction of histones	Control	Hypoxia	Erythropoietin
H1	712±53.5	1190±93.7*	1182±152.9*
H2A + H4	154±16.0	355±60.8*	375±93.8*
H2B	252±27.5	315±74.2	599±72.7*
H3	110±9.0	168±7.5*	278±82.2*

* $p < 0.01$

Table 2

Influence of exogenous erythropoietin and hypoxic hypoxia on ^{14}C acetate incorporation into histones of rabbit bone marrow. Figures give the specific activities (mean ± S. E.) in CPM/mg of protein

Fraction of histones	Control	Hypoxia	Erythropoietin
H1	291±35.8	263±26.3	314±43.9
H2A + H4	339±50.5	290±43.7	497±88.5
H2B	395±35.4	427±62.5	442±30.2
H3	482±62.2	514±21.8	843±67.5*

* $p < 0.01$

Table 3

Incorporation of ^{14}C glycine into non-fractioned histones of rabbit bone marrow after hypoxia

	Control	Hypoxia
Specific activity (mean ± S. E.) in CPM/mg of protein	1696±128.6	1803±97.3 ($p > 0.1$)

Discussion

We found five main histone fractions in the rabbit bone marrow. Some peculiarities in the fractional content of histones of haemopoietic tissue were found in avian nucleated erythrocytes which contained a cell-specific histone H5 [12]. In our electrophoretic system this histone moved between fractions H1 and H3 [18]. With this electrophoretic mobility we did not find any additional protein band. Proteolytic degradation of histones during their isolation, a possible cause of absence of the H5 fraction during electrophoresis was insignificant because fraction H1, the one most sensitive to the action of proteases [17], accounted for 15 per cent of the total histone protein; these results agree with findings of other authors who used similar methods of estimation [13].

Under the influence of exogenous and endogenous erythropoietin the phosphorylation increased of all fractions of histones in the rabbit bone marrow. Changes in the specific activity of the various fractions were similar (Table 1), the discrepancies in activity under the effect of the two stimuli may be explained by differences in the level of exogenous and endogenous erythropoietin in the sera of rabbits. Indeed, the titre of erythropoietin in the sera of hypoxic rabbits was equivalent to 0.12 I.R.P. while the exogenous hormone was administered in a dose of 40 I.R.P./kg body weight. Human urinary erythropoietin injected into normal rats and then assayed in fasted rats showed a biphasic curve with an initial 50 per cent drop in the plasma hormone level in one-half h and then a slower drop. The half-life of the second component was 2.5 h. Inasmuch as 20–25 per cent of exogenous erythropoietin remains in the circulation at least 4 after administration the stimulation of phosphorylation of some fractions of histones (Table 1) was higher than after increased endogenous erythropoietin production.

The well-defined increase in the acetylation of bone marrow histones was observed only in fraction H3 after erythropoietin administration. The selected modification of the fraction H3 under the influence of erythropoietin may be explained by the existence of two kinds of acetylation of histones whose physiological significance is different. The uptake of acetyl groups into fractions H1, H2A and H4 occurs during the synthesis of histones on the N-terminal of serine, and is characterized by metabolic stability. Acetylation of the lysine in fractions H3 and H4 takes place after the combination with DNA of the newly synthesized molecules of these histones. Such acetate groups have reversible interactions when histones function in the composition of chromatin. According to Allfrey [6] the reversible acetylation of ε-amino groups is one of the mechanisms of RNA depression. It is evident that the stimulation of acetylation of the fraction H3 was connected with the presence of ε-amino groups of lysine. We did not find any reliable stimulation of acetylation of the H3 fraction under hypoxia. The most possible explanation of this fact is the decrease in a labelled pool of CoA, a direct donor of acetyl groups under hypoxic conditions [11].

The increase in ^{32}P or ^{14}C acetate uptake may be partly a result of the increased histone synthesis and so, as an increase in the number of uptake sites of acetyl

and phosphate groups by corresponding enzymes. We did not, however, find any change in the rate of histone synthesis under the influence of endogenous erythropoietin (Table 3). These results are in agreement with the finding that histone synthesis is limited by the S-period of the cell cycle. Therefore, stimulation of histone synthesis under conditions of a non-synchronized cell population is due to an augmentation of the bulk of proliferating cells. According to Spivak [19] an increase in the synthesis of histones in the spleen of polycythaemic mice after erythropoietin administration coincided with the appearance of basophilic erythroblasts in this organ. In our experiments the synthesis of histones was investigated 4 h after baro chamber exposure when the stimulation of proliferation in the bone marrow was not well defined.

It is obvious that the action of erythropoietin [20] on acetylation and phosphorylation of histones is not the only mechanism of activation of transcription in target cells under the influence of this hormone. The mechanism of the regulation of gene expression in eucaryote cells are complex and, probably, the structural modifications of histones are one of the steps of interaction of hormones with their effectors. It is likely that histones only control the structure of DNA and they are not specific regulators. Neutralization of positive charges of histones during acetylation and incorporation of negative charged groups during phosphorylation weaken the interaction of histones with DNA, thus making less compact the fibrils of DNA when genes are activated by spedific non-histone proteins [4].

References

1. Zbarsky, I. B., Georgiev, G. P.: New data on fractionation of cell nuclei in rat liver and on chemical composition of structures. *Biokhimiya 24*, 192 (1959). (In Russian.)
2. Pavlov, A. D.: Molecular aspects of action of endogenous erythropoietin. The effect of erythropoietic stimuli on synthesis of ribonucleic acids in rabbit bone marrow. *Biochim. Biophys. Acta 195*, 156 (1969).
3. Pavlov, A. D., Soloviev, A. I., Goncharenko, Yu., D., Pashukov, E. N.: The influence of permanent magnetic field on erythron of rats. *Cosm. Biol. i Aviacosm. Med. 1*, 84 (1980). (In Russian.)
4. Khesin, R. B., Leibovich, B. A.: The structure of chromosomes, histones and activity of genes in *Drosophila melanogaster*. *Molecul. Biol. 10*, 3 (1976). (In Russian.)
5. *Chemistry and Biochemistry of Ribonucleic Acids*. I. B. Zbarsky, S. S. Dbov (eds). Meditsina, Leningrad 1968, p. 78. (In Russian.)
6. Allfrey, V. G.: Functional and metabolic aspects of DNA-associated proteins. In: *Histones and Nucleohistones*. D. M. P. Philips (ed.). Plenum Press, New York 1971, p. 241.
7. Burton, K. A.: A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J. 62*, 315 (1956).
8. Chauveau, J., Moule, J., Rouiller, C. H.: Isolation of pure and unaltered nuclei: morphology and biochemical composition. *Exp. Cell Res. 11*, 317 (1956).
9. Cotes, P. M.: Measurement of erythropoietin. In: *Kidney Hormones*. J. W. Fisher (ed.). Academic Press, New York 1971, p. 243.
10. Goldwasser, E.: Erythropoietin and the differentiation of red blood cells. *Fed. Proc. 34*, 2285 (1975).

11. Gonda, O., Quastel, J. H.: Transport and metabolism of acetate in rat brain cortex *in vitro*. *Biochem. J. 100*, 83 (1966).
12. Hnilica, L. S.: The specificity of histones in chicken erythrocytes. *Experientia 20*, 13 (1964).
13. Johns, E. W.: The electrophoresis of histones in polyacrylamide gel and their quantitative determination. *Biochem. J. 104*, 78 (1967).
14. Krantz, S. B., Goldwasser, E.: On the mechanism of erythropoietin induced differentiation. II. The effect on RNA synthesis. *Biochim. Biophys. Acta 103*, 325 (1965).
15. Oliver, D., Sommer, K. R., Panyim, S., Spiker, S., Chalkley, R.: A modified procedure for fractionating histones. *Biochem. J. 129*, 349 (1972).
16. Ord, M. G., Stocken, L. A.: Further studies on phosphorylation and the thiol/disulphide ratio of histones in growth and development. *Biochem. J. 112*, 81 (1969).
17. Panyim, S., Chalkley, R.: High resolution acrylamide gel electrophoresis of histones. *Arch. Biochem. Biophys. 130*, 337 (1969).
18. Panyim, S., Bilek, D., Chalkley, R.: An electrophoretic comparison of vertebrate histones. *J. Biol. Chem. 246*, 4206 (1971).
19. Spivak, J. L.: Effect of erythropoietin on chromosomal protein synthesis. *Blood 47*, 581 (1976).
20. Stohlman, F. Jr., Howard, D.: Humoral regulation of erythropoiesis. IX. The rate of disappearance of erythropoietin from the plasma. In: *Erythropoiesis*. L. O. Jacobson, M. Dovle (eds). Grune and Stratton, New York 1962, p. 120.

Haematologia 17 (1), pp. 115–124 (1984)

Unusual Bone Marrow Hyperplasia in a Case of Thalassemia Intermedia

P. MASSARO, FRANCA RADAELLI, MARIANGELA COLOMBI, P. BREGANI, E. POGLIANI

Istituto di Clinica Medica I, Università di Milano, via F. Sforza, 35, 20122 Milan, Italy

(Received 20 February 1982; revised 2 September 1982; accepted 8 February 1983)

A case of β thalassemia intermedia is described. Beside the typical hematologic picture and extramedullary erythropoiesis in the liver always present in such a clinical form, atypical masses due to foci of erythropoiesis were found at paravertebral, parasternal and subcostal sites. The size of these masses caused problems in differential diagnosis; they have been solved by computerized axial tomography.

Keywords: β thalassemia, computerized axial tomography, extramedullary erythropoiesis, iron overload.

*

The thalassemia syndromes may be defined as congenital or hereditary diseases due to the partially or completely suppressed synthesis of one or more of the normal hemoglobin chains [1]. There is thus an excess of the unaffected chains synthesized normally, which precipitate inside the erythrocytes [2], and the greater this imbalance, the more reduced is erythrocyte survival [3–6]. The thalassemia syndromes, therefore, vary from forms with almost completely ineffective erythropoiesis to mild, subclinical forms. The term 'thalassemia intermedia' was introduced for the intermediate clinical forms [7]; it includes similar clinical forms with different genotypes [4, 8–10].

Intense extramedullary erythropoiesis may be observed in patients with thalassemia intermedia, usually in the liver and spleen and rarely in other organs [11]. There have been occasional reports of erythropoietic thoracic foci outside bone limits as mediastinal masses, sometimes simulating tumors [12] in adult patients with thalassemia major [11]. Recent studies performed with computerized axial tomography (CAT) in thalassemia patients have confirmed that the intrathoracic tissue derives from the posterior ends of the ribs [12].

This paper reports a case of thalassemia intermedia with atypical foci of erythropoiesis outside the normal bone limits.

Case Report

A 37-year-old man was admitted to the Emergency Department with diffuse abdominal pain which had started 48 h previously, accompanied by a sense of acute abdominal tension and worsening jaundice, but since there were no signs of acute abdomen the patient was transferred to our Department.

The patient reported that he had 'Mediterranean anemia' for which he had been splenectomized at the age of 12 and had received a total of 7 blood transfusions. Since then he had lived a practically normal life and has two children. At the time of admission he was not following any treatment but the evening before the onset of symptoms he had taken a herbal laxative.

On admission to our Department he had marked pigmentation of the skin and scleras due to jaundice; distended abdomen; clearly enlarged liver (12 cm from the costal arch) with a smooth surface, rounded margins and increased consistency, and palpation caused acute pain. No other objective signs of note were found except for a protomesosystolic heart murmur which could be heard at all points, radiating to the neck, axillae and mesocardium. Blood chemistry tests showed a picture of cholestatic jaundice, which however resolved spontaneously and completely during hospitalization. Standard chest X rays revealed the

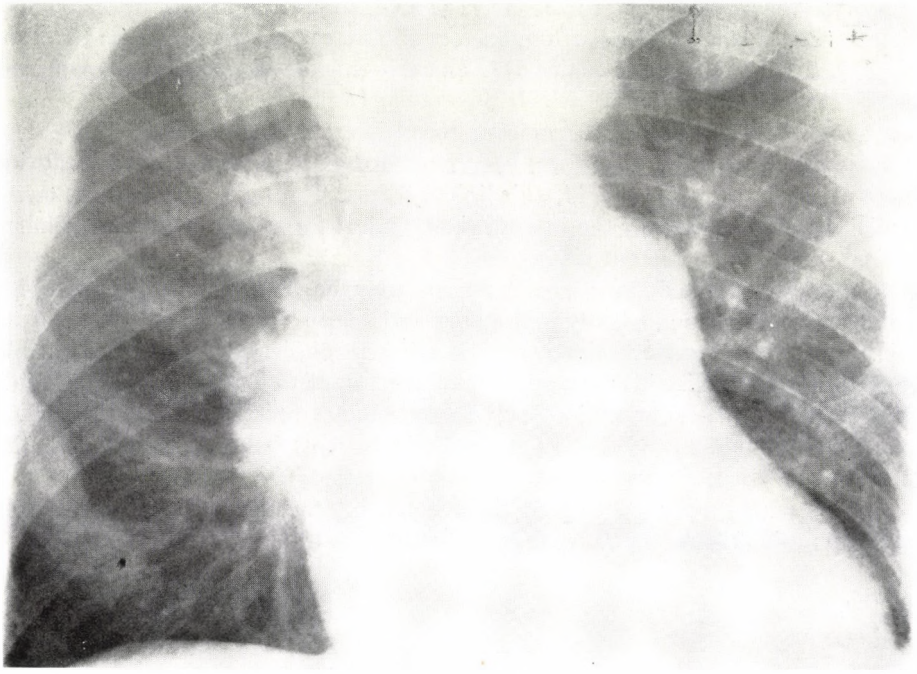


Fig. 1. Chest X ray, anteroposterior projection: roundish radiopaque images, mainly in the left pulmonary field

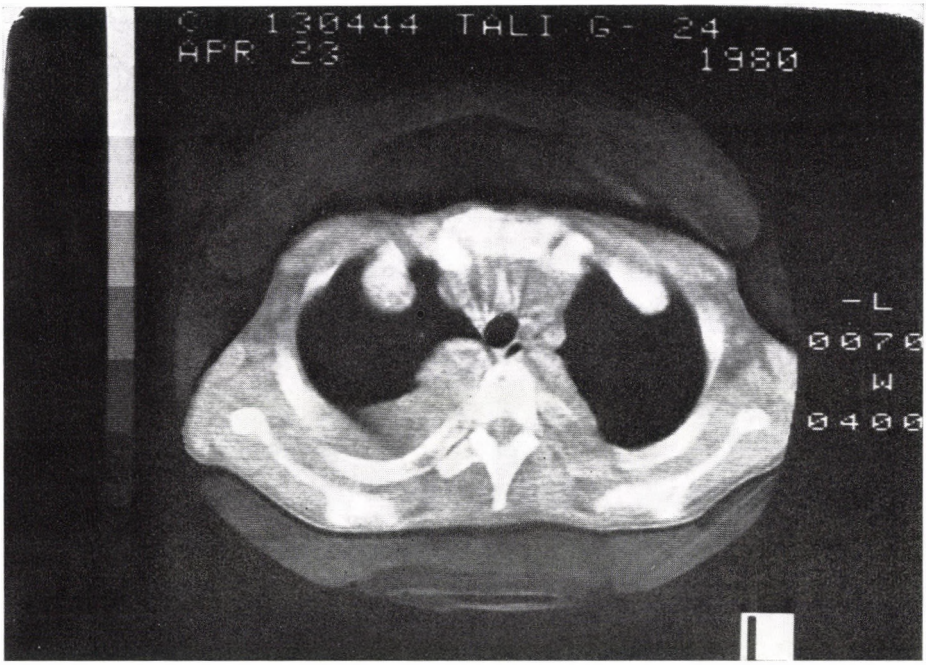


Fig. 2. CAT: masses of extramedullary erythropoiesis in the paravertebral fossae and at parasternal and subcostal sites

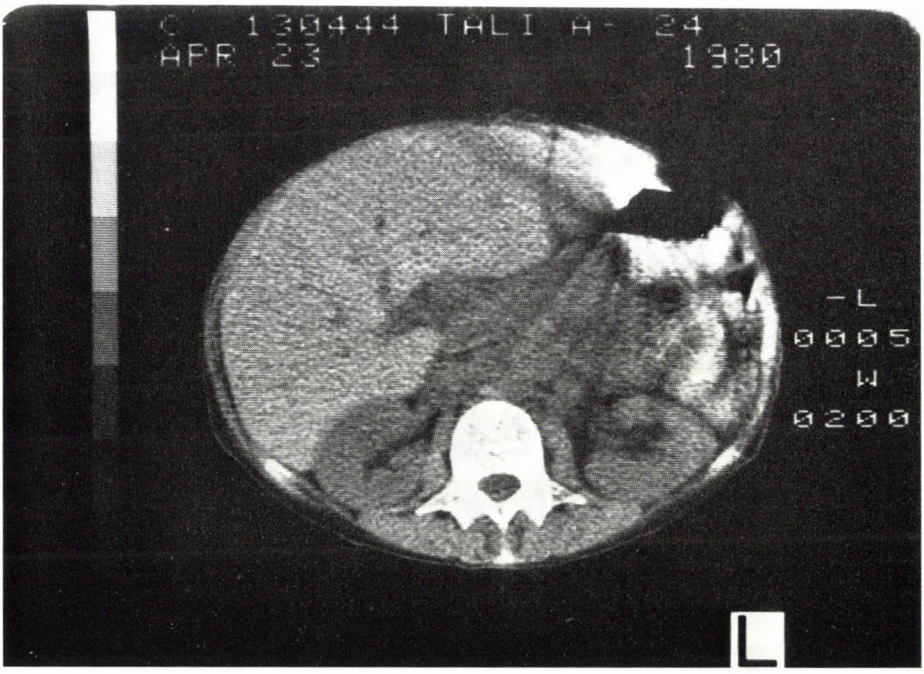


Fig. 3. CAT: relative hypodensity of renal parenchyma compared with hepatic parenchyma

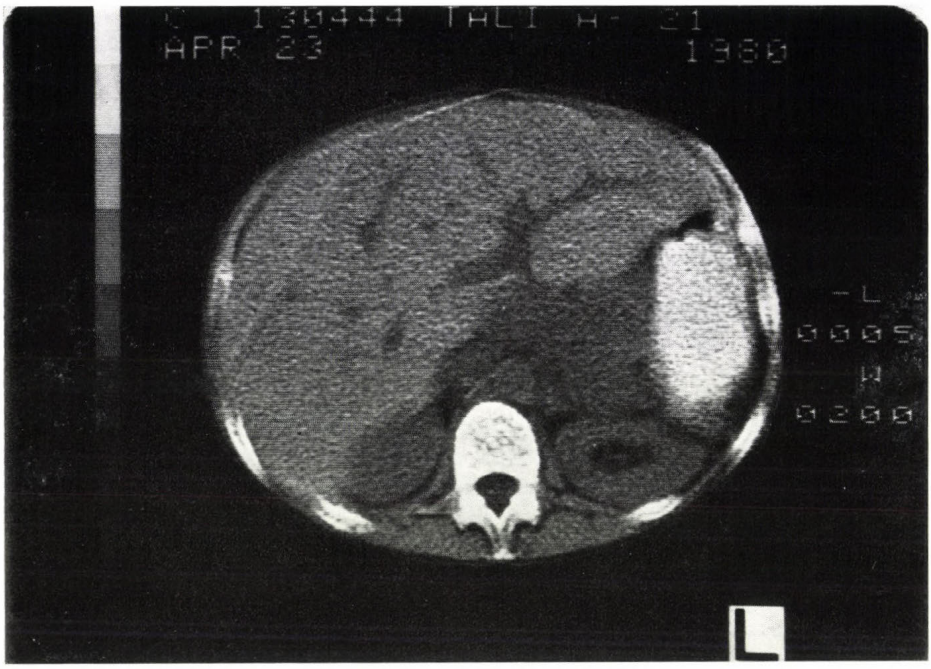


Fig. 4. CAT: considerably dilated biliary ducts inside the hepatic parenchyma

presence of roundish, clearly outlined, radiopaque images of the size of tangerines, projecting mainly in the right pulmonary field and left subclavian region; the middle arch of the left margin of the mediastinum was markedly deformed assuming a curvilinear shape (Fig. 1). There were no signs of increased flow or pulmonary stasis, ECG and echocardiogram were normal. Thoracoabdominal CAT showed abnormally dense masses; after excluding cardiac, pulmonary and bone pathogenesis, these formations were considered to be foci of erythropoiesis outside normal bone limits, which had developed in the parasternal and retrocostal regions on both sides as well as at both paraspinal fossae (Fig. 2). After Gastrografin administration, there was a significant increase in density of the liver (see relative hypodensity of renal parenchyma for comparison, Fig. 3), a typical result of siderosis. The bile ducts were considerably dilated (Fig. 4), and the gallbladder too seemed to be increased in size without images referable to gallstones. Mixed-type alterations of the bone structure in the spongiosa of the vertebral bodies were evidenced by CAT (Figs 3 and 4) and completed the picture shown by standard X rays in which diffuse skeletal decalcification and porous bones of the face and cranial base were observed. Bone densitometry performed with Gambro Bone Densitometer connected in line with Wang computer 600 provided another significant finding a marked reduction in density (radius 0.46 g/cm^2 and ulna 0.45

Table 1

	Father	Mother	Patient	Wife	Son	Daughter
Age	68	65	37	34	8	4
Hb g/dl						
N. V. ♀ 14±2						
♂ 16±2	13.4	10.8	9.5	13.8	11.4	11.2
Hmt. %						
N. V. ♀ 41±5						
♂ 45±7	44	37	33.7	42	38	36
RBC× 10^{12}/l						
N. V. ♀ 4.8±0.6						
♂ 5.4±0.9	6.3	5.6	3.98	4.5	5.7	5.9
MCV μm^3						
N. V. 84±7	71	67	79	93	65	64
MCH pg						
N. V. 29±3	21.1	19.3	24.3	30	19.8	21.9
MCHC g/dl						
N. V. 34±2	30.1	29.1	30.2	32.4	30.3	31.1
Reticul. %	1.5	1.2	6.9	1	1.1	1.2
Erythrobl.× 10^9/l	—	—	32.8	—	—	—

Routine hematological examinations were carried out according to Dacie and Lewis [13]

N. V. = normal value

g/cm^2; normal values 0.60–1.10 g/cm^2). Liver biopsy performed after the rapid regression of the jaundice evidenced foci of diffuse erythropoiesis and severe (grade IV) parenchymal siderosis (Figs 5 and 6).

The patient and his family were investigated to determine the genetic picture; the results of this investigation and hematologic data are shown in Tables 1 and 2.

The family tree shows that the patient's parents and children all carry the β thalassemia trait whereas his wife is normal. This confirmed the suspected diagnosis of homozygous β thalassemia intermedia.

Discussion

The present case is similar to descriptions in the literature of hemolytic anemias and homozygous or intermediate thalassemia which present intense erythropoiesis outside normal bone limits [11, 12]. Interesting aspects of this case were the size and localization of the erythropoietic foci. In fact, unlike the

Table 2

	Father	Mother	Patient	Wife	Son	Daughter
Age	68	65	37	34	8	4
Serum iron μg/dl						
N. V. ♀ 50–150						
♂ 60–160	100	105	197	68	92	81
TIBC μg/dl	—	—	224	—	—	—
UIBC μg/dl	—	—	27	—	—	—
Serum ferritin ng/ml						
N. V. ♀ 30–400						
♂ 30–500	—	—	20 000	—	—	—
Osmotic fragility	↓	↓	↓↓	normal	↓	↓
Hb electrophoresis*						
Hb A %	92	90	31	97	91	85
Hb A_2 %	7.2	8	2	2	6	9.6
N. V. 3 ± 0.9						
Hb F%	1	2	67	1	3	6.4
N. V. < 1						
Globin synthesis**						
α/β ratio	1.22	1.31	5.7	0.91	1.4	1.24
$\alpha/\beta + \gamma$ ratio	—	—	2.13	—	—	—
Genotype	β thal. trait	β thal. trait	β^+ thal. $\beta^\times$thal.	normal	β thal. trait	β thal. trait

* Hemoglobin electrophoresis was carried out according to Marengo-Rowe [14], and HbF by the alkaline denaturation procedure according to Singer et al. [15]

** Globin chain synthesis was determined by the method of Kan et al. after incubating peripheral blood for 2 h with ^{14}C-leucine [16]. Globin chain separation was performed according to Clegg et al. [17]

other cases reported in which thoracic extramedullary erythropoiesis was revealed by CAT next to the posterior part of the ribs [12, 18], in our patient it was also found at the front part of the ribs and behind the sternum.

The persistence of anemia due to the highly ineffective erythropoiesis and early lysis of erythrocyte production favors a gradual expansion of the erythropoietic tissue itself, and masses are formed with polycyclic outlines, simulating neoplasms, next to the thoracic wall and in anatomic continuation with the bone marrow [12, 19, 20]. These formations occur mostly in young adults, as in our case and in those reported in the literature [11].

The pathogenesis of such thoracic masses could be attributed to the combination of the pressure of the erythroblastic tissue itself which would lead to a herniation of the bone cortex, and the effect of aspiration determined by the physiologically negative intrathoracic pressure [11]. Another unusual feature of

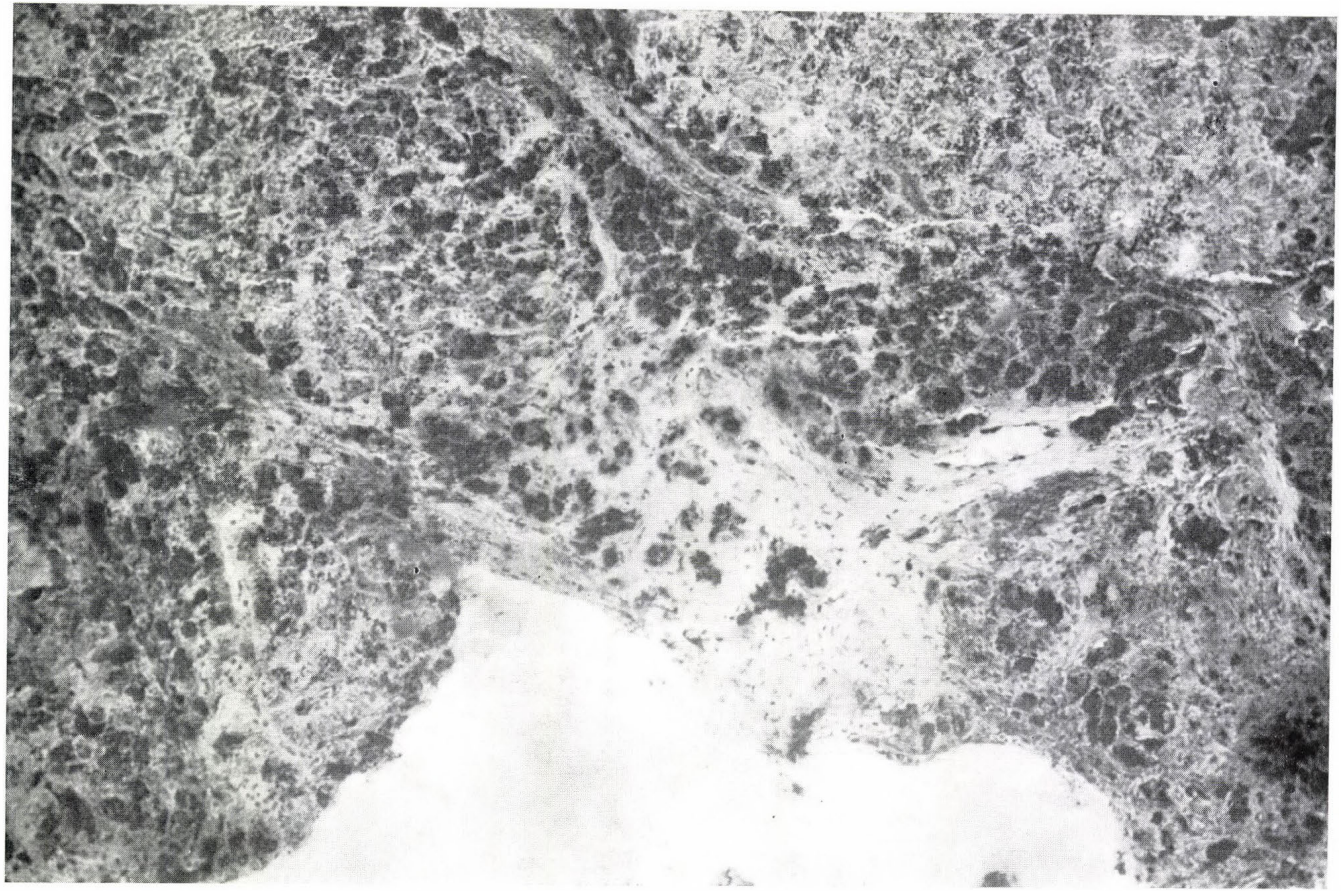


Fig. 5. Liver biopsy (Perls stain): grade IV siderosis, with diffuse accumulation especially inside the hepatocytes

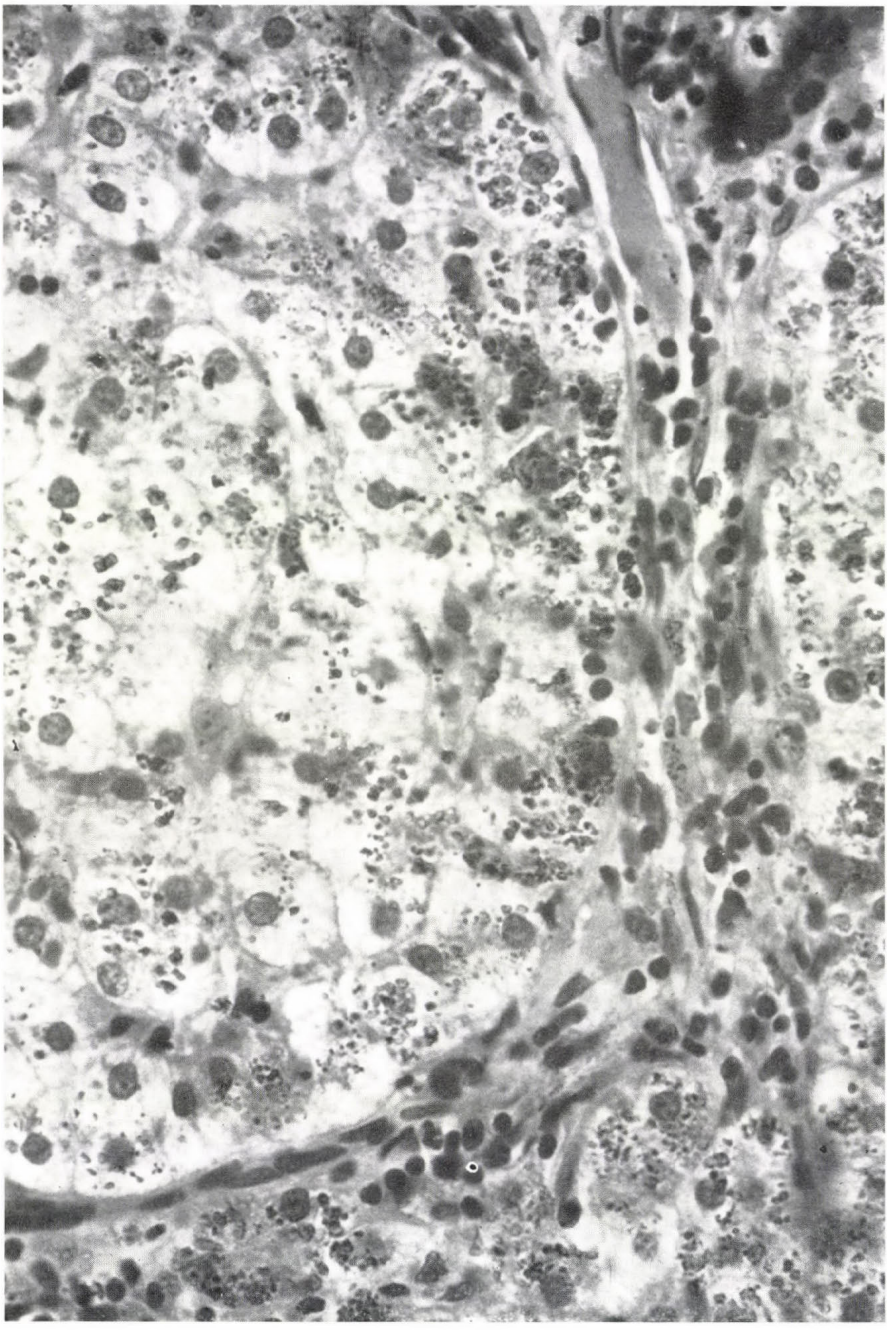


Fig. 6. Liver biopsy (hematoxylin-eosin stain): foci of diffuse erythropoiesis

this case was the degree of exclusively hepatic siderosis evidenced by CAT and confirmed histologically by needle biopsy. While this is in agreement with findings in splenectomized patients, in whom increased transferrin saturation and marked hepatic siderosis have been observed, it differs from published reports of patients with thalassemia major, in whom iron overload occurs earlier and is more generalized [21–25].

In conclusion, this case again raises the question of whether or not to treat thalassemia intermedia patients with transfusions; furthermore, the use of CAT may be considered necessary in these patients so that any foci of erythropoiesis outside normal bone limits may be better identified and iron accumulation in the various organs followed.

References

1. Weatherall, D. J., Clegg, J. B., MacCanlay, H.: The clinical and chemical heterogeneity of the β thalassemias. *Ann. N.Y. Acad. Sci. 232*, 88 (1974).
2. Bargellesi, A., Pontremoli, S., Menini, G., Conconi, F.: Excess of alpha globin synthesis in homozygous beta thalassemia and its removal from the red blood cell cytoplasm. *Eur. J. Biochem. 3*, 364 (1968).
3. Weatherall, D. J., Clegg, J. B., Na-Nakoku, S., Wasi, P.: The pattern of disordered haemoglobin synthesis in homozygous and heterozygous beta thalassemia. *Brit. J. Haemat. 16*, 251 (1969).
4. Chalevelakis, G., Clegg, G. B., Weatherall, D. J.: Imbalanced globin chain synthesis in heterozygous β thalassemic bone marrow. *Proc. Natl. Acad. Sci. USA 72*, 3853 (1975).
5. Nathan, D. G., Gunn, R. B.: Thalassemia: the consequences of unbalanced hemoglobin synthesis. *Amer. J. Med. 41*, 815 (1966).
6. Nathan, D. G., Stassel, T. B., Gunn, R. B., Zarkowski, H. S., Laforet, M. T.: Influence of hemoglobin precipitation on erythrocyte metabolism in alpha and beta thalassemia. *J. Clin. Invest. 48*, 33 (1969).
7. Kattamis, C., Ladis, V., Metaxatou Mavranati, A.: Hemoglobin F and A_2 in Greek patients with homozygous β and $\beta/\delta\beta$ thalassemia. In: *Abnormal Haemoglobin in Thalassemia Intermedia—Diagnostic Aspects*. R. M. Schmidt (ed.). Academic Press, New York 1975, p. 212.
8. Brancati, C., Baglioni, C.: Homozygous $\delta\beta$-thalassemia ($\delta\beta$-microcythaemia). *Nature 212*, 262 (1966).
9. Gallo, E., Massaro, P., Miniero, R., David, O., Tarella, C.: The importance of the genetic picture and globin synthesis in determining the clinical and haematological features of thalassemia intermedia. *Brit. J. Haemat. 41*, 211 (1979).
10. Ross, P., Cogan, W.: Roentgen findings in extramedullary haematopoiesis. *Amer. J. Roentgenol. Rad. Nucl. Med. 106*, 604 (1969).
11. Long, J. A. Jr., Doppman, J. L., Nienhus, A.: Computed tomographic studies of thoracic extramedullary hemopoiesis. *J. Comput. Assist. Tomogr. 4*, 67 (1980).
12. Ben-Bassat, I., Hertz, M., Selzer, G., Ramot, B.: Extramedullary hematopoiesis with multiple tumor-simulating mediastinal masses in a patient with β thalassemia intermedia. *Israel J. Med. Sci. 13*, 1206 (1977).
13. Dacie, J. V., Lewis, S. M.: *Practical Haematology*. 5th ed. Churchill Livingstone, Edinburgh 1975.
14. Marengo-Rowe, A. J.: Rapid electrophoresis and quantitation of hemoglobins on cellulose acetate. *J. Clin. Path. 18*, 790 (1965).

15. Singer, K., Chernoff, A. I., Singer, L.: Studies on abnormal hemoglobins. Their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation. *Blood 6*, 413 (1951).
16. Kan, Y. W., Schwartz, E., Nathan, D. G.: Globin synthesis in the alpha thalassemia syndromes. *J. Clin. Invest. 47*, 2515 (1968).
17. Clegg, J. B., Naughton, M. A., Weatherall, D. J.: Abnormal human haemoglobins. Separation and characterization of the α and β chains by chromatography and the determination of two new variants, Hb Chesapeake and Hb J (Bangkok). *J. Molec. Biol. 19*, 91 (1966).
18. Fuochi, C., Taddei, L.: Eritropoiesi extramidollare nel mediastino posteriore: considerazioni clinico-radiografiche su una osservazione personale. *Radiol. Med. 66*, 25 (1980).
19. Long, J. A. Jr., Doppman, J. L., Nienhus, A. V., Mills, S. R.: Computer tomographic analysis of β thalassemia syndromes with hemochromatosis: pathologic findings with clinical and laboratory correlations. *J. Comput. Assist. Tomogr. 4*, 158 (1980).
20. Malamos, B., Papavisiliou, C., Avramidis, A.: Tumor simulating intrathoracic extramedullary hematopoesis. *Acta Radiol. 57*, 227 (1962).
21. Ellis, J. T., Schulman, I., Smith, C. H.: Generalized siderosis with fibrosis of liver and pancreas in Cooley's anemia. *Amer. J. Path. 30*, 287 (1953).
22. Okon, E., Levis, I., Rachmilewitz, F. A.: Splenectomy, iron overload and liver cirrhosis in β thalassemia major. *Acta Haemat. 56*, 142 (1976).
23. Pippard, M. J., Callender, S. T., Warner, G. T., Weatherall, D. G.: Iron absorption and loading in β thalassemia intermedia. *Lancet 2*, 821 (1979).
24. Wasi, P.: Adverse effects of splenectomy. *J. Med. Ass. Thailand 55*, 1 (1972).
25. Witzleben, C. L., Wisatt, J. P.: The effect of long survival on the pathology of thalassemia major. *J. Path. Bact. 82*, 1 (1961).

Haematologia 17 (1), pp. 125–130 (1984)

A Case of 'Acute' Waldenström Macroglobulinaemia

J. Malý, M. Tichý, M. Bláha, O. Široký, J. Vaňásek, L. Jebavý, Z. Hrnčíř

Second Department of Internal Medicine, Faculty of Medicine,
Charles University, 500 36 Hradec Králové, Czechoslovakia

(Received 28 April 1982; revised 19 July 1982; accepted 10 January 1983)

A patient with histologically confirmed malignant lymphoma of the non-Hodgkin type with lymphoplasmocellular hyperplasia showed clinical symptoms of Waldenström macroglobulinaemia. A rapid and important increase was observed in the level of IgM lambda paraprotein possessing both cryo- and pyroprecipitative properties. This rise in paraprotein concentration was accompanied by a severe haemorrhagic syndrome. The course of disease was rapid; only six weeks passed from observing the first clinical symptoms to the death of the patient. The failure of combined cytostatic treatment allowed to conclude that the case was one of 'acute' Waldenström macroglobulinaemia.

Keywords: cryoglobulin, haemorrhagic syndrome, pyroglobulin, Waldenström macroglobulinaemia.

Introduction

In 1944 Waldenström described macroglobulinaemia as a condition different from multiple myeloma [9]. According to contemporary opinions, Waldenström macroglobulinaemia is classified into the group of malignant lymphoma from B lymphocytes [5]. According to the so-called immunological classification of Lukes and Collins [4], it is a lymphoplasmacytoid lymphoma. The disease is characterized by: (i) infiltration of the bone marrow with plasmacytoid lymphocytes; (ii) circulating IgM paraprotein of high concentration; (iii) hyperviscosity of the serum; (iv) disorder of haemostasis of the dysproteinaemic type. Monoclonal immunoglobulin of class M usually occurs in an amount larger than 15 per cent of total protein and its concentration in the plasma often exceeds 1000 mg/dl. It is considered to be a disease of advanced age. The first symptoms appear at about 64 years of age and the mean survival time is about 4 years. Krajny and Pruzanski [3] found in their patients a mean survival time of 49.5 months from the diagnosis, the life span being 5–132 months.

The case reported here had an unusually rapid course with numerous complications.

Report of a case

The patient Z. J., a 43 years old male, was admitted to our department for subfebrile temperatures, a marked fatigue syndrome and high ESR. The symptoms had appeared 10 days before. On admission on 8 May, 1980, his anaemic appearance was obvious, the spleen reached 4 cm below the costal margin. The other somatic findings were adequate. The laboratory findings on admission showed haemoglobin, 83.2 g/l; haematocrit, 0.28 u; leukocytes, 5.4×10^9/l; ESR, 32/46; thrombocytes, 152×10^9/l; X-ray examination of the lungs revealed a hilar nodular syndrome, scintigraphy of the liver and spleen showed splenomegaly. A test of blastic transformation of lymphocytes with phytohaemagglutinin showed a spontaneous higher activity of lymphocytes. Histological bone marrow examination revealed a focal multiplication of the cells of the lymphoreticular series and marked eosinophilia. Other laboratory

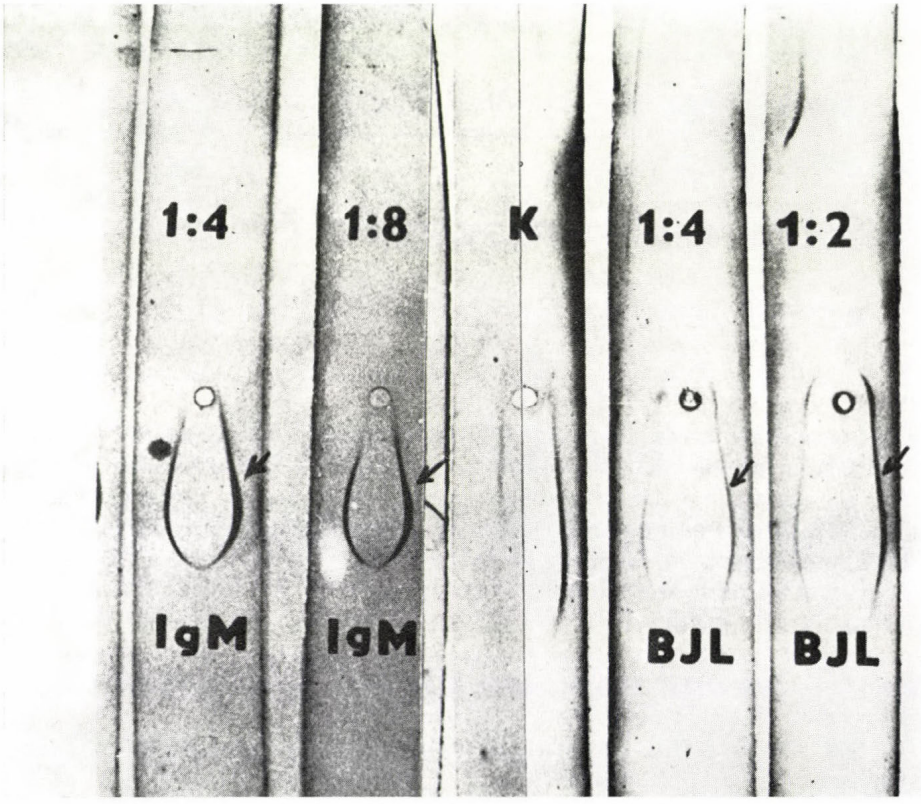


Fig. 1. Immunoelectrophoresis of serum with monospecific rabbit antisera (Sevac, Prague, Czechoslovakia) against IgM and light chains of the lambda type. The arrow indicates deformation of the precipitation line

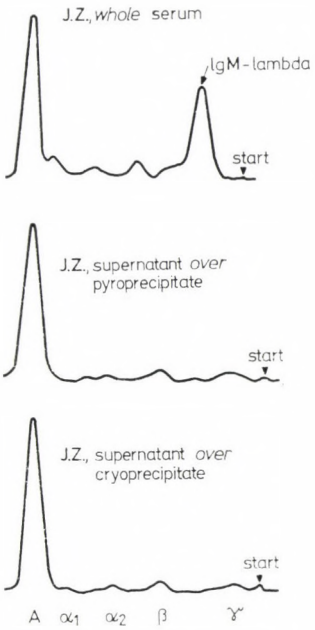


Fig. 2. Densitometric electrophoresis of *(a)* whole serum (the arrow indicates M-gradient of IgM lambda paraprotein), supernatant over *(b)* pyro- and *(c)* cryoprecipitate

findings including immunoelectrophoresis, coagulation examinations and isotopic pelvic lymphography yielded normal findings. With regard to the nodular syndrome, splenomegaly, elevated temperatures and the pathological bone marrow histology, the condition was diagnosed as suspect of malignant lymphoma and cytostatic treatment (cyclophosphamide, vincristine, prednisone) was started. The ESR gradually increased to beyond 100/h. Immunocomplexes as determined by the polyethyleneglycol 6000 method increased from the original 55 $_{\mu}$ to 550 μ, and simultaneously thrombocytopenia occurred (Fig. 4).

On 31 May, 1980, immunoelectrophoresis was repeated; it demonstrated IgM lambda paraprotein (Fig. 1). Sedimentation analysis by ultracentrifuge (MOM, Budapest) revealed pathological macroglobulin of $S_{20,w} = 18$ in an amount of 35.7 per cent of the total serum protein, corresponding to 26 g/l (Fig. 3). The results of electrophoresis on 8 May, 1980, were albumin, 0.50 $_{\mu}$; alpha-1 globulin, 0.10 $_{\mu}$; alpha-2 globulin, 0.09 $_{\mu}$; beta globulin, 0.12 $_{\mu}$; gamma globulins, 0.18 $_{\mu}$. The results on 31 May, 1980, were albumin, 0.50 $_{\mu}$; alpha-1 globulin, 0.04 $_{\mu}$; alpha-2 globulin, 0.07 $_{\mu}$; beta globulin, 0.09 μ; gamma globulins, 0.30 $_{\mu}$. Electrophoresis on 31 May yielded a marked M-gradient. In the serum of 31 May, cryoglobulin was demonstrated; the cryocrit value was 18 mm and pyroglobulin-serum was irreversibly precipitated on heating at 56 °C. Electrophoresis of super-

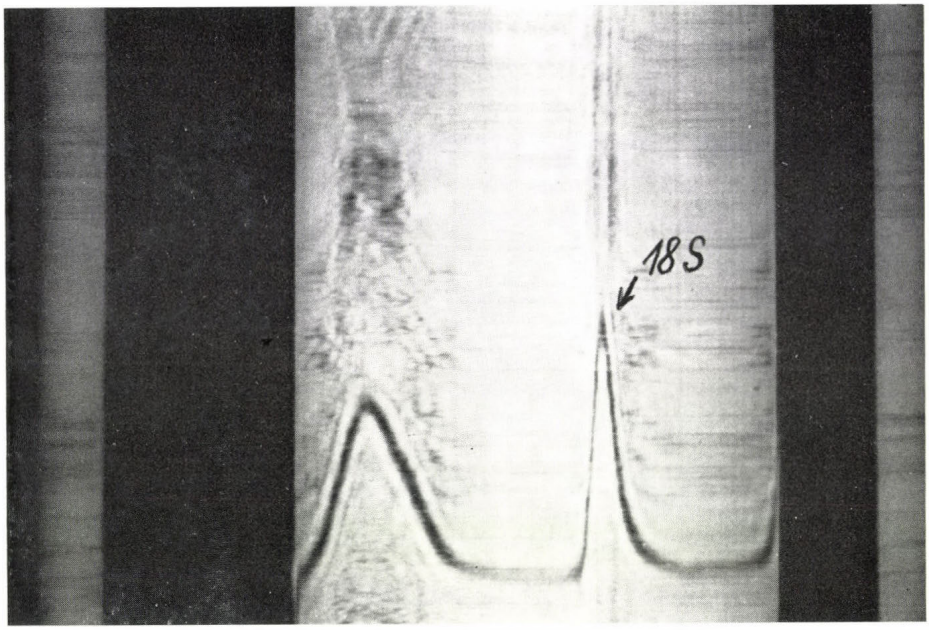


Fig. 3. Sedimentation analysis of the serum in ultracentrifuge. The arrow indicates the macroglobulin peak of IgM lambda paraprotein

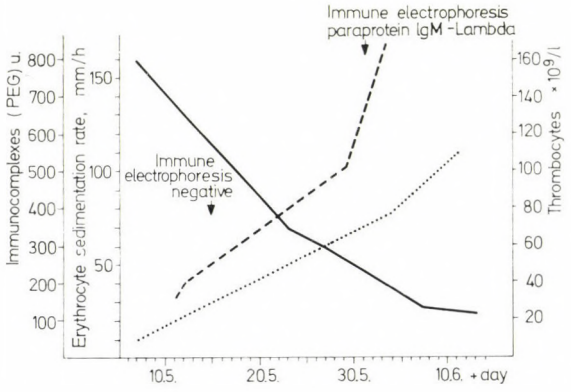


Fig. 4. ——— thrombocytes, ····· immunocomplexes, — — — erythrocyte sedimentation rate

natants over the cryo- and pyroprecipitate did not indicate the presence of M-gradient (Fig. 2). By Sephadex G-200 (Pharmacia, Sweden), cryo- and pyroglobulin were demonstrated in the first macroglobulin peak; this indicated that the cryo- and pyroprecipitative capacity of the serum was connected to IgM

lambda paraprotein. The relative viscosity of serum was 2.6 μ (the normal one being 1.4 μ). In the urine, Bence–Jones protein of the lambda type was demonstrated electrophoretically. Together with an increase in pathological proteins, relevant bleeding from gums, epistaxis and subcutaneous suffusions occurred. Haemocoagulation tests revealed thrombocytopenia, prolonged prothrombin and reptilase times, hypocoagulative thromboelastogram, and a decreased serum fibrinogen concentration. The patient had a significant leukopenia of 1.2×10^9/l with a thrombocytopenia of 15×10^9/l. The patient died on 12 June, 1980. Post-mortem examination revealed a malignant lymphoma of the non-Hodgkin type with blastic infiltration of the liver (4100 g), the spleen (940 g), of the lymph nodes of the mediastinum and porta hepatis, and of the para-aortic lymph nodes.

Discussion

The disease had a very rapid, acute course. Not more than six weeks passed from the appearance of the symptoms to the patient's death. According to the survival time of patients suffering from Waldenström macroglobulinaemia, Braun et al. [1] classified the disease into a form with slow course, with 32.2 months mean survival and a rapid form, where the mean survival is not more than 8.6 months. In the literature available no such acute course has been found.

In the present case a rapid increase occurred in the amount of IgM paraprotein in the serum and Bence–Jones protein in the urine, though they were negative at the beginning of the disease. The laboratory tests demonstrated a rapid increase in ESR, a rapid multiplication of circulating immunocomplexes and gamma globulins. IgM lambda paraprotein possessed simultaneously cryo- and pyroprecipitative properties and caused a typical paraproteinaemic purpura. Cryoglobulinaemia is usually observed in about 15 per cent of paraproteinaemic sera [5, 7, 10] and pyroglobulins occur in 2–3 per cent in larger sets of paraproteins [2, 8]. In contrast to cryoprecipitation which occurs also in polyclonal gammopathies, pyroprecipitation is found solely in monoclonal immunoglobulins [8]. The finding of simultaneous cryo- and pyroprecipitation as the property of the same paraprotein is very rare. Such a combination was reported by Invernizzi et al. [2] and Watanabe et al. [10]. The haemorrhagic syndrome belongs to the clinical picture of Waldenström macroglobulinaemia and thrombocytopenia accompanies this disease in 30 per cent of the cases [6]. The cause of the pathological haemorrhagic condition in Waldenström macroglobulinaemia is most probably an interaction of pathological protein with the components of the haemocoagulation system. The patient had severe haemorrhages the origin of which correlated chronologically with the increase in paraprotein concentration.

The hyperacute course of the disease, the rapid increase in the pathological proteins and the failure of cytostatic treatment, considered together with other haematological malignancies, led to the conclusion that the case was one of acute Waldenström macroglobulinaemia.

References

1. Braun, H. J., Bruchhaus, K. F., Aly, F. W.: Monoklonale Makroglobulinämien. *Dtsch. Med. Wschr. 98*, 1935 (1973).
2. Invernizzi, F., Cattaneo, R., Rosso, V.: Pyroglobulinemia. *Acta Haemat. 50*, 65 (1973).
3. Krajny, M., Pruzanski, W.: Waldenström macroglobulinemia: review of 45 cases. *Canad. Med. Ass. J. 116*, 899 (1976).
4. Lukes, R., Collins, R.: Immunologic characterisation of human malignant lymphoma. *Cancer 34*, 1488 (1974).
5. Messmore, H. L., Fareed, J., Silberman, S., Gawlik, G. M., Bermes, E. W.: Macroglobulinemia of Waldenström. Diagnosis and management. *Ann. Clin. Lab. Sci. 8*, 310 (1978).
6. Nilsson, I. M.: *Haemorrhagic and Thrombotic Diseases.* Wiley, London 1974.
7. Ritzmann, S. E., Daniels, J. C.: *Serum Protein Abnormalities.* Little, Brown and Co., Boston 1975.
8. Tichý, M., Hrnčíř, Z., Mraček, J., Hrnčířová, L., Matěja, F.: Immunochemical and clinical characteristics of a series of 516 paraproteinic patients. *Neoplasma 25*, 477 (1978).
9. Waldenström, J.: Incipient myelomatosis or 'essential' hyperglobulinemia with fibrinogenopenia—a new syndrome? *Acta Med. Scand. 117*, 216 (1944).
10. Watanabe, A., Kitamura, M., Shimizu, M.: Immunoglobin A (IgA) with properties of both cryoglobulin and pyroglobulin. *Clin. Chim. Acta 52*, 231 (1974).

Abstracts

An improved procedure for the preparation and measurement of ($Na^+ + K^+$)-ATPase in human erythrocytes. B. M. Charalambous, M. Afzal Mir (Department of Medicine, Metabolism and Diabetes Unit, Welsh National School of Medicine, University Hospital of Wales, Cardiff, S. Glamorgan, UK). *Biochim. Biophys. Acta 691*, 71 (1982).

Sodium and potassium-stimulated adenosine triphosphatase (($Na^+ + K^+$)-ATPase) activity and membrane permeability as measured by ^{22}Na influx were compared in erythrocyte membranes prepared by hypotonic and detergent (ionic and non-ionic) haemolysis. The detergent-treated erythrocytes showed significantly ($P < 0.001$) greater ($Na^+ + K^+$)-ATPase and permeability to ^{22}Na compared with erythrocyte membranes prepared by hypotonic haemolysis. In addition, increased enzyme activities were exhibited when membranes initially prepared by hypotonic haemolysis were subsequently exposed to 1 % (w/v) saponin in the reaction mixture. A maximum level of ($Na^+ + K^+$)-ATPase was achieved with 1.5 mg/ml sodium deoxycholate haemolysing agent. A method for erythrocyte vesicle preparation with sodium deoxycholate as haemolysing agent was developed. This method yielded high and reproducible ($Na^+ + K^+$)-ATPase activity, which could be related to a new additional index of vesicle count. Biological variations and dietary habits influenced day to day levels of ($Na^+ + K^+$)-ATPase activity but these fluctuations were kept to a minimum when blood samples were collected under identical conditions after an overnight fast, and a strictly standardized protocol of erythrocyte vesicle preparation was followed. Reproducibility of ($Na^+ + K^+$)-ATPase activity from assay to assay could be predicted by monitoring the completeness of haemolysis, vesicle size-distribution graphs, vesicle count and vesicular protein concentrations. The initial packed erythrocyte volume and the enzyme assay volume yielded the most reliable indices of ($Na^+ + K^+$)-ATPase activity, though vesicle count could also be used as an index.

Ágnes Enyedi

Rate constants for calmodulin binding to Ca^{2+}-ATPase in erythrocyte membranes. O. Scharff, B. Foder (Department of Clinical Physiology, Finsen Institute, Copenhagen, Denmark). *Biochim. Biophys. Acta 691*, 133 (1982).

The Ca^{2+}-ATPase (ATP phosphohydrolase, EC 3.6.1.3) in human erythrocyte membranes, which is part of the Ca^{2+} pump, can be activated by binding of calmodulin. Rate constants (k_1) for association of calmodulin and enzyme, which depends on the Ca^{2+} concentration, have been determined by the aid of an enzyme model. K_1 increased from $0.25 \cdot 10^6$ to $17.3 \cdot 10^6$ M^{-1} min^{-1} (70 times) when the free Ca^{2+} concentration was raised from 0.7 to 20 μM. The binding of calmodulin to the Ca^{2+}-ATPase is reversible. The rate constants (k_{-1}) for dissociation of enzyme-calmodulin complex decreased from 6.0 to 0.044 min^{-1} (135 times) when the free Ca^{2+} concentration was increased from 0.1 to 2–20 μM. The apparent dissociation constant

$K_d = k_{-1}/k_1$ accordingly increased from 2.5 nM to 25 μM (or higher) when the Ca^{2+} concentration was reduced from 20 to 0.1 μM. Therefore, at 10^{-7} M free Ca^{2+} most of the Ca^{2+}-pump enzyme will not bind calmodulin. For the intact cell the time dependences of activation and deactivation of the Ca^{2+}-pump enzyme have been estimated from the rate constants above. The results suggest that the Ca^{2+}-pump is well suited to maintain a cytosolic concentration of 10^{-7} M free Ca^{2+} (or lower) in the unstimulated cell and, when the cell is stimulated, to allow transient Ca^{2+} signals up to approx. 10^{-5} M in the cytosol.

Ágnes Enyedi

Influence of cholesterol on the rotation and self-association of Band 3 in the human erythrocyte membrane. T. Mühlebach, R. J. Cherry (Eidgenössische Technische Hochschule, Laboratorium für Biochemie, ETH-Zentrum, Zürich, Switzerland). *Biochemistry 21*, 4225 (1982).

The cholesterol/phospholipid mole ratio (C/P) in the human erythrocyte membrane was varied by incubating cells with liposomes. The rotational mobility of band 3 proteins was measured in these membranes by observing flash-induced transient dichroism of the triplet probe eosin maleimide. Measurements were performed with membranes in which associations of band 3 with cytoskeletal proteins were removed by mild proteolysis with trypsin. It was found that decreasing C/P resulted in a more rapid decay of the flash-induced anisotropy. The anisotropy decay curves were analyzed by curve-fitting procedures, which indicated the existence of different sized small aggregates of band 3. The changes in the decay curves with varying C/P can be explained by an effect of cholesterol on the size distribution of these aggregates. The experiments suggest a possible role of cholesterol in regulating associations between integral membrane proteins.

G. Gárdos

The interaction of hemoglobin with isolated band 3 cytoplasmic fragments. I. Zilber, N. Shaklai (Department of Chemical Pathology, Sackler School of Medicine, Tel Aviv University, Israel). *Biochemistry Internat. 4*, 297 (1982).

The ability of band 3 cytoplasmic fragments to interact with hemoglobin was studied using the capacity of its heme group to quench the fluorescence intensity of the band 3 dissected peptides. At pH 6 and 5 mM phosphate buffer, a binding constant of $K_d = 2.8 \times 10^7\ M^{-1}$ was calculated. This binding constant is in the same order of magnitude as the one reported previously for the high affinity sites of the red blood cell membrane. The interaction of hemoglobin and band 3 cytoplasmic fragments was also characterized by its pH and ionic strength dependencies and was found to behave similarly to that of the RBC-Hb system.

G. Gárdos

The phosphorylation of intact erythrocytes by exogenously added cAMP-dependent protein kinase. T. M. Chiang, E. S. Kang, A. H. Kang (The Veterans Administration Medical Center, Departments of Medicine, Biochemistry, and Pediatrics at the University of Tennessee Center for the Health Sciences, Memphis, TN, USA). *Biochem. Biophys. Res. Comm. 107*, 1446 (1982).

Intact human erythrocytes incubated with exogenously added cAMP-dependent protein kinase and [γ-^{32}P]ATP resulted in the phosphorylation of several proteins as analyzed by sodium dodecylsulfate-slab gel electrophoresis and radioautography. Bands with apparent molecular weights of 220K, 200K, 180K, 67K, 58K and 29K were phosphorylated by this process. The 220 and 200K components appear to be glycoproteins by their staining characteristics using stain-all. They also precipitate with fibronectin monoclonal antibodies. The identity of the other phosphoproteins is not known. The results indicate that fibronectin and other proteins are substrates for exogenously added

cAMP-dependent protein kinase located on the external surfaces of intact human erythrocytes.

G. Gárdos

Electrokinetic behavior of inside-out vesicles from human red cell membranes. W. S. Yen, R. W. Mercer, B. R. Ware, Ph. B. Dunham (Department of Chemistry and Biology, Syracuse University, Syracuse, N.Y. USA). *Biochim. Biophys. Acta 689*, 290 (1982).

The electrokinetic behavior of red cell membrane vesicles of normal (ROV) and inverted (IOV) sidedness has been characterized using the laser Doppler technique of electrophoretic light scattering (ELS). At neutral pH ROV have an (approx. 25%) higher electrophoretic mobility than IOV and the two peaks can be resolved in the ELS spectrum to provide a quantitative estimate of the IOV/ROV ratio which is consistent with the ratio determined by assay of the activity of acetylcholinesterase. The ROV peak coincides with the mobility of fresh red blood cells and of resealed ghosts. Neuraminidase treatment reduces the ROV mobility by a factor of 2.6, while the IOV peak is reduced only slightly ($< 5\%$). Treatment with trypsin results in a single narrow ELS peak at about 60% of the mobility of ROV. Treatment of IOV with phospholipase C leaves the electrophoretic mobility unaltered, whereas treatment with phospholipase D increases their mode mobility by 22%. The mobility titration curve of IOV from pH 2 to pH 10 reveals three distinct inflection points which may be assigned to chemical groups on the cytoplasmic surface of the red cell membrane.

G. Gárdos

Glycophorin incorporation increases the bilayer permeability of large unilamellar vesicles in a lipid-dependent manner. A. T. M. Van der Steen, B. De Kruijff, J. De Gier (Laboratory of Biochemistry and Institute of Molecular Biology, State University of Utrecht, Utrecht, The Netherlands). *Biochim. Biophys. Acta 681*, 13 (1982).

The effect of glycophorin incorporation on the permeability properties of lipid bilayers is investigated by employing a new method, in which the trap of a small permeant and a high molecular weight, nonpermeable molecule are simultaneously monitored. Glycophorin induces a high permeability to potassium and glucose in large dioleoylphosphatidylcholine vesicles. The glycophorin-induced enhanced permeability is found to be lipid dependent. By monitoring the % ratio of the glucose (permeant)/dextran (impermeant) trap in large unilamellar glycophorin-containing vesicles, it was determined immediately after removal of non-enclosed marker, that glycophorin-containing vesicles composed of dioleoylphosphatidylcholine were highly permeable to glucose (% glucose/dextran trap = 1.4), whereas glycophorin-containing vesicles prepared from total human erythrocyte lipids demonstrated a greatly reduced permeability in glucose (% glucose/dextran trap = 50). The glycophorin-induced increased permeability appears to be related to transbilayer movement of lipid molecules. Glycophorin induces a fast lipid-transbilayer movement of lysophosphatidylcholine and a high permeability to potassium and glucose in dioleoylphosphatidylcholine vesicles, whereas glycophorin-containing vesicles comprised of total human erythrocyte lipids show no lipid-transbilayer movement and only a slight permeability enhancement to glucose, as compared to the protein-free vesicles.

Ilma Szász

Antibodies directed toward human erythrocyte Ca^{2+}-ATPase: Effect on enzyme function and immunoreactivity of Ca^{2+}-ATPases from other sources. A. K. Verma, J. P. Gorski, J. T. Penniston (Mayo Clinic/Foundation, Department of Cell Biology, Section of Biochemistry, Rochester, Minnesota, USA). *Arch. Biochem. Biophys. 215*, 345 (1982).

Antibodies directed against purified human erythrocyte Ca^{2+}-ATPase were raised in rabbits. In competitive radioimmunoassay tests of immunological cross-reactivity, human erythrocyte Ca^{2+}-ATPase shows a con-

sistent pattern of immunological similarity to the Ca^{2+}-ATPases derived from cell surface fractions of other species, such as rat and dog erythrocyte ghosts, rat corpus luteum plasma membranes, and rat brain synaptic plasma membranes. On the other hand, a purified Ca^{2+}-ATPase preparation from rabbit skeletal muscle sarcoplasmic reticulum failed to show any immunological similarity to the human enzyme. The amount of Ca^{2+}-ATPase protein in the erythrocyte ghosts was estimated to be about 0.6 μg/mg ghost protein, which was not too different from the calculated value of 1.2 ± 0.2 μg/mg ghost protein (mean ± SD, $n = 6$) based on the calmodulin binding studies of the erythrocyte ghosts. Anti-Ca^{2+}-ATPase immunoglobulin G inhibited enzyme activity and calcium transport, showing that at least one subpopulation of antibodies can block the active site of the enzyme. The antibodies had no effect on the binding of calmodulin to erythrocyte membranes.

Ilma Szász

Cytoskeletal influence on merocyanine 540 receptors in the plasma membrane of erythroleukemic cells. R. C. Hunt, J. A. Hood (Department of Biochemistry, The University of Mississippi Medical Center, Jackson, MS., USA). *Biochim. Biophys. Acta 720*, 106 (1982).

When human erythroleukemic cells are induced to differentiate in vitro, the lipids in the plasma membrane that bind the fluorescent dye merocyanine 540 are redistributed into a cap at one pole of the cell. This capping phenomenon can also be observed in uninduced cells that have been incubated with cytochalasin B, an agent which disrupts actin-containing microfilaments or with local anesthetics which act on both microfilaments and microtubules. Colchicine which acts on microtubules, however, has no effect. This suggests that the uniform distribution seen in uninduced cells is maintained by the cytoskeletal microfilaments and that loss of these structures leads to spontaneous redistribution of merocyanine 540-binding sites.

Ilma Szász

Evidence for coupling of phosphatidic acid formation and calcium influx in thrombin-activated human platelets. A. Imai, Y. Ishizuka, K. Kawai, Y. Nozawa (Department of Biochemistry, Gifu University School of Medicine, Gifu 500, Japan). *Biochem. Biophys. Res. Comm. 108*, 752 (1982).

The time-sequential relationship between Ca^{2+} flux, phospholipid metabolism and platelet activation have been examined. Thrombin-activation caused a marked enhancement in $^{45}Ca^{2+}$ influx and a decrease in extracellular Ca^{2+} concentration measured by murexide dye, which occurred in parallel with the conversion of 1,2-diacylglycerol (DC) to phosphatidic acid (PA). The incorporated $^{45}Ca^{2+}$ was located mainly in cytosolic fraction. The influx of Ca^{2+} was observed to commence prior to the onset of lysophospholipids formation and subsequent liberation of arachidonic acid. These data provide evidence which indicates a coupling between the rapid PI-turnover and the active Ca^{2+} influx, in which phosphatidic acid (PA) may serve as a Ca^{2+} ionophore.

Ilma Szász

Book Review

Bone Marrow Biopsy Revisited. A New Dimension for Haematologic Malignancies. By R. Bartl, B. Frisch and R. Burkhardt. S. Karger, Basel 1982, 94 p., 52 figs, 8 cpl., 13 tab., hard cover, SFr. 58.— (DM 69.—)

Based on the investigation of over 2000 patients with haematologic malignancies the book presents information certain to broaden the range of clinical applications for bone marrow histology. Data on more than 3000 bone marrow biopsies as well as on undercalcified plastic-embedded semithin sections are analyzed. Each of the nine chapters are followed by detailed literary references.

The first chapter is an introduction which discusses the importance of bone marrow biopsy in general. The second chapter focuses on patients and methods, on the techniques of bone marrow aspiration, and preparation of histological sections. Chapter three considers structure and function of the normal bone marrow.

Subsequent chapters decribe bone marrow findings in specific haematological diseases. These include myeloproliferative disorders, adult acute leukaemias, multiple myeloma, non-Hodgkin's lymphomas and Hodgkin's disease. Current knowledge, the authors' own experiences and practical points are successfully brought together. The authors also discuss the usefulness of bone marrow biopsy in the early stages of haematological diseases, in histological classification and staging of bone marrow involvement, prognostic value and in evaluation of therapeutical consequences. The colour and black and white picture and the outstanding schematic illustrations greatly help comprehension.

It is hoped that the book will not only facilitate recognition of the vast clinical utility of bone marrow biopsy but also become part of the mandatory reading for haematologists, oncologists and pathologists everywhere.

A warm foreword is written by Professor D. A. G. Galton from London's Hammersmith Hospital.

Júlia Tamáska

An announcement by the International Committee for Standardization in Haematology*

Expression of results of haemoglobin determination

On the occasion of the Congresses of the International Society of Haematology and the International Society of Blood Transfusion in Budapest (August 1982), the Board and the General Assembly of the International Committee for Standardization in Haematology approved the following resolution:

"In physiological, biochemical or metabolic studies expressing results of haemoglobin determination as amount of substance is acceptable, but for classification, diagnostic and/or therapeutic purposes the continued use of mass concentration is recommended. However, for the sake the uniformity in reporting it may be useful to include also the result expressed as amount of substance.

When Hb is reported as mass concentration it should be expressed in g/l. When reported as substance concentration the elementary entity should be defined as the haemoglobin monomer, and clearly specified in the component name, e.g. Hb(Fe), HbCO(Fe), MCH(Fe)."

---

* President: S. R. Hollán, MD
NIHBT Budapest

Executive Secretary: Dr. R. L. Verwilghen
University Hospital
Capucijnenvoer 33
B-3000 Leuven, Belgium y
Telephone: 32 16 20 2015
Ext 2018

Chairman of Secretariat: Dr. S. M. Lewis
Royal Postgraduate Medical School
Ducane Road
London W1 2 OHS, UK
Telephone: 44 1 743 2030
Ext 511
Telex: 935589 MRC CYL G

INSTRUCTIONS TO CONTRIBUTORS

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

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24
H-1113 Budapest, Hungary

Three copies of the manuscript should be submitted. They should be typed double-spaced on one side of a good quality paper with proper margins. The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which the work has been carried out; (4) name and full postal address of the author to whom communications regarding the manuscript should be directed; (5) a short title not to exceed 40 characters (including space) to be used as a running head. The second page should contain an **abstract** of 50—100 words, summarizing the reasons for the study, the methods used, the results, and the major conclusions. This page should also contain 4—8 **keywords** placed in alphabetical order. Original papers should not exceed 15 printed pages including tables, figures, and references. Case reports should not be more than four, technical notes and Letters to the Editor not more than two printed pages in length. In the manuscripts the approximate location of tables and figures should be indicated in the margin. The manuscript of original papers should be divided into summary, introduction, materials and methods, results, discussion, acknowledgements and references. Review articles should also be appropriately divided. SI units should be used in the manuscript, except that, for the time being, litre (l) may be used as a unit of volume.

References: Only papers closely related to the author's work should be referred to. In the text citations should include the name of the authors and/or the reference number in parentheses. A list of references, numbered according to the order of their occurrence in the text, should be compiled at the end of the paper. It should be typewritten, double-spaced, on a separate sheet.

References to periodicals should be typed double-spaced and mention: (1) name(s) and initials of all the authors; (2) title of the paper; (3) international abbreviation of the periodical; (4) volume; (5) first page number; (6) year of publication in parentheses. Thus: 4. Liebermann, J. E., Gordon-Smith, E. C.: Red cell pyrimidine 5'-nucleotidase and glutathione in myeloproliferative and lymphoproliferative disorders. *Brit. J. Haemat. 44*, 425 (1980).

References to books should include: (1) author(s)' name(s); (2) title; (3) editor(s); (4) publisher; (5) place and year of publication. Thus: 5. Guyton A. C., Taylor, A. E., Granger, H. J.: *Circulatory Physiology. II. Dynamics and Control of Body Fluids.* Saunders, Philadelphia 1975. Or: 6. Rappaport, J. M.: Granulocyte transfusion. In: *Haematology of Infancy and Childhood.* D. G. Nathan, F. A. Oski (eds). Saunders, Philadelphia 1974, p. 875.

Illustrations must be cited and numbered in the order they appear in the text. All line drawings should be submitted as clear, glossy, black and white originals or good quality photographs. The author's name and the title of the paper together with the serial number of the figure should be written on the back of each print. Legends for illustrations should be typewritten, double-spaced, on a separate sheet, and included at the end of the manuscript. Tables, each bearing a title, should be self-explanatory and numbered consecutively.

Authors will receive one set of proofs which must be corrected and returned to the Editor-in-Chief *within three days of receipt*. Major alterations of the text cannot be accepted.

Authors are entitled to 50 reprints free of charge.

HAEMATOLOGIA
VOLUME 17 · NUMBER 1 **1984**

Contents

Index: 26861

VOLUME 17 • NUMBER 2 • 1984

HU ISSN 0017-6

HAEMATOLOGIA

International Quarterly of Haematology

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.

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

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24.
H-1113 Budapest
Hungary

Technical Editor:
R. J. Valenti

Distributor for

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

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

For all other countries subscriptions should be sent to

VNU SCIENCE PRESS
P.O.B. 2073
3500 GB Utrecht, The Netherlands

Subscription price for volume 17

DM 279 incl. surface mail
DM 305 incl. air mail

VOLUME 17 · NUMBER 2

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

VNU SCIENCE PRESS, UTRECHT, THE NETHERLANDS

AKADÉMIAI KIADÓ, BUDAPEST

1984

Printed in Hungary
Akadémiai Nyomda, Budapest

Haematologia 17 (2), pp. 137–149 (1984)

Prostanoid Activation of Erythropoiesis

J. W. FISHER, P. K. NELSON, M. BELEGU, M. HAGIWARA, BARBARA BECKMAN

Department of Pharmacology, Tulane School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112, USA

(Received 27 January 1983; accepted 17 March 1983)

A model is proposed for the role of the kidney in the control of erythropoietin production in which the initial trigger is an oxygen deficit created by anemia, hypobaria or ischemia. It is postulated that hypoxia creates a decrease in the oxygen level in a critical renal sensor cell, perhaps in the glomerular tuft, which eventually leads to the production of prostacyclin. It is possible that the endothelial cell in the glomerular tuft responds to this oxygen deficit to produce prostacyclin to trigger erythropoietin production. Recent studies on prostaglandin synthesis by human isolated glomeruli indicate that the most abundant prostanoid synthesized by the glomerular tuft cells was 6-keto $PGF_{1\alpha}$, a metabolite of prostacyclin (PGI_2). PGI_2 has also been reported to be produced by isolated vascular endothelial cells. The mechanism by which hypoxia may initiate the synthesis and/or release of prostaglandins and prostacyclin in the renal cell has not been elucidated. Significant to erythropoietin production is the production by hypoxia of prostacyclin which eventually leads to the production of the metabolite 6-keto PGE_1. We further propose that 6-keto PGE_1 is the prostanoid which activates a specific cell membrane adenylate cyclase, causing the conversion of ATP to cyclic AMP. This is a very critical step in that there must be a sufficient amount of ATP remaining to generate cyclic AMP in order for erythropoietin biosynthesis to occur with the reduced level of ATP which may have caused a perturbation of the cell membrane. The elevated cyclic AMP leads to the activation of protein kinases which are essential in phosphorylating the lysosomal hydrolases released by hypoxia into the cytosol of the cell and may be the precursors of erythropoietin. Neutral proteases and lysosomal hydrolases, documented triggers of erythropoietin production, have been demonstrated to be elevated in the kidney after hypoxia. The mechanism of labilization and release of these enzymes from the renal lysosomes has been postulated to be related to increases in cyclic GMP levels in a renal cell. Hypoxia causes the release of renal lysosomal hydrolases which then undergo phosphorylation through activation by protein kinases following prostanoid stimulation of renal adenylate cyclase to generate cyclic AMP, resulting in increased biosynthesis of erythropoietin.

Keywords: erythropoiesis, erythropoietin, hypoxia, kidney, prostacyclin, prostaglandins, prostanoid, 6-keto prostaglandin E_1.

Introduction

We have been interested for several years in the role of an oxygen deficit as the fundamental stimulus of renal erythropoietin production. The kidney was established as the primary site of erythropoietin production with the finding by

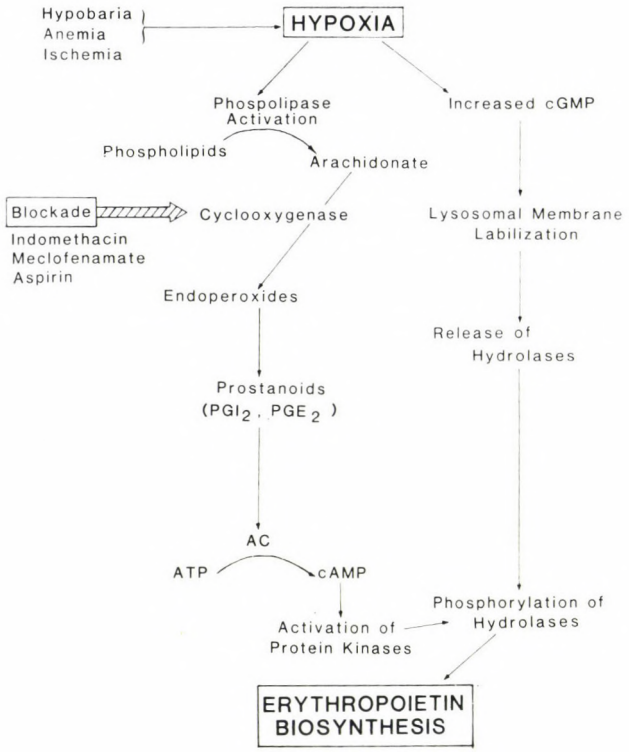


Fig. 1. A schematic model for hypoxic stimulation of renal production of erythropoietin (Fisher, J. W. Ref. 31)

Jacobson et al. [1] in 1957 that bilateral nephrectomy abolished the erythropoietic response of rats to bleeding. More direct evidence for the role of the kidney in erythropoietin production was provided in the reports by Kuratowska et al. [2] and Fisher and Birdwell [3] in 1961 when these investigators demonstrated that erythropoietin could be produced in the isolated perfused kidney following hypoxemic perfusion of the rabbit kidney [2] and the isolated dog kidney perfused with blood containing cobalt [3]. Fisher et al. [4] demonstrated later that cobalt and hypoxemic perfusion of the isolated perfused dog kidney produced an apparent synergistic effect by enhancing erythropoietin production in the kidney. The mechanism for the control of renal erythropoietin production is not well understood but is postulated to be regulated by the oxygen level in a critical renal sensor cell, and has been the subject of several investigations and reviews [5–13].

This paper will focus primarily on experiments which involve the role of prostaglandins and prostacyclin in hypoxic stimulation of renal of erythropoietin production as well as their effects directly on erythroid progenitor cells in the bone marrow compartment. The present studies center around the prostaglandins and prostacyclin, especially the prostacyclin metabolite 6-keto PGE_1, and their effects

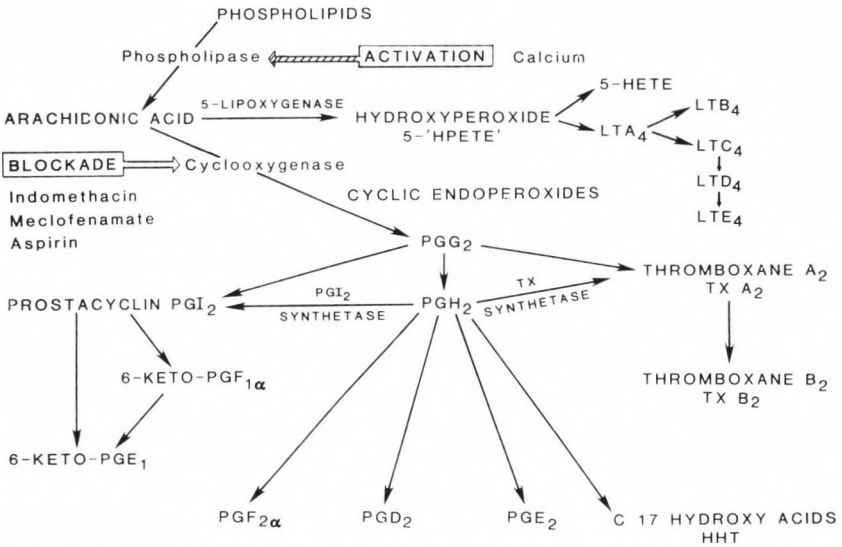


Fig. 2. Pathways of phospholipid, arachidonate, endoperoxide, prostacyclin, prostaglandins, lipoxyenases and thromboxane metabolism. The intermediate cyclic endoperoxides (PGG_2 and PGH_2), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) are unstable with half lives at pH 7.4 and 37 °C of less than 5 min. Indomethacin, meclofenamate and aspirin will block the biosynthesis of all of these intermediates and the stable prostaglandins

on the production of erythropoietin in the kidney as well as the enhancement of the erythroid progenitor cell compartment by PGE_2 and PGD_2. We have found that the effects of beta-2 adrenergic agonist drugs like albuterol [11] and renal artery constriction (ischemia) [12] on erythropoietin production can be blocked by the prostaglandin synthesis inhibitors indomethacin and meclofenamate, which suggests that their effects on erythropoietin production are through a prostaglandin mechanism. The mechanisms of erythropoietin biosynthesis in the kidney are really a "black box" and it is still not clear whether a proerythropoietin, "so-called" erythrogenin, or a lysosomal hydrolase released under the influence of hypoxia, are involved, as an early step in the biosynthesis of erythropoietin. Thus, two postulated mechanisms of prostanoid activation of erythropoiesis are: 1) activation of the erythroid progenitor cells directly in the bone marrow by prostaglandins E_2 and D_2, and 2) stimulation of renal erythropoietin production by prostacyclin especially its metabolite 6-keto PGE_1.

Model for hypoxia and the control of erythropoiesis

The finding that hypoxia of the isolated perfused kidney leads to an elevation in erythropoietin and the prostacyclin metabolite 6-keto $PGF_{1\alpha}$ [10, 14] supports our hypothesis that prostacyclin and/or its intrarenal metabolite, 6-keto PGE_1 [15,

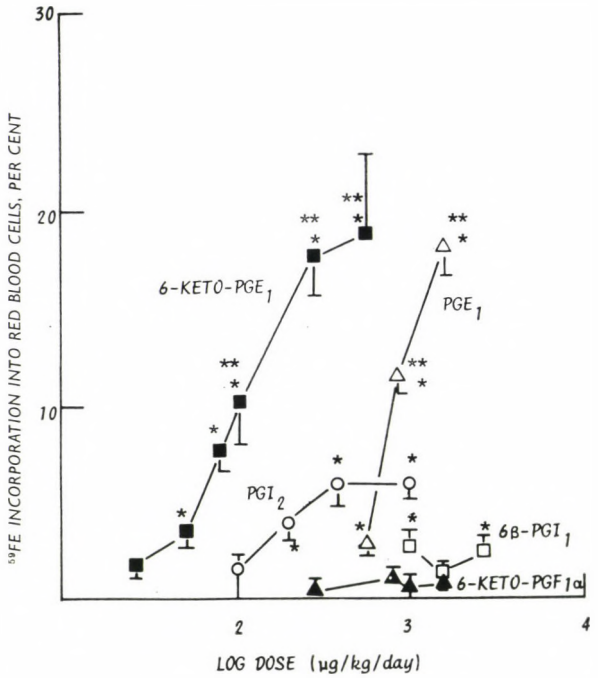


Fig. 3. Comparative effects of PGI_2 (○), 6-keto PGE_1 (■), PGE_1 (△), 6-keto $PGF_{1\alpha}$ (▲), 6β-PGI_1 (□) on ^{59}Fe incorporation into newly formed red blood cells of exhypoxic polycythemic mice (EPM). Each value expresses a mean ± S.E.M. The percentage of ^{59}Fe incorporation observed after analogous treatment with saline and Tris buffer (20 mM) were 0.67% and 0.74%, respectively. *Significantly different from control value. **Significantly different from PGI_2 (400 μg/kg/day) (Nelson, P. K. et al., *J. Pharmacol. Exp. Ther.* 226 : 493-499, 1983).

16], may actually be involved as the trigger for the adenylate cyclase system to initiate erythropoietin production. It seems clear that prostacyclin or its metabolite 6-keto PGE_1 play some physiologic and/or pathophysiologic role in renal production of erythropoietin. Prostacyclin, or its metabolite 6-keto PGE_1, may be released in response to hypoxia [17, 18] to trigger adenylate cyclase [19] leading to an initiation of the erythropoietin biosynthetic cascade according to the model shown in Figure 1. This model involves an oxygen deficit created by anemic or hypobaric hypoxia resulting in the release of prostacyclin and its metabolite 6-keto PGE_1 or the release of PGE_2 with ischemic hypoxia. Prostacyclin, 6-keto PGE_1 or PGE_2 activation of adenylate cyclase, may produce an increase in cyclic AMP which causes the activation of a protein kinase and the phosphorylation of a hydrolase leading to increased biosynthesis of erythropoietin.

Figure 2 shows a simplified metabolic pathway for arachidonate and the prostanoid cascade. Arachidonate is released by phospholipases under the influence of hypoxia and cyclooxygenase leads to the production of the cyclic endo-

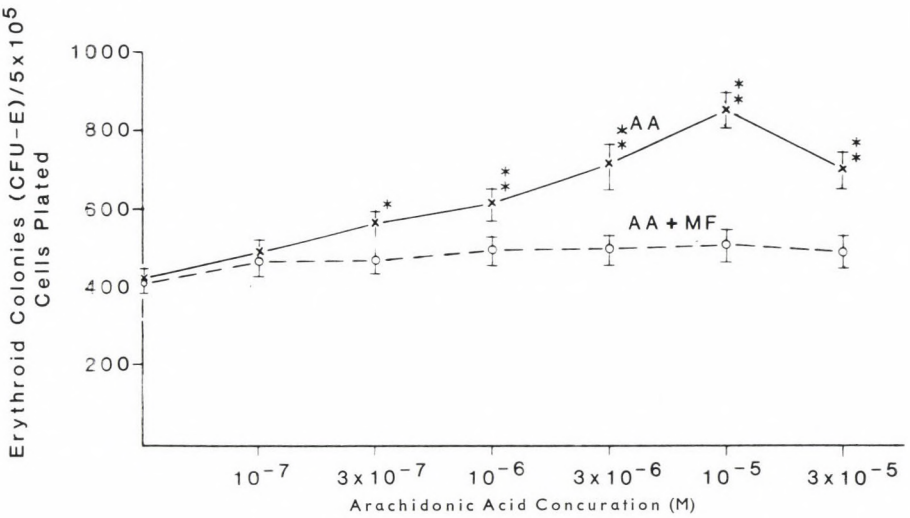


Fig. 4. Erythroid colony formation (CFU-E) in normal mouse bone marrow cultures (5×10^5 cells/plate) treated with several concentrations of arachidonic acid. Each culture plate contained 0.2 U erythropoietin. * Significantly different from erythropoietin alone ($P < 0.05$) ** Significantly different from erythropoietin alone ($P < 0.001$) (Belegu, M. et al., *Am. J. Physiol.* 245 : (Cell Physiol. 14) : c322–c327, 1983).

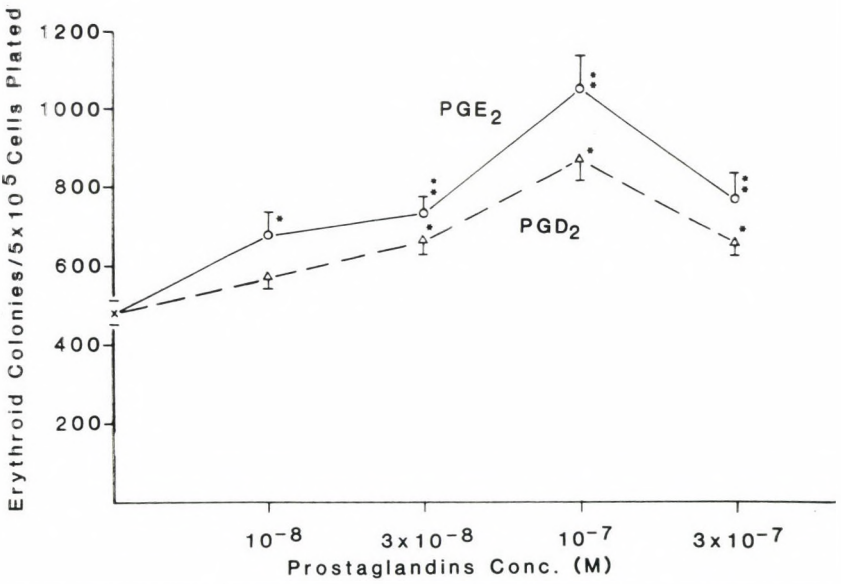


Fig. 5. Erythroid colony formation (5×10^5 cells/plate) in normal mouse bone marrow cultures treated with several concentrations of prostaglandin E_2 and prostaglandin D_2. Each culture plate contained 0.2 unit erythropoietin. * Significantly different from erythropoietin alone ($P < 0.05$) ** Significantly different from erythropoietin alone ($P < 0.001$) (Belegu. M. et al., *Am. J. Physiol.* 245 : (Cell Physiol. 14) : c322–c327, 1983).

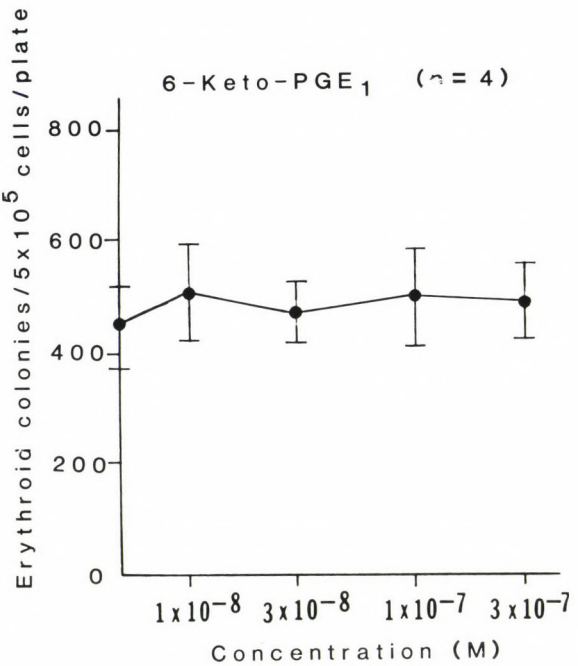


Fig. 6. Effects of 6-keto PGE_1, the stable metabolite of prostacyclin, on erythroid colony forming cells (CFU-E) in mouse bone marrow cultures using the methyl cellulose system of Iscove et al. (27). The mean $\pm$ S.E.M. is given for 4 experiments in which duplicate plates were counted in each experiment (unpublished data)

peroxides. Very important to our model is that PGE_2 and D_2 seem to be the primary prostaglandins produced by the bone marrow [25] and exert moderate effects on erythroid progenitor cells directly in the marrow [26]; whereas prostacyclin is probably produced in response to hypoxia by the kidney, perhaps a vascular endothelial cell [18], to trigger an erythropoietin producing cell in the kidney [29] either directly or through its ultimate metabolite 6-keto PGE_1 to increase erythropoietin biosynthesis.

Figure 3 shows the effects of several prostanoids on radioiron incorporation in red cells of exhypoxic polycythemic mice where we were mainly interested in the E type prostaglandins [17] and prostacyclin (PGI_2) has moderate stimulatory effects on radioiron incorporation in exhypoxic polycythemic mice. One metabolite of prostacyclin is the more stable 6-keto $PGF_{1\alpha}$ which has no significant effect on radioiron incorporation presumably because of its structural characteristics. The 6-beta form of PGI_1, a more stable form of prostacyclin, was also evaluated but was not found to have a significant effect on radioiron incorporation. On the other hand, 6-keto PGE_1, a stable metabolite of PGI_2, had potent erythropoietic

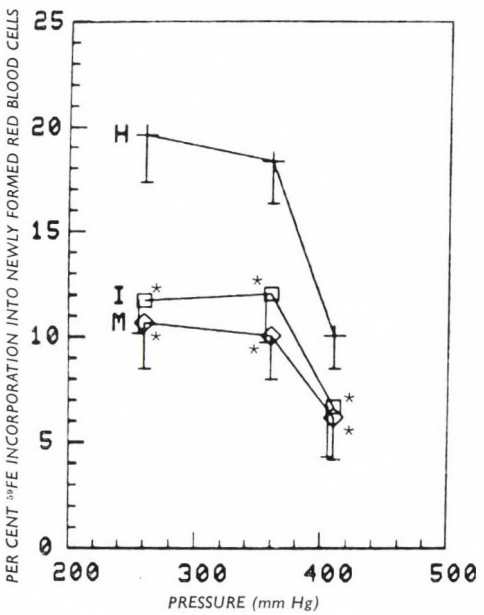


Fig. 7. ^{59}Fe incorporation in exhypoxic polycythemic mice exposed to 260, 360 and 410 mmHg (hypobaric hypoxia) following pretreatment with Na_2CO_3 vehicle (H), indomethacin (I) (5 mg/kg i.p.) or sodium meclofenamate (M) (5 mg/kg i.p.). Each value expresses mean $\pm$ S.E.M. for 8–15 mice/group. *Significantly ($p < 0.05$) different from the respective pressure for the hypoxia (H) vehicle treated controls and indomethacin (I) plus hypoxia or meclofenamate (M) plus hypoxia (Nelson, P. K. et al., *J. Pharmacol. Exp. Ther.* 226:493–499, 1983).

effects when compared with prostacyclin and was 3–4 times more active than PGE_1 [10, 17, 30]. Therefore, we have focused on 6-keto PGE_1 for further study as a possible prostanoid involved in erythropoietin production.

Direct effects of prostanoids on erythropoiesis

We have studied the direct effects of prostaglandins in enhancing the formation of erythroid colony forming cells, the so-called CFU-E compartment, in mouse bone marrow cultures. The murine bone marrow erythroid colonies (CFU-E) were cultured in methylcellulose using the technique of Iscove et al. [27]. In a recent paper by Kojima [25] the mouse bone marrow was demonstrated to produce primarily prostaglandins E_2 and D_2.

Figure 4 shows some of the work carried out by Belegu et al. [26] that arachidonic acid produced a significant increase in erythroid colony forming cells (CFU-E) in the marrow. This effect was blocked by the addition of the cyclooxygenase inhibitor meclofenamate implicating a possible prostaglandin

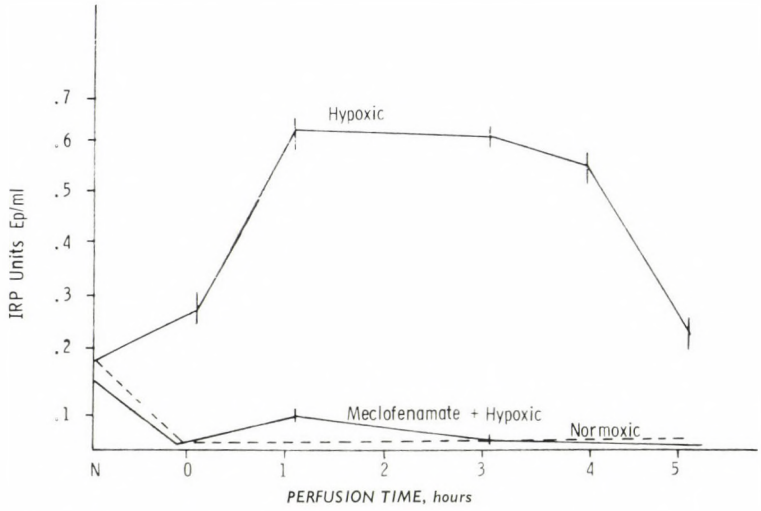


Fig. 8. Erythropoietin titers in the perfusates of the hypoxemic perfused isolated dog kidney (4 h posthypoxic) with and without meclofenamate (1 μg/ml) in comparison with a normoxic perfused kidney. Bars indicate S.E.M. of 5 experiments (Fisher, J. W., et al. (Ref. 10)).

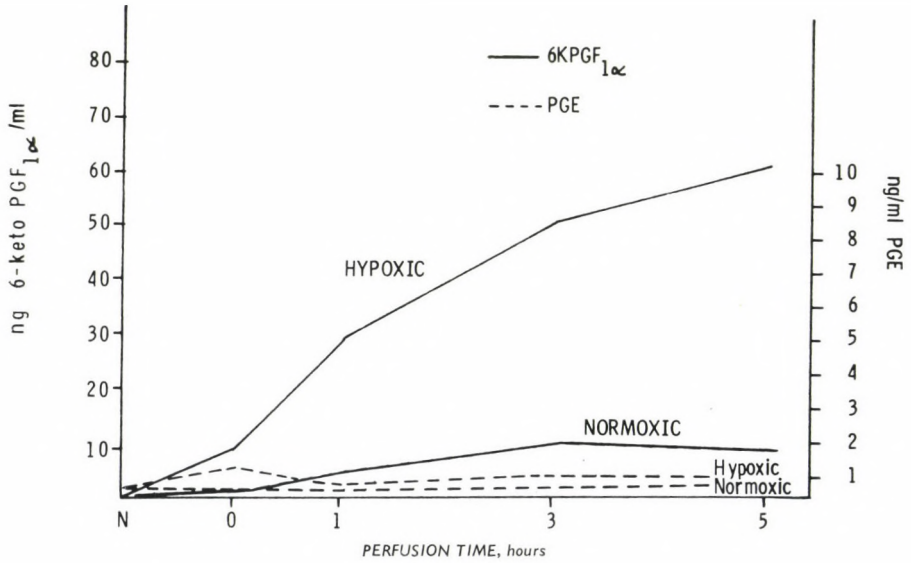


Fig. 9. PGE and 6-keto prostaglandin $PGF_{1\alpha}$ levels in the perfusates of the isolated dog kidneys (4 h, posthypoxic) perfused with blood at normal (normoxic) and low (hypoxic) oxygen tensions. — Each point represents the mean of 5 experiments (Fisher, J. W., et al. (Ref. 10)).

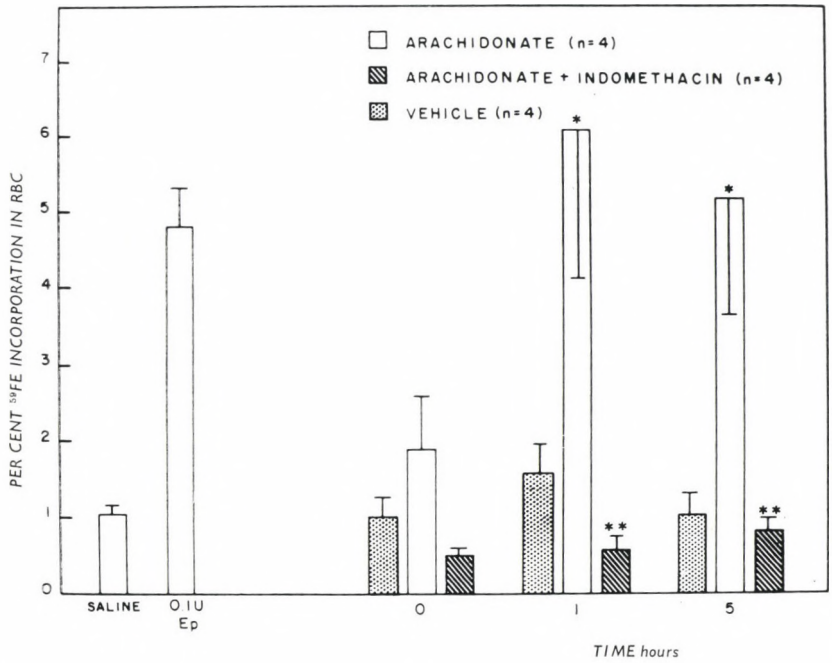


Fig. 10. The effect of arachidonate infusion (80 μg/min) on erythropoietin production in the posthypoxic isolated perfused dog kidney. Each bar represents the mean ± S.E.M. * Significant difference ($P < 0.05$) between vehicle and arachidonate-infused kidneys. ** Significant difference ($P < 0.05$) between arachidonate-infused kidneys with and without pretreatment with indomethacin (Foley, J. E. et al., *J. Pharmacol. Exp. Ther. 207*, 402–409, 1978).

activation of erythropoiesis in the marrow. Note in Figure 5 that PGE_2 and D_2 both exerted a significant effect in increasing erythroid colony forming cells of the CFU-E type in mouse bone marrow cultures. However, as noted in Figure 6, 6-keto PGE_1, a stable metabolite of prostacyclin, did not exert a significant effect on erythroid colony forming cells. These findings further confirm our hypothesis that the effect of 6-keto PGE_1 in the exhypoxic polycythemic mouse assay must be predominantly to enhance renal production of erythropoietin. The mechanism by which arachidonate is triggered to form PGE_2 and PGD_2 in the marrow to enhance erythropoiesis is not known but may also involve an hypoxic mechanism. Therefore, we postulate that prostacyclin and its metabolite 6-keto PGE_1 are probably more involved with hypoxic stimulation of renal erythropoietin production and perhaps prostaglandins E_2 and D_2 act primarily by activating erythroid progenitor cells directly in the marrow. It would appear that the bone marrow cell is programmed to produce PGE_2 and PGD_2 while the kidney is programmed to produce prostacyclin, $PGF_{2\alpha}$, 6KPGE$_1$ and PGE_2.

Hypoxic stimulation of renal erythropoietin production

Our further studies were aimed at determining the role of prostanoids in hypoxic stimulation of renal erythropoietin production. Note in Figure 7 that in mice hypoxia alone produced a significant increase in radioiron incorporation in polycythemic mice at various degrees of hypobaric pressure. On the other hand, pretreatment with the cyclooxygenase inhibitors meclofenamate and indomethacin significantly inhibited the response of polycythemic mice to hypoxia as measured by radioiron incorporation [17]. Therefore, it would appear that the response to hypoxia was inhibited by blocking the synthesis of prostanoids such as prostacyclin.

In order to more clearly elucidate the role of hypoxia in erythropoietin production, we next turned to the effect of hypoxemic perfusion of the isolated kidney on erythropoietin biosynthesis as it relates to prostacyclin production and inhibition of the cyclooxygenase system with the use of meclofenamate. The isolated dog kidney was perfused for five hours with blood at low oxygen tension. As noted in Figure 8 the erythropoietin titers were significantly elevated as early as one hour following hypoxemic perfusion; whereas the addition of meclofenamate to the perfusate completely blocked the rise in erythropoietin production following hypoxia [14]. Normoxic perfusion did not significantly increase erythropoietin titers. The metabolite of prostacyclin, 6-keto $PGF_{1\alpha}$, was measured by RIA in this system and this prostacyclin metabolite was markedly elevated during hypoxemic perfusion; whereas PGE_2 was not increased (Fig. 9).

In order to prove the role of prostanoids in a biological process one should demonstrate that arachidonate itself, the precursor of the prostanoids has biological effects. As noted in Fig. 10 arachidonate was found to enhance erythropoietin production in the isolated perfused kidney and was blocked by pretreatment of the dog with indomethacin, a cyclooxygenase inhibitor [28]. Note also in Fig. 10 that arachidonic acid perfusion of the isolated dog kidney produced a significant increase in erythropoietin titers after one and five hours. On the other hand, when the normoxic perfused kidney was perfused with blood containing arachidonate plus pretreatment with indomethacin, no significant rise in erythropoietin titers occurred. Normoxic perfusion alone did not increase the erythropoietin titers.

Erythropoietin has been localized in the glomerular tuft of the sheep kidney utilizing the indirect fluorescent antibody technique [24]. Busuttil et al. [20, 21] have demonstrated intense fluorescence in the glomerular tufts of anemic human and hypoxic dog kidneys. They localized the fluorescence in the peripheral portion of the glomerular tuft of the anemic human [20] and the hypoxic dog kidney [21] and suggested that the localization was in the epithelial cells of the tuft. Burlington et al. [22] produced erythropoietin in renal glomerular cultures and found an overgrowth of epithelial cells in the glomerular tuft. In contrast, Kurtz et al. [23] found an increased level of erythropoietin in the culture media of an isolated rat glomerular preparation but the cells in their subcultures which produced erythropoietin were reported to be mesangial cells. Incubation of anti-Ep with highly puri-

Table 1

PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ production rates by isolated glomeruli

	Basal	With arachidonic acid (5 μg/ml)
PGE_2	2079± 201 (34)	7497± 655 (27)
$PGF_{2\alpha}$	1642± 307 (10)	3526± 738 (6)
6-keto-$PGF_{1\alpha}$	1371± 197 (5)	2689± 82 (5)

Results are the means ± SEM of individual assays (number in brackets) performed on different days. Each of these assays represents the mean of 6 determinations performed at increasing dilutions of the incubation medium. Results are expressed as PG synthesized per mg glomerular protein and per hour (Sraer, J. et al. *FEBS Letters 104:* 420-424, 1979).

Table 2

Effect of 3-week-pools of culture medium on the growth of erythroid precursor cells (CFU-E) expressed as percentage of maximal number of colonies

Oxygen	Control	Subculture: Day 6 (epithelial cells)	Subculture: Day 21 (mesangial cells)
20%	43± 4	33± 5	62± 2
3.5%	43± 4	36± 4	98± 8

Data represent mean ± SEM (n = 3) (Kurtz, A. et. al. *FEBS Letters 137:* 129–132, 1982)

fied erythropoietin blocked the fluorescence seen in the glomerular tufts of the human [20] and the dog [21] kidneys.

Table 1 shows the results of studies by Sraer et al. [29] in which they have found that the rat glomerular tuft is capable of generating in vitro PGE_2, $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$, a metabolite of prostacyclin. In that prostacyclin can be converted to 6-keto PGE_1 by the kidney [16, 18], we feel that the chances are very good that $6KPGE_1$ can also be produced in this cell system under the influence of hypoxia.

As noted in Table 2, Kurtz et al. [23] used a subculture method for growing glomerular mesangial and epithelial cells and found that hypoxia triggers the isolated glomerular cell cultures to produce erythropoietin and that the mesangial cells rather than epithelial cells produce Ep.

References

1. Jacobson, L. O., Goldwasser, E., Friend W., Plzak, L.: Role of the kidney in erythropoiesis. *Nature* (Lon.) *179*, 633 (1957).
2. Kuratowska, Z., Lewartowski, B., Michalak, E.: Studies on the production of erythropoietin by the isolated perfused organs. *Blood 18*, 527 (1961).
3. Fisher, J. W., Birdwell, B. J.: The production of an erythropoietic factor by the in situ perfused kidney. *Acta Haemat.* (Basel) *26*, 224 (1961).
4. Fisher, J. W., Langston, J. W.: The influence of hypoxemia and cobalt on erythropoietin production in the isolated perfused dog kidney. *Blood 29*, 114 (1967).
5. Nelson, P. K., Fisher, J. W., Gross, D. M., Folwy, J. E.: A concept for the control of kidney production of erythropoietin (Ep). The role of prostaglandins (PG) and cyclic nucleotides. *Haematologia 63*, 620 (1978).
6. Fisher, J. W.: Erythropoietin, pharmacology, biogenesis and control of production. *Pharmacol. Rev. 24*, 459 (1972).
7. Gordon, A. S., Kaplan, S. M.: *Erythrogenin* (REF). In: Fisher, J. W. (ed.): *Kidney Hormones*. Academic Press, London 1977, Vol. II, p. 187.
8. Erslev, A. J.: *The clinical usefulness of erythropoietin measurements*. In: Fisher, J. W. (ed.): *Kidney Hormones*. Academic Press, London 1977, Vol. II, p. 571.
9. Fisher, J. W., Gross, D. M.: *Renal prostaglandins and kidney production of erythropoietin*. In: Fisher, J. W. (ed.). *Kidney Hormones*. Academic Press, London 1977, Vol. II, p. 357.
10. Fisher, J. W., Nelson, P. K., Beckman, B., Burdowski, A.: *Kidney control of erythropoietin production*. In: Dunn, M. (ed.): *Renal Endocrinology*. William & Wilkins, Baltimore 1983, p. 142–180.
11. Radtke, H. W., Jubiz, W., Smith, J. B., Fisher J. W.: Albuterol-induced erythropoietin production and prostaglandins release in the isolated perfused dog kidney. *J. Pharm. Exp. Ther. 214*, 467 (1980).
12. Mujovic, V. M., Fisher, J. W.: The effects of indomethacin on erythropoietin production in dogs following renal artery constriction. I. The possible role of prostaglandins in the generation of erythropoietin by the kidney. *J. Pharm. Exp. Ther. 191*, 575 (1974).
13. Fisher, J. W.: Prostaglandins and kidney erythropoietin production. *Nephron 24*, 111 (1979).
14. Burdowski, A. J., Fisher, J. W.: Prostanoids and erythropoietin production in programmed isolated perfused kidneys. *Exp. Hemat. 8* (Suppl. 7), 206 (1980) (abstract).
15. Ferreri, N. R., McGiff, J. C., Miller, M. J. S., Spokas, E. G., Wong Py-K.: *The kidney and arachidonate metabolism*. In: Samuelsson, B., Paoletti, R., Ramwell, P. (eds.): *Advances in Prostaglandin, Thromboxane and Leukotriene Research*. Raven Press, New York 1983, Vol. II, p. 481.
16. Hassid, A., Sebrosky, A., Dunn, M. J.: *Prostaglandin metabolism in human kidney. Synthesis of 6-keto-prostaglandin E_1 from prostaglandin I_2*. V Int. Conf. Prostaglandins, Florence, Italy, p. 472 (1982).
17. Nelson, P. K., Brookins, J., Fisher, J. W.: Erythropoietic effects of Prostacyclin PGI_2 and Its Metabolite 6-keto-PGE_1. *J. Pharm. Exp. Ther.* 226 : 493–499 (1983).
18. Gerritsen, M. E.: *Prostaglandin synthesis by microvessel endothelial cells*. V Int. Conf. Prostaglandins, Florence, Italy, p. 557 (1982).
19. Gorman, R. R., Hamilton, R. D., Hopkins, N. K.: *Prostacyclin and thromboxane A_2 biosynthesis and regulation of adenylate cyclase in human diploid cell lines*. In: Vane, J. R., Bergstrom, S. (eds.): *Prostacyclin*. Raven Press, New York 1979, p. 85.
20. Busuttil, R. W., Roh, B. L., Fisher, J. W.: The cytological localization of erythropoietin in the human kidney using the fluorescent antibody technique. *Proc. Soc. Exp. Biol.* (N. Y.) *137*, 327 (1971).
21. Busuttil, R. W., Roh, B. L., Fisher, J. W.: Further evidence for the production of erythropoietin in the dog kidney. *Acta Haemat.* (Basel) *47*, 238 (1972).

22. Burlington, H., Cronkite, E. P., Beinecke, U., Zanjani, E. D.: Erythropoietin production in cultures of goat renal glomeruli. *Proc. Nat. Acad. Sci.* (Wash.) *69*, 3547 (1972).
23. Kurtz, A. W., Jelkmann, W., Bauer, C.: Mesangial cells derived from rat glomeruli produce an erythropoiesis stimulating factor in cell culture. *FEBS Letters 137*, 129 (1982).
24. Fisher, J. W., Taylor, G., Porteous, D. D.: Localization of erythropoietin in the glomeruli of sheep kidney using a fluorescent antibody technique. *Nature* (Lon.) 205, 611 (1965).
25. Kojima, A., Shiraki, M., Takahashi, R., Orimo, H., Morita, I., Minota, S.: Prostaglandin D_2 is the major prostaglandin of arachidonate metabolism in rat bone marrow homogenate. *Prostaglandins 20*, 171 (1980).
26. Belegu, M., Beckman, B., Fisher, J. W.: Effects of beta adrenergic blocking drugs on erythroid colony (CFU-E) stimulation by prostaglandins E_2 and D_2. *J. Cell Physiol.* 245 (Cell Physiol. 14) : c322–c327 (1983).
27. Iscove, N. N., Seiber, F.: Erythroid progenitors in mouse bone marrow detected by microscopic colony formation in culture. *Exp. Hemat. 3*, 32 (1975).
28. Foley, J. E., Gross, D. M., Nelson, P. K., Fisher, J. W.: The effects of arachidonic acid on erythropoietin production in exhypoxic polycythemic mice and the isolated perfused canine kidney. *J. Pharm. Exp. Ther. 209*, 402 (1978).
29. Sraer, J., Foidart, J., Chansel, D., Mahien, P., Kouznetzova, B., Ardailou, R.: Prostaglandin synthesis by mesangial and epithelial glomerular cultured cells. *FEBS Letters 104*, 420 (1979).
30. Fisher, J. W., Radtke, H. W., Jubiz, W., Nelson, P. K., Burdowski, A.: Prostaglandins activation of erythropoietin production and erythroid progenitor cells. In: Lange, R. D., Dunn, C. D. R., Heim, L. R., Fisher, J. W. (eds.): *Experimental Hematology*, 1980, Vol. 8, Suppl. 8, p. 65.
31. Fisher, J. W.: Control of Erythropoietin Production. *Proc. Soc. Exp. Biol. Med. 173 :* 289–305 (1983).

Haematologia 17 (2), pp. 151–160 (1984)

Polyamine Induced Suppression of Erythropoiesis in Uremia

W. RADTKE, E. SCHEUERMANN, H. DESSER*

Department of Nephrology, Div. Internal Medicine, University Hospital, Theod.-Stern-Kai 7, D-6000 Frankfurt/M, Federal Republic of Germany, and Ludwig Boltzmann Institute for Leukemia Research and Hematology,* Vienna, Austria

(Received 5 January 1983; accepted 14 March 1983)

The effect of the polyamine spermine previously shown to suppress erythropoiesis in vitro was investigated in a long term animal experiment. Continuous application of spermine by intraperitoneally implanted microperfusion pumps caused a mild hypoproliferative microcytic anemia in normal rats. Furthermore, in 12 patients with advanced renal insufficiency spermine was shown to be markedly elevated. This, together with the results obtained in animal experiments, suggests that hyperspermineemia is the reason for the impaired erythropoiesis in anemic uremic patients.

Keywords: erythropoiesis, polyamine, renal anemia, spermine, uremic toxin

Introduction

Since hemodialysis has become a worldwide and largely applied method to treat patients with end-stage renal failure successfully, the long lasting search for uremic toxins has been intensified considerably. However, in spite of all efforts, not a single substance accumulating in patients with renal insufficiency and at least partially removed by hemodialysis has been identified as an uremic toxin. One reason for this failure might be that uremia is not a simple disorder, but rather a large group of very different symptoms.

To ease our task, we looked only at one symptom, known to be most constantly associated with uremia, namely the anemia of chronic renal failure. Whereas some causes for the development of the anemia of uremia are well understood, others are still under discussion. On the one hand, bleeding from uremic epithelial lesions and increased hemolysis of red cells dwelling in the uremic environment causes a continuous blood loss [1, 2], which on the other hand cannot adequately be compensated for by an increase of red cell production in the uremic patient's bone marrow. The failure to produce erythrocytes at a rate sufficient to meet the increased demand might be due to two major factors. The first of these is the deficiency of the hormone erythropietin (Ep), which is produced in the kidney [3, 4], and the second are some uremic toxins which might act as inhibitors of erythropoiesis, accumulating in plasma and body fluids of patients with end-stage renal failure [5, 6].

Some years ago, Ohno and Fisher demonstrated that uremic sera impaired in vitro erythroid progenitor cell growth of erythroid burst forming units (BFU-E)

and erythroid colony forming units (CFU-E) in rabbit bone marrow cell cultures [7]. In the same laboratory we have recently found that this uremic inhibitor of in vitro erythropoiesis was small in molecular size, about 200 daltons, alkaline in nature and might be identical with the polyamine spermine [8]. When spermine was added to normal human sera at concentrations common in uremic patients and studied in fetal mouse liver cell culture and normal human bone marrow cell culture a dose-related inhibition of erythroid colony formation was noted. Furthermore, the inhibitory effect on erythroid colony formation of crude uremic sera, uremic serum dialysate, and fractions of uremic serum dialysate from gel filtration chromatography, was completely abolished by the addition of a specific rabbit antiserum to spermine [8].

It was the purpose of the present study to characterize the polyamine induced inhibition of erythropoiesis and to extend the in vitro observations to experiments in vivo.

Material and Methods

Sera were obtained (*a*) from 8 severely uremic anemic patients immediately before the onset of regular hemodialysis treatment and (*b*) from 12 patients with advanced renal insufficiency who had not yet had hemodialysis therapy (Table 1).

Table 1

Plasma polyamine concentration in patients with end-stage renal failure (nmol/l)

Patient	Putrescine	Spermidine	Spermine	Hematocrit, vol%	Creatinine, mg/dl
G. E.	131	56	60	27	8.6
N. O.	145	586	1430	28	7.9
S. C.	96	81	108	26	11.8
N. A.	96	60	83	36	7.8
A. L.	87	33	71	22	16.0
L. I.	101	69	71	22	9.5
M. O.	168	387	967	31	6.5
H. E.	153	66	52	32	13.1
W. O.	74	76	61	33	8.9
S. I.	233	73	64	27	5.3
F. O.	61	35	43	28	7.7
W. E.	61	82	67	37	7.1
Mean	117	134	256		
Mean ± SD of 38 normal controls	98+39	82+42	28+22		

Sera were heat inactivated at 56 °C for 30 min and stored in the deep-freeze unit assayed. For polyamine determination the sera were deproteinized, lyophilized and measured as described in detail elsewhere [9].

Fetal mouse liver cell technique

Fetal mouse liver cells from fetuses 13 day old or less were prepared according to the method of Iscove et al. [10]. Liver cells were disaggregated and suspended as single cells at a concentration of 100 000 cell/ml in a culture medium in methylcellulose, 30% fetal calf serum, 100 mU/ml of human urinary erythropoietin (Ep), 0.1 μmol of mercapto-ethanol, 100 U penicillin/ml, and 100 μg streptomycin/ml. 1 ml of cell culture was plated in 35 × 10-mm Petri dishes and incubated at 37 °C for 48 h in a humidified atmosphere of 95% air and 5% CO_2. After staining the plates with diaminobenzidine. CFU-E of 8 or more cells were counted in 3 replicate culture plates.

BFU-E colonies of 50 or more cells were cultured in the same way with the exception that Ep concentration was increased to 500 mU/ml and incubation time extended to 7 days.

Human bone marrow cell technique

Bone marrow was aspirated from the posterior iliac crest of normal male human volunteers and collected in an equal volume of Hank's balanced salt solution with 5 U/ml heparin. Under sterile conditions, an equal volume of disaggregated cell suspension was layered over Ficoll-Paque and centrifuged at 400 *g* at 18 °C for 30 min. After transferring the mononuclear cells to a clean tube, they were washed twice in 2 vol of Hank's balanced salt solution by centrifuging at 100 *g* for 10 min and the washed pellet was resuspended in alpha medium. The final concentration was 0.74% methylcellulose, 30% normal human AB serum, 2×10^4 cell/ml, 200 U penicillin/ml, 200 μg streptomycin/ml and 0.1 mol mercaptoethanol. Human urinary Ep (200 mU/ml) was added to each microtiter well before plating 0.1 ml culture medium. The plates were incubated at 37 °C in 5% CO_2 and 95% air for 7 days. CFU-E colonies were scored directly at × 100 after staining with benzidine.

Animal preparation

In 5 normal non uremic male Wistar rats a microperfusion pump (Alzet-Osmotic-Pump) was implanted intraperitoneally on days 0 and 10, which delivered continuously 0.5 μmol/h of spermine over a period of 3 weeks. Five sham operated rats were investigated for comparison. In both groups, hematocrit was measured on days 0, 7, 14 and 21, and plasma spermine concentration on day 21.

For statistical analysis, Student's paired and unpaired *t* test was used.

Results

Effect of uremic sera on normal human bone marrow culture

Uremic sera added to normal human bone marrow cultures at a concentration of 20% (v/v) significantly inhibited human CFU-E formation, as shown in Fig. 1. This demonstrates that the CFU-E inhibition by uremic sera previously observed in fetal mouse liver cell cultures is also true for human bone marrow cultures, proving the validity of the fetal mouse liver cell technique in testing inhibitors of erythropoiesis.

Interaction between the uremic inhibitor and Ep

Figure 2 shows the relation between the uremic inhibitor of erythropoiesis and Ep. Increasing concentrations of an uremic serum were tested at different Ep concentrations. As shown on the left panel, the uremic serum exhibited the same

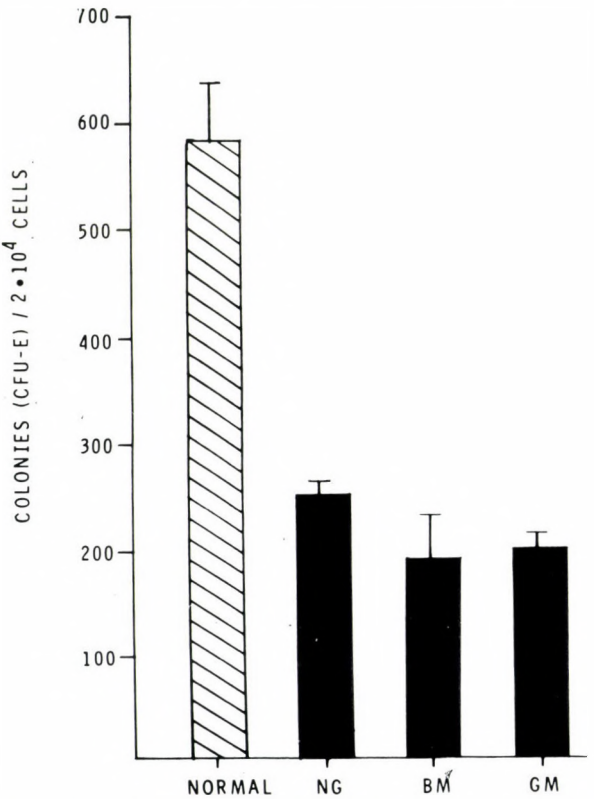


Fig. 1. Effect of uremic sera on normal human bone marrow culture (CFU-E). Bars indicate SEM of 3 replicates

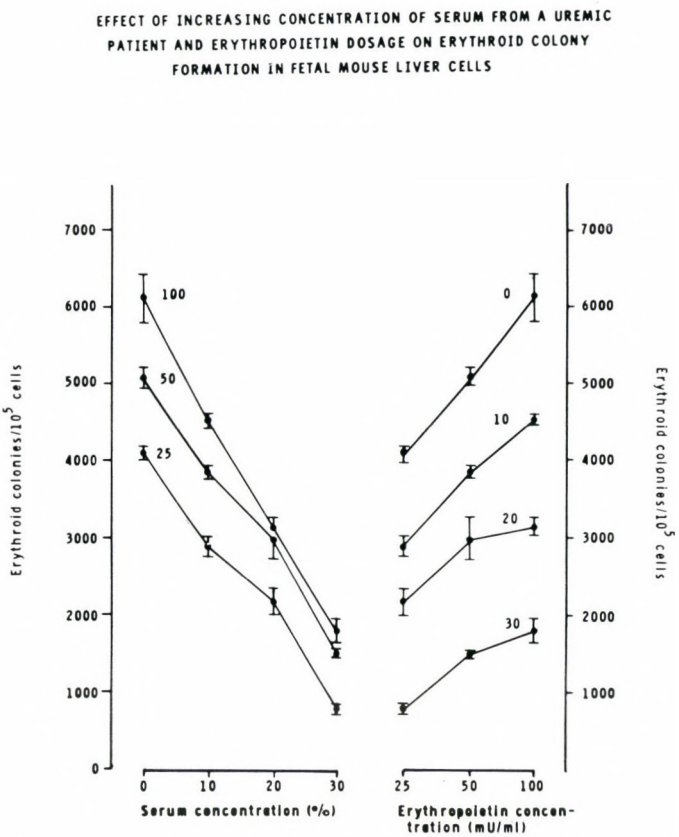


Fig. 2. Relation between the uremic inhibitor of erythropoiesis and Ep in fetal mouse liver cell culture. Increasing concentrations of an uremic serum in the presence of different Ep concentrations (left panel), and Ep dose–response curves at different uremic serum concentrations (right panel). Bars indicate SEM of 3 replicates

inhibition in the presence of 100, 50 and 25 mI Ep/ml. The right panel shows the corresponding Ep dose–response curves at different uremic serum concentrations. They become less steep at higher concentrations of the uremic serum, but it is obvious that the uremic serum inhibitor of erythropoiesis does not act as an anti-Ep, since the effect of Ep is still demonstrable, even when the inhibitor is present in excess.

Effect of spermine on fetal liver BFU-E formation

Since we have shown recently that the polyamine spermine inhibits fetal mouse liver CFU-E formation at very low concentrations, the effect of 3 mol per

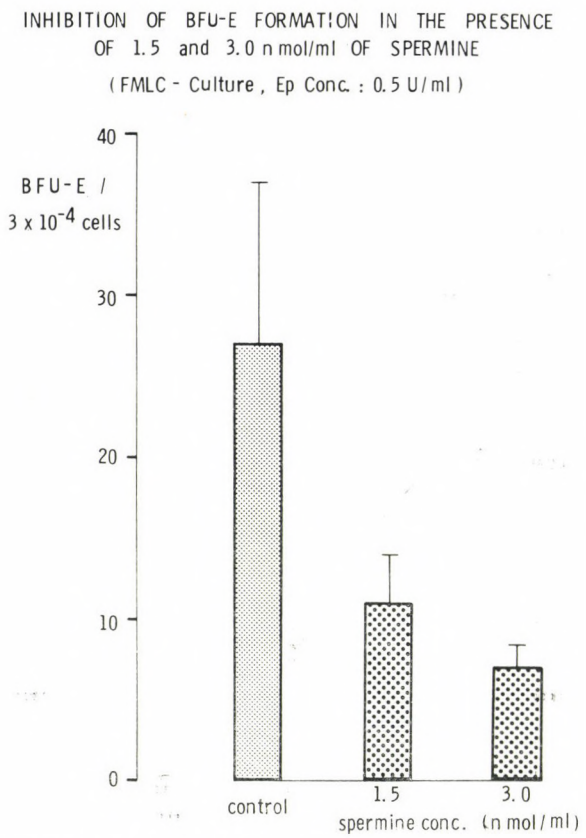


Fig. 3. Effect of spermine on fetal mouse liver BFU-E formation. Bars indicate SEM of 3 replicates

ml of spermine was tested on fetal mouse liver BFU-E formation. As shown in Fig. 3 spermine inhibited BFU-E formation in the same way as it inhibited CFU-E formation in the fetal liver.

Putrescine, spermidine and spermine concentrations in patients with chronic renal failure

By an iron-exchange column in a high performance liquid chromatography (HPLC) device the serum concentrations of the polyamine putrescine, spermidine and spermine were simultaneously measured in 12 patients suffering from advanced chronic renal insufficiency who had not had hemodialysis therapy. As shown in Table 1, serum creatinine concentration in these patients ranged from 5.6 to 16.0 mg/dl and the hematocrit from 22 to 37%. Of all polyamines, spermine con-

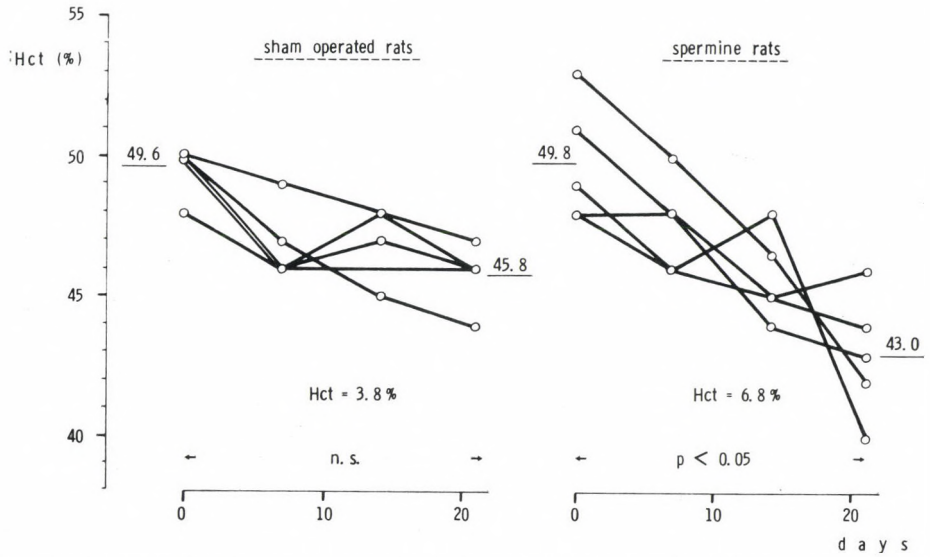


Fig. 4. Decrease of hematocrit under the influence of continuous spermine infusion of 0.5 μmol/h

centration was the only one elevated significantly ($p < 0.01$), to approximately 9 times above the normal controls and reaching occasionally levels as high as 1000 mol/l.

In vivo effect of continuous spermine application in normal rats

The effect of spermine continuously delivered over a period of 3 weeks by an intraperitoneally implanted micropump was investigated in 5 normal non uremic rats. As shown in Fig. 4, the mean hematocrit dropped from 50 to 43% ($p < 0.01$) while the hematocrit of 5 sham-operated animals decreased from 50 to 46% (n.s.). Thus, continuous spermine administration caused a mild anemia which was normochromic and microcytic in nature, resembling the anemia of chronic renal failure.

Discussion

Although neither urea nor creatinine are toxic by themselves, these substances serve widely as crude measures of the degree of kidney impairment and uremic intoxication. This is due to the fact that the "uremic toxin(s)" are unknown and it is assumed that urea and creatinine accumulate parallel with the unknown sub-

stance(s) under discussion. This only shows how desperately we need to know what the uremic toxins are in order to be able to compare different methods of kidney substitution therapy such as hemodialysis, hemofiltration and continuous ambulatory peritoneal dialysis (CAPD), and for the purpose of invention of some devices for new effective detoxification therapy.

For a long period of time the idea that so-called middle molecules, substances of a molecular weight between 500 and 5000 daltons comprise the toxins responsible for all or most of the uremic symptoms has been favoured [11]. But since hemofiltration which clears most of the middle molecules does not ameliorate the symptoms of uremia convincingly, this hypothesis has increasingly been questioned. And indeed, the present study suggested that small molecules play an important role in the inhibition of erythropoiesis.

Among the small molecular substances accumulating in uremia and accused to generate uremic symptoms, several candidates would match the size of our inhibitor such as indols, phenols, aromatic amino acids, aromatic amines and polyamines [11]. The latter are the most suspicious ones because polyamines are known to play an important role in the regulation of cell replication [12]. At higher concentrations they have been shown to suppress cell proliferation in vitro [13] and to inhibit the activity of such important enzymes as adenylate cyclase [14] and Na–K–ATPase [15].

Little is known about plasma polyamine levels in uremic patients. Campbell et al. found that the plasma spermine level in uremic patients was 4 times above normal, whereas free spermidine concentrations were only moderately elevated [16]. This is in good agreement with our preliminary data of 12 uremic patients with spermine levels of approximately 9 times above normal. Putrescine and spermine levels were in the same range as in normal subjects. Interestingly enough, spermine levels in uremic patients do not behave homogeneously, since two patients exhibited very high spermine levels. These patients were neither the most anemic ones nor did they show the highest creatinine levels, and the reason for their hypersperminemia was not known. These 12 patients were still in a stable clinical condition and had not yet reached the terminal phase of uremia, where the hematocrit usually decreases dramatically to values as low as 12 or 15%, and the signs of uremic intoxication call for immediate institution of hemodialysis therapy. One may assume that a pronounced accumulation of spermine occurs in end-stage renal failure, which might be the major cause of the gross deterioration of anemia. This is based on the fact that spermine has a very low clearance rate, much below that of inulin. In a preliminary experiment we have measured long term spermine clearance rates in three male Wistar rats in a metabolic cage by use of a microperfusion pump with ^{14}C-spermine. As shown in Table 2 renal spermine clearance was on 1–4% of that of inulin which might explain the plasma level increasing parallel with the decreasing excretory renal function. The reason for the low clearance rate of this small molecular substance is not clear, the polycationic nature of spermine might inhibit its diffusion across the glomerular membrane. Its diffusion across the membrane of the hemodialysis is also poor, since a 6 h hemodialysis

Table 2

Spermine clearance rate in 3 male Wistar rats
(body weight 250–300 g)

1.	0.02 ml/min/g	kidney
2.	0.04 ml/min/g	kidney
3.	0.01 ml/min/g	kidney

For comparison:
Inulin clearance of normal rats ~ 1.0 ml/min/g kidney

decreases the plasma spermine level only by some 30%, as has been shown by Campbell et al. [16].

To answer the important question whether the inhibition of erythropoiesis by spermine could be observed in vivo too, we have performed animal experiments. In contrast to sham operated rats, application of 05 μmol/h of spermine for three weeks produced a steady decrease of the hematocrit in all animals, which was not very pronounced in absolute terms but still significant statistically. Considering the about 100 day normal life span of red cells, the experimental period of three weeks might have been too short to allow the development of a more severe anemia. Clearly, more experimental work is necessary to confirm that spermine can inhibit the proliferation and maturation of erythroid cells and produce a hypoproliferative anemia in normal animals.

References

1. Mason, E. E.: Gastrointestinal lesions occurring in uremia. *Ann. Intern. Med. 37*, 96 (1952).
2. Shaw, A. G.: Haemolysis in chronic renal failure. *Brit. Med. J. 2*, 213 (1967).
3. Caro, J., Brown, S., Miller, O., Murray, T., Erslev, A. J.: Erythropoietin levels in uremic nephric and anephric patients. *J. Lab. Clin. Med. 93*, 449 (1979).
4. Radtke, H. W., Claussner, A., Erbes, P. M., Scheuermann, E. H., Schoeppe, W., Koch, K. M.: Serum erythropoietin concentration in chronic renal failure: relationship to degree of anemia and excretory renal function. *Blood 54*, 877 (1979).
5. Fisher, J. W., Moriyama, Y., Rege, A. B., Lertora, J. J. L.: The role of inhibitors of heme synthesis and bone marrow erythroid colony forming cells in the mechanism of the anemia of renal insufficiency. *Proc. Clin. Dial. Transplant. Forum 4*, 276 (1974).
6. Wallner, S., Kurnick, J., Ward, H., Vautrin, R., Alfrey, A. C.: The anemia of chronic renal failure and chronic diseases: in vitro studies of erythropoiesis. *Blood 47*, 561 (1976).
7. Ohno, Y., Rege, A. B., Fisher, J. W., Barona, J.: Inhibitors of erythroid colony forming cells (CFU-E and BFU-E) in sera of azotemic patients with anemia of renal disease. *J. Lab. Clin. Med. 92*, 916 (1978).
8. Radtke, H. W., Rege, A. B., LaMarche, M. B., Bartos, D., Bartos, F., Campbell, R. A., Fisher, J. W.: Identification of spermine as an inhibitor of erythropoiesis in patients with chronic renal failure. *J. Clin. Invest. 67*, 1623 (1981).

9. Desser, H., Lutz, D., Krieger, O., Stierer, M.: Rapid detection of polyamine in the sera of patients with colorectal carcinoma by liquid ion-exchange chromatography. *Oncology 37*, 376 (1980).
10. Iscove, N. N., Sieber, F., Winterhalter, K. H.: Erythroid colony formation in cultures of mouse and human bone marrow: Analysis of requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin. *J. Cell. Physiol. 83*, 309 (1974).
11. Bergström, J., Fürst, P.: Uremic toxins. *Kidney Int. Suppl. 8*, S9 (1978).
12. Bloomfield, V. A., Wilson, R. W.: Interactions of polyamines with polynucleotides. In: Morris, D. R., Marton, L. J. (eds): *Polyamines in Biology and Medicine.* Dekker, New York 1981, p. 183.
13. Grahl, W. A., Changus, J. W., Pitot, H. C.: The effect of spermine and spermidine on proliferation in vitro of fibroblasts from normal and cystic patients. *Pediat. Res. 12*, 5 (1976).
14. Atmar, V. J., Kuehn, G. D.: Effects of polyamines in adenylate cyclase activity and acidic nuclear protein phosphorylation in physarum polydephalum. *Fed. Proc. 36*, 686 (1977).
15. Quarforth, G., Ahmed, K.: Sites of action of polyamines on Na, K-ATPase. *Fed. Proc. 36*, 360 (1977).
16. Campbell, R., Talwalker, Y., Bartos, D., Bartos, F., Musgrave, J., Harner, M., Puri, H., Grettie, D., Donley, A. M., Logan, B.: Polyamines, uremia, and hemodialysis. In: Campbell, R. A. (ed.): Polyamines. Raven Press, New York 2, 319 (1978).

Haematologia 17 (2), pp. 161–168 (1984)

Prostaglandin Synthesis Inhibitors in Erythropoiesis

V. PAVLOVIĆ-KENTERA, D. SUŠIĆ, L. BILJANOVIĆ-PAUNOVIĆ, P. MILENKOVIĈ

Institute for Medical Research, Bulevar JNA 10, P. O. Box 721, 11001 Belgrade, Yugoslavia

(Received 10 February 1983; accepted 15 March 1983)

PG synthesis inhibitors were used to study the role of PG in modulating Ep effects on erythroid progenitors. The stimulating effect of exogenous Ep on both ^{59}Fe incorporation into RBC and CFU-E number in polycythaemic mice was inhibited with indomethacin and aspirin treatment during the posthypoxic period. The inhibitory effect of indomethacin on CFU-E derived colonies was demonstrated only when cultured bone marrow and spleen cells were obtained from mice stimulated by prolonged exposure to hypoxia. The results implicate the role of PG in the Ep dependent regulation of the erythroid progenitor cell compartment size and Ep responsiveness in vivo.

Keywords: CFU-E; erythroid progenitors; erythropoiesis; erythropoietin; prostaglandins; PG synthesis inhibitors

Introduction

Prostaglandins (PG), a ubiquitous family of fatty acids, are known to influence the function of different organs and tissues. PG are known to inhibit granulopoiesis and stimulate erythropoiesis. It has been postulated that PG enhance erythropoiesis by two mechanisms, by stimulating erythropoietin (Ep) production and by a direct effect on erythroid progenitors [1]. Recently we have shown that the PG synthesis inhibitors: aspirin and indomethacin inhibit the synthesis of Ep in rats exposed to acute hypoxia [2, 3]. The present work was oriented toward the study of the effects of the PG synthetase inhibitors aspirin and indomethacin, on the size of a late stage erythroid progenitor cell compartment (CFU-E). In order to gain more information about the role of PG in modulating Ep effects on erythroid progenitors we have studied the effects of exogenous Ep on erythropoiesis in polycythaemic mice pretreated with PG synthesis inhibitors. In addition, the ability of erythroid progenitors to form colonies when stimulated with Ep in vitro in the presence of the PG synthetase inhibitors was determined. The results obtained indicate that PG augment the effect of Ep, the fundamental regulator of erythropoiesis.

Material and Methods

Three months old female CBA mice either normal or polycythaemic were used in the experiments.

Polycythaemia was achieved by exposure of the mice to chronic hypoxia in a silicone rubber membrane enclosure with about 7% O_2 [4]. After two weeks in hypoxia the mice were divided into four groups of 10 mice each. Groups 1 and 2 were controls. In group 3 the mice were injected s.c. on days 2, 3, 4 and 5 with 40 μg of indomethacin in 0.1 ml of 0.1 M Na_2CO_3. The mice in group 4 were given 0.5 mg of aspirin in 0.2 ml H_2O intragastrically on days 2, 3, 4 and 5. On the 6th posthypoxic day mice from groups 2, 3 and 4 were injected with a test dose of exogenous Ep (3 mg of crude human urinary Ep with 1.5 units/mg). Seven mice from each of the four groups were used for erythropoietin responsive cell (ERC) estimation and three mice for determination of CFU-E in the bone marrow and spleen. To estimate ERC, the test dose of Ep was followed after 48 hours by an injection of 0.5 μCi ^{59}Fe and 48 hour radioiron incorporation into red blood cells (RBC) was measured [5]. CFU-E in the bone marrow and spleen was determined 24 hours after the Ep test dose. Response to Ep was determined by measuring 48 hour ^{59}Fe incorporation in polycythaemic mice injected with 3 different doses of Ep on the 6th and 7th posthypoxic day [4].

CFU-E assay was performed by the slightly modified method described by Iscove et al. [6]. 1.0 ml with 1×10^5 marrow and 2×10^5 spleen cells was plated in plastic tissue culture dishes 35 mm in diameter. The mixture consisted of 30% fetal calf serum in DMEM with 10^{-4} M alpha thioglycerol, 0.2 units of Ep and 0.8% methylcellulose in DMEM. The Ep used was from plasma of mice irradiated and treated with phenylhydrazine as described by Tambourin et al. (8). The Ep level was 10.0 U/ml and 20μl/culture was added. The same amount of normal mouse plasma was added to the control.

For in vitro studies indomethacin was dissolved in ethanol and stock solutions were diluted in Dulbecco's Modified Eagle's Medium (DMEM) 10 : 100 v/v as suggested by Rossi et al. [7]. The doses added to the cultures were 2.5×10^{-5} M, 5×10^{-5} M and 10^{-4} M/ml. Control plates contained 1 μl/dish of ethanol. Aspirin was dissolved in DMEM and 7×10^{-3} M, 3.5×10^{-2} M and 7×10^{-2} M/dish in 75 μl were added to the cultures at the beginning of the incubation period, same as indomethacin.

Statistical treatment of the results involved the calculation of sample mean, standard deviation (SD) and standard error of the mean (SEM). The significance of the differences was tested by means of Student's *t*-test.

When indomethacin or aspirin were used to study the in vitro effect, bone marrow and spleen cells were obtained either from normal mice or mice during chronic exposure to hypoxia.

Results

Indomethacin administered daily to mice with hypoxic polycythaemia, initiated on the 2nd day after hypoxia and continued for four days induced a decrease of the Ep response to a high Ep dose (0.9 unit) but not to lower doses (0.27 and 0.37 units) (Fig. 1).

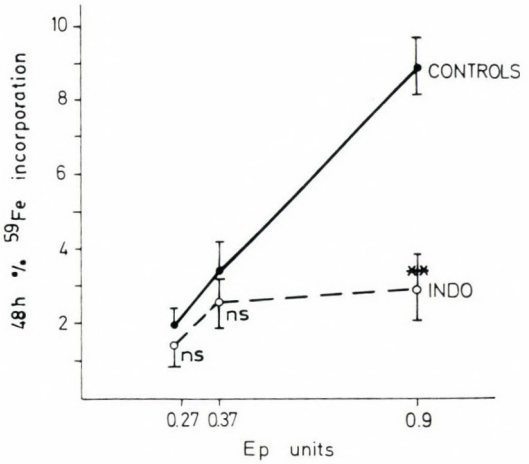


Fig. 1. Effect of indomethacin given on days 2, 3, 4 and 5 after hypoxia on Ep response in mice with posthypoxic polycythaemia. Ep was injected on the 6th and 7th day after hypoxia both to controls and indomethacin treated animals. The results are mean ± SEM for two experiments in seven mice each. ** different from controls at 0.01 level

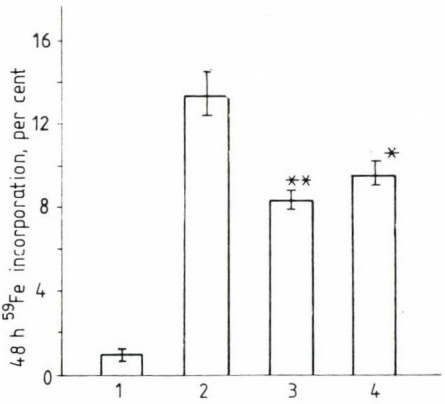


Fig. 2. Radioiron incorporation into RBC of mice with posthypoxic polycythaemia. The results are mean ± SEM for two experiments in seven mice each. Group 1: polycythaemic mice given ^{59}Fe on the 8th day after hypoxia. Group 2: same as 1, with Ep given on day 6. Group 3: same as 2 but pretreated for four days with indomethacin. Group 4: — same as 2 but pretreated with aspirin for four days. Different from Group 2 at 0.05 level *, at 0.01 level **, at 0.001 level ***

Table 1

Number of CFU-E derived colonies from the bone marrow (10^5 cell) and spleen (2×10^5 cell) of normal mice incubated with different doses of indomethacin and aspirin added at the beginning of the incubation period. Control values are for the same cell suspension incubated with Ep and vehicle only. The results are mean ± SD for four dishes/assay

No.	Control	Indomethacin			Aspirin		
		10^{-4} M	5×10^{-5} M	2.5×10^{-5} M	7×10^{-2} M	3.5×10^{-2} M	7×10^{-3} M
Bone marrow							
1	524±33			304±164*	0	49±33***	170±24***
2	638±64	426±149	392±149*		0	55±10***	229±28***
3	507±58	553±51	579±29	518±38			
4	598±38			566±41			
5	758±30		694±34	775±28			
6	926±28		324±34				
7	550±39		459±55	468±17			
Spleen							
1	218±10			186±25*	0	44±25***	115±30***
2	312±75	261±54	261±27	237±64	0	60±25**	175±40*
3	303±25	202±23***	289±35	313±38			
4	364±54			213±30**			
5	338±40		328±13				
6	320±66		222±40*	288±32			

Significance of the differences as in Fig. 2

Table 2

Number of CFU-E derived colonies from the bone marrow (10^5 cells) and spleen (2×10^5 cells) of mice on 8th day of hypoxia, incubated with different doses of indomethacin and aspirin added at the beginning of the incubation period. Control values are from the same cell suspensions incubated with Ep and vehicle only. The results are mean ±SD for four dishes/assay

No.	Control	Indomethacin			Aspirin		
		10^{-4} M	5×10^{-5} M	2.5×10^{-5} M	7×10^{-2} M	3.5×10^{-2} M	7×10^{-3} M
Bone marrow							
1	705±71		385±143**	136±18***	0	66±32***	294±32***
2	1050±228	114±57***	180±47***	312±41***	0	339±50***	680±34*
3	1795±106	1746±78	1494±89**	1392±88**			
Spleen							
1	858±49		667±57**	412±39***	0	44±25***	262±32***
2	612±62	514±19*	448±49**	350±57***	0	557±185	557±65
3	922±143	533±53**	595±54	850±35**			

Significance of the differences as in Fig. 2.

The 48 hour ^{59}Fe incorporation into RBC in four groups of polycythaemic mice given a test dose of Ep on the 6th posthypoxic day is shown in Fig. 2. The decreased radioiron incorporation into RBC of mice pretreated with indomethacin and aspirin indicated a decrease in ERC in those animals.

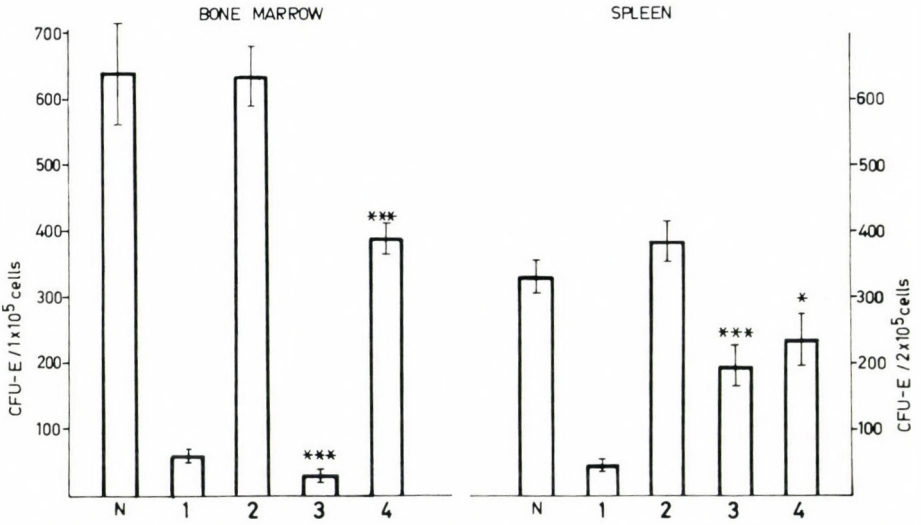


Fig. 3. Number of CFU-E derived colonies from the bone marrow (10^5 cell) and spleen (2×10^5 cells) of normal mice with posthypoxic polycythaemia. The results of two separate experiments were pooled and are now presented as mean $\pm$SEM. Three mice per group and four dishes/assay. Groups as in Fig. 2. Significance of the differences are the same as in Fig. 2

When Ep was injected into polycythaemic mice on the 6th posthypoxic day (Group 2) after 24 hours the number of CFU-E increased over the control values (Group 1) both in the bone marrow and the spleen. Pretreatment with either indomethacin (Group 3) or aspirin (Group 4) decreased the effect of the test dose of Ep on the bone marrow and spleen cell CFU-E (Fig. 3).

The results obtained in experiments with PG synthetase inhibitors added in vitro at the beginning of the incubation period are presented in Tables 1 and 2. As can be seen from Table 1 the number of CFU-E derived colonies from bone marrow and spleen cells of normal mice incubated with indomethacin and Ep did not differ significantly from those incubated only with Ep. However, addition of aspirin instead of indomethacin caused a decrease in the number of colonies. When the bone marrow and spleen cells obtained from mice during chronic exposure to hypoxia, i.e. haemopoietic cells with a high number of CFU-E, were cultured in the presence of Ep combined with indomethacin or aspirin, the decrease of CFU-E derived colonies was significant and dose dependent (Table 2).

Discussion

In the present study we have confirmed the results of other investigators [1, 9–14] in that PG enhanced the effect of Ep interacting with Ep at specific receptor sites on ERC. Our finding that inhibition of the stimulating effect of exogenous Ep on the number of CFU-E in bone marrow and spleen cultures from polycythaemic mice treated with indomethacin and aspirin during the posthypoxic period, agrees with the hypothesis that a synergetic interaction of PG with Ep occurs in the ERC compartment. The lower number of ERC found in mice pretreated with PG synthesis inhibitors could be explained by the hypothesis [9] that in the presence of PG more target cells respond to a given level of Ep. Since indomethacin and aspirin treatment was started four days before Ep injection it is postulated that the obtained effect on the number of CFU-E in vivo may be a consequence of the primary effect on BFU-E or CPU-S. This is consistent with the increased numbers of BFU-E in peripheral blood of patients given PGE_2, as observed by Ortega et al. [15], and the effect of PGE on CFU-S [16]. The fact that we have not been able to inhibit the growth of CFU-E derived colonies speaks in favour of the explanation. It is also possible that in the animals pretreated with indomethacin the effect of Ep impairs the transition of BFU-E to the CFU-E stage. We have recently shown [17] that Ep is essential for the transition of early committed cells to erythropoiesis (pre-ERC) to the ERC. Also, priming of the animals with PG synthesis inhibitors may influence the size of the erythroid committed precursor compartment, resulting in the observed low number of ERC and CFU-E.

The inhibitory effect of indomethacin and aspirin in haemopoietic tissue cultures from animals stimulated by chronic exposure to hypoxia suggests that the level of PG may be increased in these tissues as in other tissues during hypoxia [18, 19]. Beckman et al. [20] found, however, that sodium meclofenamate and indomethacin which inhibit PG synthetase had no effect on mouse bone marrow cell CFU-E grown in hypoxia. The inhibitory effect seen after hypoxic pretreatment may also indicate an expanded erythroid cell compartment capable of responding to indomethacin and aspirin.

The results presented implicate a role of PG in the Ep dependent regulation of the size of the erythroid progenitor cell compartment and Ep responsiveness. Further experiments are needed to determine whether these effects of PG are obligatory for the Ep responsiveness under different experimental conditions.

*

The authors extend a special thanks to Dr James W. Fisher for his critical review of the manuscript and helpful suggestions.

This work was supported by a grant from the Serbian Research Foundation.

References

1. Fisher, J. W., Radtke, H. W., Jubiz, W., Nelson, P. K., Burdowski, A.: Prostaglandins activation of erythropoietin production and erythroid progenitor cells. *Exp. Hemat. 8*, suppl. 8, 65 (1980).
2. Sušić, D., Milenković, P., Pavlović-Kentera, V.: The effect of aspirin on erythropoietin formation in the rat. *Proc. Soc. Exp. Biol. (N. Y.) 161*, 476 (1979).
3. Pavlović-Kentera, V., Sušić, D., Milenković, P., Biljanović-Paunović, L.: Effects of prostaglandin synthetase inhibitors, salt overload and renomedullary dissection on the hypoxia stimulated erythropoietin production in rats. *Exp. Hemat. 8*, suppl. 8, 284 (1980).
4. Biljanović-Paunović, L., Kostić, K., Milenković, P., Pavlović-Kentera, V.: Bioassay for erythropoetin. *Yugoslav. Physiol. Pharmacol. Acta 12*, 165 (1976).
5. Gurney, C. W., Lajtha, L. G., Oliver, R.: A method for investigation of stem-cell kinetics. *Brit. J. Haemat. 8*, 461 (1963).
6. Iscove, N. N., Sieber, F., Winterhalter, K. H.: Erythroid colony formation in cultures of mouse and human bone marrow: analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A. *J. Cell Physiol. 83*, 309 (1974).
7. Rossi, G. B., Migliaccio, A. R., Lettieri, F., DiRosa, M., Mastroberardino, G., Paschle, C.: In vitro interactions of PGE and cAMP with murine and human erythroid precursor. *Blood 56*, 74 (1980).
8. Tambourin, P. E., Wendling, F., Gallien-Lartigue, O., Huaulme, D.: Production of high plasma levels of erythropoietin in mice. *Biomedicine 19*, 112 (1973).
9. Dukes, P. P., Shore, N. A., Hammond, D., Ortega, J. A., Detta, M. C.: Enhancement of erythropoiesis by prostaglandins. *J. Lab. Clin. Med. 82*, 704 (1973).
10. Dunn, C. D. R.: Prostaglandins and erythropoiesis: structure/action relationships and identification of prostaglandin responsive cells. *Blut 42*, 307 (1981).
11. Golde, D. W.: Hormonal modulation of erythropoiesis in vitro. In: M. J. Murphy Jr. (ed): *In vitro aspects of erythropoiesis*. Springer Verlag, Berlin 1978, p. 81.
12. Schooley, J. C., Mahlmann, L. J.: Stimulation of erythropoiesis by prostaglandins and its inhibition by anti-erythropoietin. *Proc. Soc. Exp. Biol. (N. Y.) 138*, 523 (1971).
13. Razin, E., Hayari, Y., Globerson, A.: Effects of indomethacin on haematopoiesis in mice. *Prostaglandins 6*, 613 (1981).
14. Keighley, G., Cohen, N. S.: Stimulation of erythropoiesis in mice by adenosine 3'5' monophosphate and prostaglandin E_1. *J. Med. 9*, 129 (1978).
15. Ortega, J. A., Malekzadeh, M. H., Dukes, P. P., Ma, A., Shore, N. A.: A clinical trial of prostaglandin E_2 to increase erythropoiesis in anemia of chronic renal failure. *Exp. Hemat. 9*, Suppl. 9, 34 (1981).
16. Gidali, J., Feher, I.: The effect of E type prostaglandins on the proliferation of haemopoietic stem cells in vivo. *Cell Tissue Kinet. 10*, 365 (1977).
17. Pavlović-Kentera, V., Milenković, P.: Effect of erythropoietin on erythropoietin-responsive cell regeneration in polycythemic mice treated with cyclophosphamide. *Acta Haemat. (Basel) 66*, 6 (1981).
18. Wennmalm, Å.: Hypoxia induced prostaglandin release from rabbit heart. In: Roy, P. E., Rona, G. (eds): *Recent Advances in the Studies on Cardiac Structure and Metabolism, 10*. University Park Press, Baltimore 1975, p. 379.
19. Kalsner, S.: The effect of hypoxia on prostaglandin output and on tone in isolated coronary arteries. *Canad. J. Physiol. Pharmacol. 55*, 882 (1977).
20. Beckmann, B., Belegu, R. D., Belegu, M., Katsuoka, Y., Fisher, J. W.: Hypoxic enhancement of murine erythroid colony formation. In: Baum, S. J., Ledney, G. D., Thierfelder, S. (eds.): *Experimental Hematology Today — 1982*. S. Karger, Basel 1982, p. 53.

Haematologia 17 (2), pp. 169–178 (1984)

The Possible Role of Platelets in Bypassing the Contact Phase of Blood Coagulation

P. N. WALSH, G. P. TUSZYNSKI, JUDITH S. GREENGARD,* J. H. GRIFFIN*

Thrombosis Research Center, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140, and Scripps Research Institute,* La Jolla, CA., USA

(Received 5 January 1983; accepted 10 February 1983)

Data presented herein and previously support an active role for platelets in promoting the interaction and activation of the coagulation proteins of the contact phase of intrinsic coagulation. The platelet membrane, activated by ADP collagen or thrombin, can promote the proteolytic activation of factor XII to factor XIIa in the presence of kallikrein and high molecular weight kininogen. The zymogen factor XI associates with high molecular weight kininogen in plasma and becomes bound to a site on the membrane of thrombin or collagen activated platelets. Thereafter, platelet bound factor XI can be proteolytically activated to factor XIa either in the presence of factor XIIa or in the presence of kallikrein. These observations could explain the absence of bleeding complications in patients with factor XII deficiency. In addition, platelets contain a molecule which has a higher molecular weight than plasma factor XI and possibly consists of a tetramer of four identical subunits of 52 000 daltons each of which is functionally and immunologically similar to plasma factor XI. Since this molecule is present in the platelets of patients with severe plasma factor XI deficiency and no evidence of bleeding, we postulate that platelet factor XI can substitute for plasma factor XI in hemostasis and possibly account for the considerable variability in clinical severity observed in patients with factor XI deficiency.

Keywords: platelet membrane, coagulation proteins, contact phase

Introduction

A thoughtful consideration of the mechanism by which blood coagulation reactions are triggered and promoted in vivo requires that one eye be focused on the enzymatic reactions which occur between purified coagulation proteins while the other eye is focused on the patients with deficiencies of coagulation proteins and the resultant clinical consequences.

Thus, it is impossible to ignore the fact that patients with severe factor VIII, factor IX or factor X deficiency often have profound bleeding complications whereas those with deficiencies of factor XII, prekallikrein and high molecular weight kininogen almost never bleed abnormally. Variable bleeding complications, sometimes almost nonexistent and at other times moderately severe, are observed in patients with factor VII deficiency and factor XI deficiency. These clinical observations raise a number of interesting questions about the interrelationships between the extrinsic and the intrinsic mechanisms of blood coagulation and about

the functions of various coagulation proteins, particularly the contact factors which seem so important in reactions carried out in vitro and so unimportant in vivo.

Several hypotheses have been advanced in an attempt to answer some of these questions. One proposal [1, 2] focuses on the extrinsic coagulation pathway. It has been suggested that factor IX can be activated directly by the tissue factor–factor VII complex, thus bringing factor IX rather than factor X into the central role for convergence of the intrinsic and extrinsic coagulation systems. However, this proposal does not explain the moderately severe bleeding complications seen in some patients with factor XI deficiency [3]. Furthermore, the kinetic constants for the activation of factor IX by the tissue factor–factor VII complex make it unlikely that this is a preferred reaction pathway in vivo compared with factor-X activation by factor VII–tissue factor [2]. Alternatively, we have been interested for a number of years in the part that platelets might play in triggering intrinsic coagulation reactions by sequestering the coagulation proteins of the contact phase and providing an activating surface as well as in bypassing some of these reactions thus obviating the need for the plasma system of contact activation.

Specifically the hypothesis under examination is that platelets have an essential role in the initiation of intrinsic coagulation and may participate in zymogen activation mechanisms that bypass the plasma system of contact activation [4]. This hypothesis arose from previous observations [5, 6] suggesting that platelets are involved in the interactions and activations of factor XII and factor XI. In this paper we present some recent data on the interactions between isolated platelets and purified coagulation proteins which tend to support this suggestion.

Materials and Methods

Preparation of Washed Platelets

Blood was collected and platelets washed by albumin density gradient centrifugation and then gel filtered as previously described [7, 8]. Alternatively platelet concentrates were prepared by the method of Mustard et al. [9] for studies of factor XI antigen in platelets.

Purified Proteins

Factor XI and factor XII were purified and radiolabeled with ^{125}I as previously described [7]. High molecular weight (HMW) kininogen and kallikrein were prepared by published methods as described previously [7]. All proteins were more than 95% pure as judged by sodium dodecyl sulfate (SDS) gel electrophoresis and retained 100% of their coagulant activity after radiolabeling.

Gel Electrophoresis

Proteins or incubation mixtures were subjected to gel electrophoresis in SDS according to the procedures of either Weber and Osborn [10] or Laemmli [11] as described earlier [7, 8].

Immunoprecipitation

Studies of immunoprecipitation of purified human plasma factor XI or of platelet extracts were carried out using antibody to purified human plasma factor XI prepared and characterized as previously described [8].

Reagents Used in Experiments

All reagents were obtained reported previously [7, 8] and were the highest grade available.

Results

Proteolytic Cleavage of Factor XII

Since the conversion of factor XII from zymogen to active serine protease is accompanied by fragmentation of the 80 000 molecular weight molecule to heavy and light chains of 50 000 and 30 000 molecular weight respectively [12], attempts were initially made to determine whether the limited proteolytic cleavage of factor XII by kallikrein was enhanced in the presence of platelets. Washed collagen-treated platelets were incubated with high molecular weight kininogen, ^{125}I-labeled factor XII and kallikrein and the extent of cleavage of factor XII was assessed by SDS polyacrylamide gel electrophoresis. Shown in Figure 1 is a gel profile from one of ten similar experiments in which between 14% and 32% cleavage of radiolabeled factor XII occurred in the presence of collagen-treated platelets, high molecular weight kininogen and kallikrein compared with 3–7% cleavage of the molecule in similar incubation mixtures in which platelets were omitted. Control experiments with platelets absent and celite present as an activating surface demonstrated 57–72% cleavage of ^{125}I-labeled factor XII under similar experimental conditions (data not shown).

Proteolytic Cleavage of Factor XI

The conversion of the zymogen factor XI to an active serine protease involves the limited proteolytic cleavage of the native molecule which has a molecular weight of 160 000 without reduction and 80 000 in the presence of reducing agents to yield two polypeptide chains of molecular weights 48 000 and 32 000

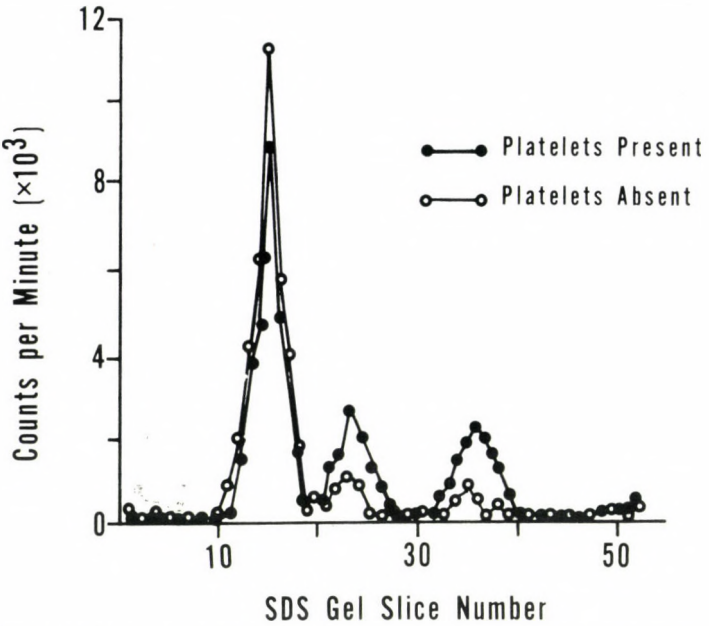


Fig. 1. Reduced SDS polyacrylamide gel electrophoresis of ^{125}I-labeled factor XII (0.2 μg) in mixtures of HMW kininogen (0.54 μg) and kallikrein (0.5 μg) in the presence (●——●) and absence (○——○) of washed platelets (5.31×10^8/ml) treated with collagen (250 μg/ml). See text for details of methods. Figure reprinted with permission from Ref. [7]

that are held together by disulfide bonds [13]. To determine whether the platelet-dependent proteolytic cleavage of factor XII by kallikrein shown in the previous experiment was associated with the appearance of enzymatic activity we incubated washed platelets or Tyrode's solution with collagen, ADP or buffer and then with purified factor XII, high molecular weight kininogen, kallikrein and radiolabeled factor XI. The radioactive gel profiles of ^{125}I-labeled factor XI on reduced SDS gels from a representative experiment are shown in Figure 2. When platelets were absent and kallikrein, factor XII, high molecular weight kininogen and radiolabeled factor XI were incubated in the presence of celite, factor XI was cleaved into heavy and light polypeptide chains as expected (Fig. 2, Panel B). When celite and platelets were absent and the purified proteins were incubated with collagen, ADP or buffer, only trace cleavage of factor XI was observed (Fig. 2, Panels A, C and D, open symbols). In contrast, when platelets were present (Fig. 2, Panels A, C and D, closed symbols), the amount of factor XI cleaved in the collagen sample (Panel A) was similar to that observed in the celite sample with platelets absent (Panel B). Enhanced platelet-dependent cleavage of radiolabeled factor XI was also observed in ADP-treated (Panel C) and buffer-treated (Panel D) samples but to a lesser extent than that in the collagen-treated sample.

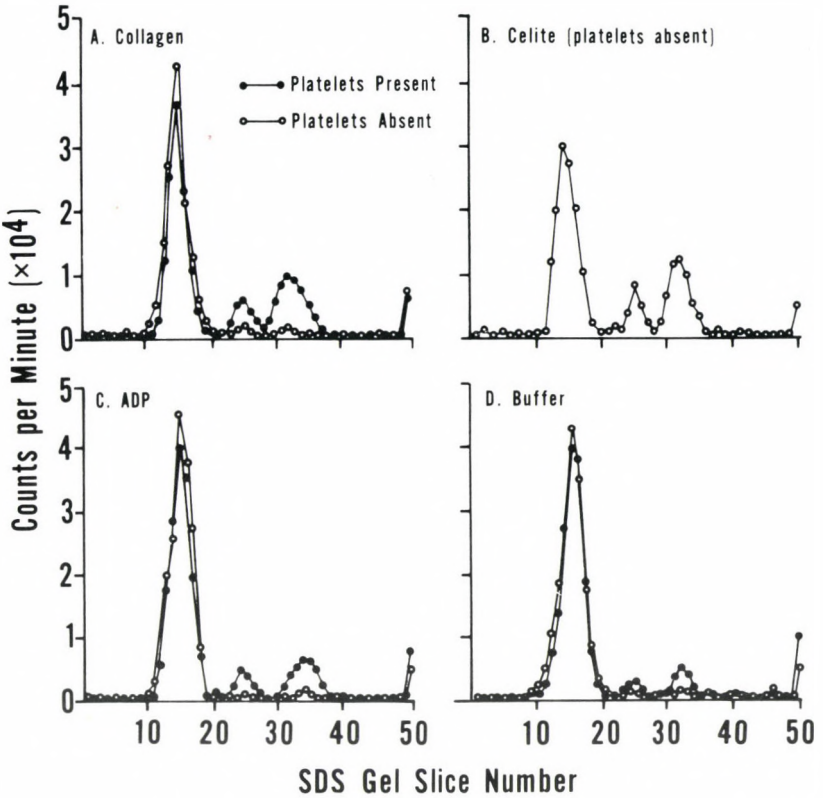


Fig. 2. Reduced SDS polyacrylamide gel electrophoresis of ^{125}I-labeled factor XI (0.15 μg) in mixtures of factor XII (0.36 μg), HMW kininogen (0.54 μg), and kallikrein (0.5 μg), in the presence (●——●) and absence (○——○) of platelets (5.58×10^8/ml) or in the presence of celite (1.57 μg/ml). See text for details. Figure reprinted with permission from Ref. [7]

Requirements for Proteolytic Activation of Factor XI

A series of experiments was carried out to determine the extent to which platelet-dependent proteolytic cleavage of factor XI was dependent upon the presence of high molecular weight kininogen, factor XII, factor XIIa and kallikrein. In the experiment shown in Table 1, the proteolytic cleavage of radiolabeled factor XI was assessed in the presence of collagen-treated platelets, collagen alone, or celite as a positive control. When high molecular weight kininogen, factor XII and kallikrein were all present, 41 ng of factor XI were cleaved in the presence of collagen-treated platelets compared with 3 ng in the presence of collagen alone and 68 ng in the presence of celite (Table 1, line 1). When factor XII was left out of the incubation mixture (Table 1, line 3), virtually no cleavage of factor XI was observed either in the presence of collagen or celite with platelets absent since

Table 1

Proteolytic cleavage of factor XI effects of HMW kininogen, factor XII and kallikrein

Incubation mixture*			Amount (ng) F XI cleaved		
HMW kininogen (0.54 μg)	F XII (0.36 μg)	Kallikrein (0.5 μg)	Platelets + collagen	Tyrode's + collagen	Tyrode's + celite
+	+	+	41	3	68
−	+	+	27	2	37
+	−	+	22	<1	<1
+	+	−	<1	<1	<1

* Each incubation mixture contained a mixture of cold and ^{125}I-labeled factor XI (0.15 μg). The concentration of platelets was 6.77×10^8/ml, collagen 300 μg/ml, and celite 1.57 mg/ml. Incubation time was 120 min.

kallikrein does not activate factor XI. In contrast, when collagen-treated platelets were present 22 ng of factor XI were cleaved, thus confirming our previous suggestion [6] that collagen-treated platelets can promote the activation of factor XI in the absence of added factor XII.

Immunoprecipitation of Platelet Extracts with Anti-Factor XI Antibody

Previous observations made in our laboratory indicated that platelets contain a factor XI-like activity that remains closely associated with platelets after many washes and is highly concentrated in the plasma membrane fraction of platelet lysates centrifuged on sucrose gradients [14, 15]. To determine the nature of the intrinsic factor XI activity of platelets we prepared a monospecific antibody to highly purified plasma factor XI. Either purified factor XI or washed platelets were labeled with ^{125}I and after the platelets suspensions were solubilized with Triton X-100 in the presence of 1 mM DFP, either the labeled platelets or purified factor XI was subjected to immunoprecipitation using Staph A and antifactor XI antibody. The immunoprecipitates were analyzed on SDS gel followed by autoradiography. When the immunoprecipitates were run on a 10% SDS gel containing 5% beta-mercaptoethanol the results shown in Figure 3 were obtained. Lane 1 is an immunoprecipitate of the platelet extract using normal rabbit serum rather than antifactor XI antibody. Lane 2 is a total platelet extract. Lane 3 is an immunoprecipitate of the platelet extract using antifactor XI antibody. Lane 4 is an immunoprecipitate of purified radiolabeled factor XI. Lane 5 is an immunoprecipitate of a platelet extract using antialbumin antibody rather than antifactor XI antibody. Thus, it would appear that whereas purified factor XI is immunoprecipitated with an expected molecular weight of 80 000, the material immunoprecipitated from platelets migrated on reduced gels with an apparent molecular weight of 52 000–55 000.

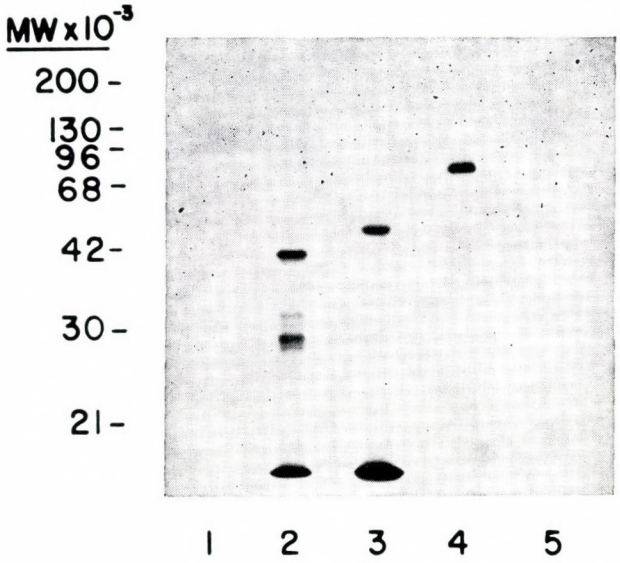


Fig. 3. Autoradiogram of 10% reduced SDS polyacrylamide gel electrophoresis of Staph A immunoprecipitates of radiolabeled platelet extracts and purified plasma factor XI. See text for methods and identification of lanes. Figure reprinted with permission from Ref. [8]

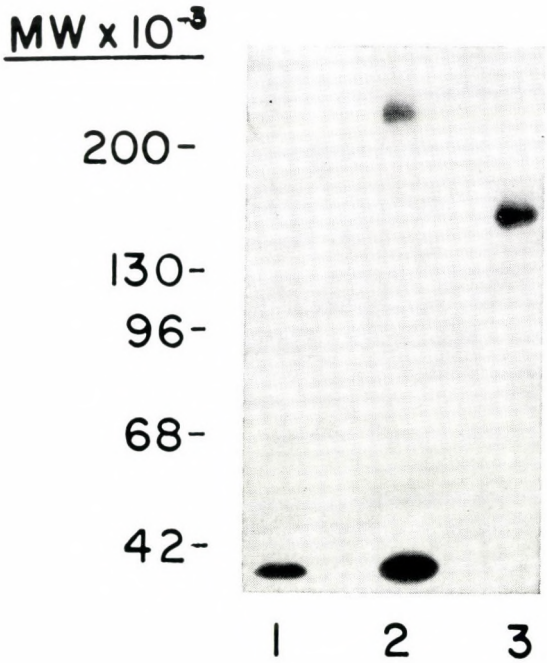


Fig. 4. Autoradiogram of 6% nonreduced SDS polyacrylamide gel electrophoresis of Staph A immunoprecipitates of radiolabeled platelet extracts and purified plasma factor XI. See text for methods and identification of lanes. Figure reprinted with permission from Ref. [8]

On nonreduced 6% SDS gels (Fig. 4) preimmune serum gave no immunoprecipitate from the platelet extract (lane 1) whereas antifactor XI antibody immunoprecipitated material from platelets with a molecular weight of approximately 220 000 (lane 2) compared with the immunoprecipitate of purified plasma factor XI (lane 3) which migrated with molecular weight of 160 000 as expected. Similar results were obtained with platelets from patients with severe plasma factor XI deficiency and no clinical evidence of bleeding.

Discussion

We have presented evidence herein and elsewhere [7] that platelets treated with ADP or collagen can promote the proteolytic activation of purified factor XII in the presence of high molecular weight kininogen and kallikrein as shown firstly by the proteolytic cleavage of factor XII, secondly by the development of factor XIIa coagulant activity and thirdly by the resultant proteolytic cleavage of added factor XI. Furthermore, platelets treated with collagen or thrombin can participate with high molecular weight kininogen and kallikrein to promote the proteolytic activation of factor XI by mechanisms both dependent upon and independent of factor XII as shown firstly by proteolytic cleavage studies of factor XI and secondly by the development of factor XIa coagulant activity. Additional data [7] indicate that the zymogen activations observed are platelet related as shown by the enhanced proteolysis and coagulant activation observed after platelet stimulation, secondly by the inhibition of proteolytic activation by inhibition of platelet responses with indomethacin, thirdly by the absence of proteolytic activation in the absence of platelets or other surfaces and fourthly by the localization of factor XI cleavage products in platelet pellets.

To determine whether a specific interaction of factor XI and platelets may underlie the functional events described above resulting in a platelet-dependent proteolytic activation of factor XI, formal binding studies of the platelet-factor XI interaction were carried out [16]. We concluded from these studies that factor XI binds specifically and tightly to activated platelets. Secondly, factor XI binding to platelets requires the presence of high molecular weight kininogen. Thirdly, factor XI binding is initially reversible and becomes irreversible with time. Fourthly, we have estimated binding constants from saturation experiments and have found a dissociation constant of approximately 1–2 nM with 500–1200 binding sites per platelet. These data are consistent with the sites on activated platelets being saturated at the normal plasma factor XI concentration which is approximately 25 nM. Finally, we have concluded that this factor XI binding may underlie the platelet role in the proteolytic activation of factor XI in the presence of either factor XIIa or kallikrein.

In addition to these studies on the interaction of platelets with plasma proteins active in the contact phase of intrinsic coagulation the studies presented above and those published previously [8] indicate that platelets contain a mole-

cule which is functionally and immunologically similar to plasma factor XI but different in molecular weight, ionic charge and subunit structure. Platelets appear to contain a molecule which has a higher molecular weight and possibly consists of a tetramer of four identical subunits of 52 000 daltons each of which is functionally and immunologically similar to plasma factor XI. Since this molecule is present in the platelets of patients with severe plasma factor XI deficiency and no evidence of bleeding, we postulate that platelet factor XI can substitute for plasma factor XI in hemostasis and possibly account for the considerable variability in clinical severity observed in patients with factor XI deficiency. Validation of this hypothesis will require the demonstration that patients with plasma factor XI deficiency and bleeding complications have less platelet factor XI antigen than hemostatically normal patients deficient in plasma factor XI only.

In conclusion, we believe that platelets can participate in contact activation reactions by sequestering contact phase proteins and promoting the conversion of coagulation zymogens to enzymes. In addition, platelets may also promote coagulation reactions that bypass the contact phase of plasma coagulation thereby possibly explaining the lack of hemostatic defects in certain patients with congenital deficiencies of factor XII, high molecular weight kininogen, prekallikrein and in some instances, factor XI. Further studies will be required to investigate the existence and nature of these postulated alternative mechanisms for initiating intrinsic coagulation.

*

This work was supported in part by DHEW grants HL-14217, HL25661, HL-16411, HL28149 and by a grant from the Council for Tobacco Research (No. 1389). The authors are grateful to Ms. Patricia Pileggi for excellent secretarial assistance.

References

1. Østerud, B., Rapaport, S. I.: Activation of factor IX by the reaction product of tissue factor and factor VII: Additional pathway for initiating blood coagulation. *Proc. Nat. Acad. Sci USA. 74*, 5260 (1977).
2. Zur, M., Nemerson Y.: Kinetics of factor IX activation via the extrinsic pathway. Dependence of Km on tissue factor *J. Biol. Chem. 255*. 5703 (1980).
3. Stern, D. M., Nossel, H. L., Owen, J.: Acquired antibody to factor XI in a patient with congenital factor XI deficiency. *J. Clin. Invest. 69*, 1270 (1982).
4. Walsh, P. N.: Platelet coagulant activities and hemostasis: An hypothesis. *Blood 43*, 597 (1974).
5. Walsh, P. N.: The role of platelets in the contact phase of blood coagulation. *Brit. J. Haemat. 22*, 237 (1972).
6. Walsh, P. N.: The effects of collagen and kaolin on the intrinsic coagulant activity of platelets. Evidence for an alternative pathway in intrinsic coagulation not requiring factor XII. *Brit. J. Haemat. 22*, 393 (1972).
7. Walsh, P. N., Griffin, J. H.: Contributions of platelets to the proteolytic activation of blood coagulation factors XII and XI. *Blood 57*, 106 (1981).

8. Tuszynski, G. P., Bevacqua, S. J., Schmaier, A. H., Colman, R. W., Walsh, P. N.: Factor XI antigen and activity in human platelets. *Blood 59*, 1148 (1982).
9. Mustard, J. F., Perry, D. W., Ardlie, N. G., Packham, M. A.: Preparation of suspensions of washed platelets from humans. *Brit. J. Haemat. 22*, 193 (1972).
10. Weber, K., Osborn, M.: The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem. 244*, 4406 (1969).
11. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* (*Lond.*) *227*, 680 (1970).
12. Griffin, J. H.: The role of surface in the surface-dependent activation of Hageman factor (blood coagulation factor XII). *Proc. Nat. Acad. Sci. USA. 75*, 1998 (1978).
13. Bouma, B. N., Griffin, J. H.: Human blood coagulation factor XI. Purification, properties, and mechanism of activation by activated factor XII. *J. Biol. Chem. 252*, 6432 (1977).
14. Walsh, P. N.: Albumin density gradient separation and washing of platelets and the study of platelet coagulant activities. *Brit. J. Haemat. 22*, 205 (1972).
15. Lipscomb M. S., Walsh, P. N.: Human platelets and factor XI. Localization in platelet membranes of factor-XI-like activity and its functional distinction from plasma factor XI. *J. Clin. Invest. 63*, 1006 (1979).
16. Greengard, J. S., Walsh, P. N., Griffin, J. H.: Binding of factor XI to human platelets. *Blood. 58*, 194a (1981).

Haematologia 17 (2), pp. 179–186 (1984)

Post-Translational Modifications of Hemoglobin

H. F. BUNN

Howard Hughes Medical Institute Laboratory, Hematology Division, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA

(Received 5 April 1983; accepted 11 April 1983)

Post-translational modifications of hemoglobin can provide special insights into metabolic disorders. A variety of small molecules in health and disease can form covalent adducts with hemoglobin. The most abundant and best understood of these non-enzymatic modifications is the glycosylation of hemoglobin at the N-terminus of the β chain (Hb A_{Ic}) as well as at the N-terminus of the α chain and at certain lysine residues. Glycosylated hemoglobins are elevated in diabetics and offer a useful way of monitoring diabetic control. Moreover, non-enzymatic glycosylation of other tissues may contribute significantly to the long term complications of diabetes. Additional minor hemoglobin components can be formed from other small reactive compounds. For example, cyanate, a breakdown product of urea, reacts with hemoglobin to form distinct minor components in red cells of uremic patients. Acetaldehyde can form hemoglobin adducts in red cells of alcoholics. Thus, hemoglobin can be viewed as a "reporter molecule", revealing metabolic perturbations in a variety of diseases.

Keywords: glycosylated hemoglobin, hemoglobin, minor hemoglobin components, post-translational modifications, reporter molecule

*

Normal human red cells contain one major hemoglobin component, HbA ($\alpha_2\beta_2$), and two minor components, Hb A_2 ($\alpha_2\delta_2$) and Hb F ($\alpha_2\gamma_2$), proteins which are encoded by four different globin genes. Additional minor components arise because of post-translational modifications, the most prevalent being non-enzymatic glycosylation. The most abundant minor component in human red blood cells is Hb A_{Ic} which normally comprises about 3% of the total but is elevated 2–3 fold in the red cells of patients with diabetes. The red cell also contains smaller amounts of more negatively charged hemoglobins which have arisen by means of other types of post-translational modifications.

Glycosylated Hemoglobins

Structure – Chromatography of human hemolysate on BioRex 70, a cation exchange resin, reveals 4 negatively charged minor components, which are designated Hbs A_{Ia1}, A_{Ia2}, A_{Ib}, and A_{Ic}, in order of their elution [1, 2]. Their electrophoretic separation is shown schematically in Figure 1. The only component which has undergone a complete structural analysis is Hb A_{Ic} [3–5]. There is rather

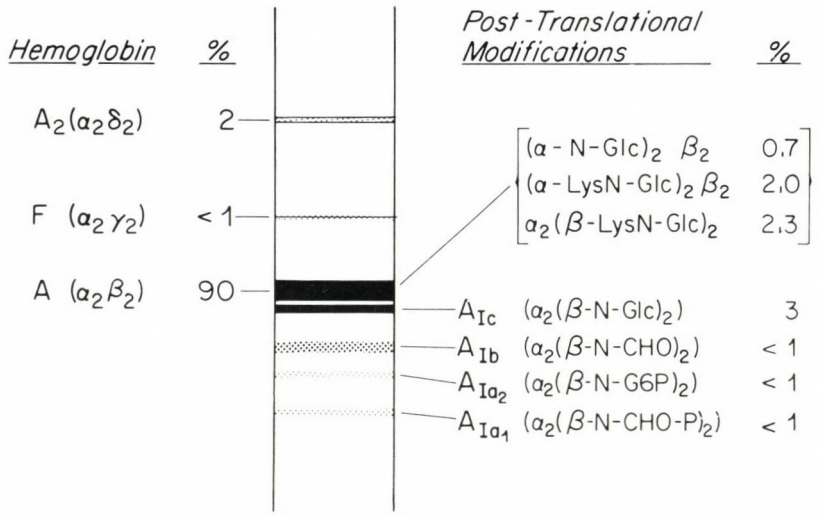
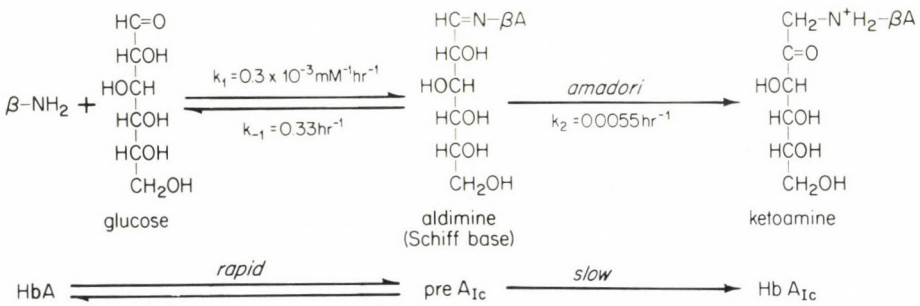


Fig. 1. Analysis of hemoglobin components in a normal human hemolysate by gel-electrofocusing

convincing evidence that this hemoglobin is identical to Hb A except that glucose is attached to the N-terminus of the β chain by a Schiff base which undergoes an Amadori rearrangement to a more stable ketoamine linkage.



Hbs A_{Ia1}, A_{Ia2}, and A_{Ib} are also modifications at the N-terminus of the β chain [6]. Hb A_{Ic2} may be formed by the reaction of hemoglobin with glucose-6-phosphate.

In addition to its binding at the β-N-terminus, glucose can also form ketoamine linkages with the N-terminus of the α chain and the ε amino of certain lysine residues [7]. These other adducts differ from Hb A_{Ic} in that they cannot be separated from the non-glycosylated major component (Hb A) by ordinary chromatographic or electrophoretic techniques. However, these components are increased in diabetics in parallel with Hb A_{Ic}.

Biosynthesis – According to the reaction sequence shown above, Hb A_{Ic} is formed by the simple bimolecular condensation of the aldehyde function of glucose with the N-terminal amino group of the β chain. The formation of glycosylated minor components was precisely analyzed in vivo by injecting a human volunteer with ^{59}Fe-transferrin which labels a cohort of developing erythrocytes within the bone marrow [8]. Measurement of the specific radioactivity of these minor hemoglobin components relative to that of the major component, Hb A_0, showed that Hbs A_{Ia}, A_{Ib}, and A_{Ic} were all formed slowly, continuously and nearly irreversibly during the 120-day life span of the red cells (Figure 2). Therefore, the level of the glycosylated hemoglobins is greater in old red cells than in young ones [9]. Furthermore, Hb A_{Ic} is reduced in patients with hemolysis [8]. The slow rate of formation of these hemoglobins suggests a non-enzymatic process. If so, it should be possible to synthesize Hb A_{Ic} by prolonged incubation of Hb A_0 with glucose under solvent conditions which mimic those of the erythrocyte. In fact, hemoglobin which has chromatographic and functional properties identical to those of Hb A_{Ic} can be synthesized in vitro by the incubation of glucose and hemoglobin [10].

A number of factors influence the formation of ketoamine adducts on proteins exposed to sugars. These include the concentration of the sugar, the permea-

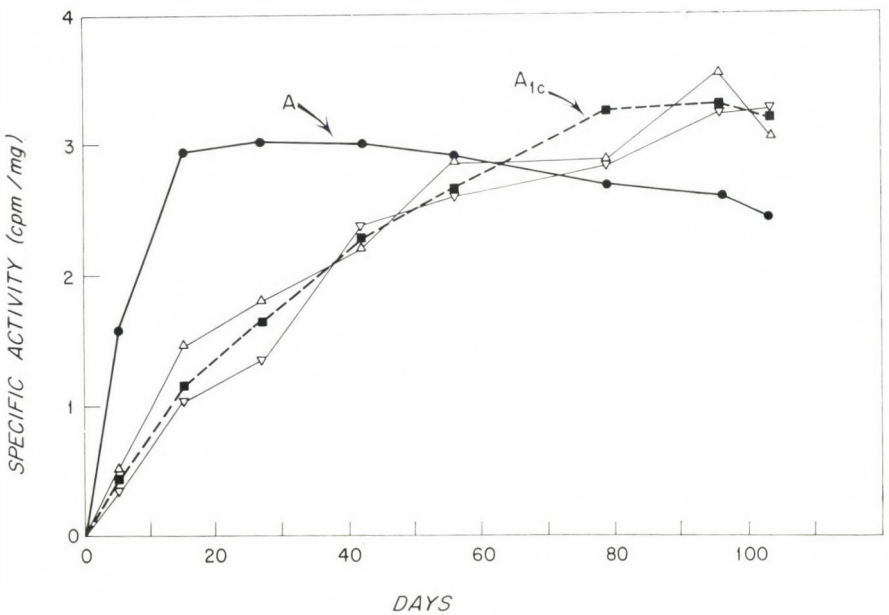


Figure 2. Biosynthesis of minor hemoglobin components in vivo. A normal volunteer was given an intravenous injection of ^{59}Fe-transferrin. At intervals during the next 100 days, blood samples were analyzed by chromatography on BioRex 70 cation exchange resin. The specific activities of the negatively charged components (Hbs A_{Ia}, A_{Ib} and A_{Ic}) increased slowly and continuously over the period of observation

bility of the cell to the sugar [12], the equilibrium between the sugar's open and ring forms [13], the turnover time of the protein, the pKa of its reactive amino groups, and the rate of conversion of the initial aldimine linkage to the ketoamine [11].

Function — Several laboratories have completed a detailed analysis of the functional properties of the glycosylated hemoglobins [14–16]. Hbs A_{Ia1} and A_{Ia2}, presumed to have sugar phosphates linked to the β-N-terminus, have much lower oxygen affinity than Hb A and are unresponsive to the addition of organic phosphate. Hb A_{Ic} has similar oxygen affinity to Hb A and decreased, but not absent response to added organic phosphate.

Although the glycosylated minor hemoglobin components have abnormal functional properties, it is very unlikely that they have a significant influence on oxygen transport in vivo. Even in diabetics, who often have a 2–3 fold increase in Hb A_{Ic}, the whole blood oxygen dissociation curve is only slightly displaced to the left [17]. Despite claims to the contrary [18], there is no evidence that such a small increase in oxygen affinity causes any impairment in oxygen unloading.

Other Post-Translational Modifications

Although glucose adducts are by far the most common and abundant type of post-translation modification of hemoglobin, other small molecules are also capable of forming covalent linkages with hemoglobin and may reflect significant metabolic perturbations.

Carbamylated Hemoglobin

Urea is normally present in the plasma in a concentration of about 2.5 mM. However patients with renal failure may have plasma levels as high as 40 mM. Urea is in equilibrium with ammonium and cyanate ions:

$$H_2N\underset{\underset{O}{\|}}{C}NH_2 \rightleftharpoons NH_4^+ + CNO^-$$

Cyanate can combine with amino groups on proteins to form irreversible covalent adducts:

$$HbNH_2 + CNO^- + H^+ \longrightarrow HbNH\underset{\underset{O}{\|}}{C}NH_2$$

when hemoglobin is incubated with cyanate in vitro, the primary sites of modification are the N-terminal amino groups of the α and β chains [19]. Because carbamylation of hemoglobin S greatly reduces its tendency to polymerize, cyanate has been used in the treatment of sickle cell disease [20].

The presumed presence of increased levels of cyanate in plasma of uremic patients raises the possibility that their hemoglobin becomes carbamylated in vivo. Increased amounts of "Hb A_I" have been observed in uremics, despite normal levels of glycosylated hemoglobin [21, 22]. Amino acid analyses demonstrated that this additional negatively charged hemoglobin was carbamylated at the N-terminal amino of the globin chains [21]. Patients on hemodialysis had about 1–2% carbamylated hemoglobin while those who had not received dialysis had levels as high as 4%. This minor degree of modification would have no significant effect on hemoglobin function. However, other proteins may become carbamylated in uremia and perhaps contribute to the clinical manifestations of the uremic syndrome.

Acetaldehyde-Hemoglobin Adducts

When ethanol is metabolized in vivo it is oxidized to acetaldehyde. This highly reactive compound forms Schiff base linkages with amino groups on proteins:

$$RNH_2 + CH_3\overset{\overset{O}{\|}}{C}H \rightleftharpoons RN{=}CCH_3$$

When hemoglobin is incubated with acetaldehyde, negatively charged minor components are formed having the chromatographic behavior of Hbs A_{Ia+b} [23, 24]. Alcoholics have increased levels of negatively charged hemoglobin which does not appear to be glycosylated [23, 25]. It is likely that this hemoglobin component is comprised of acetaldehyde adducts but convincing structural analysis has not yet been performed. Since acetaldehyde-protein adducts are readily reversible, it is unlikely that this modification causes any long-term impairment of protein function.

Acetylated Hemoglobin

A wide variety of proteins are acetylated at the NH_2-terminus. The acetylation event is catalyzed by an acetyltransferase enzyme using acetyl-CoA as substrate [26] and occurs early in the life of a protein molecule, perhaps during growth of the nascent polypeptide chain [27]. The NH_2-terminal acetylation of proteins appears to depend in part on primary structure. Proteins having alanine or serine at the NH_2-terminus are commonly acetylated, while those having glycine are acetylated somewhat less readily. Proteins having other residues at their NH_2-termini generally escape acetylation.

Hemoglobins are acetylated much less readily than most other proteins. Among mammalian hemoglobins, only three are known to be acetylated: human and primate hemoglobin F_1 [28], hemoglobin Raleigh [29], and cat hemoglobin [30]. In each case the NH_2-terminal residue is one that favors acetylation. The

hemoglobin variant, Hb Raleigh, involves the substitution β1 Val → Ala. The NH_2-terminal residue of the β chain of cat hemoglobin B is serine. Acetylation of these hemoglobins appears to be complete: the NH_2-terminus of the β chain is totally blocked. In contrast, only about 10–15% of the γ chains of human Hb F are acetylated. The NH_2 terminal residue of the γ chain is glycine. Thus, the extent of acetylation of these hemoglobins is consistent with their NH_2-terminal residues. It is likely that during molecular evolution hemoglobins have been selected that have N-terminal sequences resistant to acetylation since these regions of the molecule are important sites for anion binding.

When hemoglobin or red cells are incubated with acetylsalicylic acid (aspirin), a significant amount of acetylated hemoglobin is formed [31, 32]. However, unlike Hb F_1, the acetyl groups are located at several lysine residues on the α and β chains [32, 33]. Patients who are on high doses of aspirin appear to have a slight increase in negatively charged hemoglobin [32] but this finding needs to be documented with structural analyses.

Hemoglobin as a Reporter Molecule

It is likely that under various circumstances other reactive compounds in the plasma can modify hemoglobin. For example, vitamins, drugs or toxins may form adducts with hemoglobin. One component of the most negatively charged hemoglobins in normal hemolysate (Hb A_{Ia}) may be an adduct with pyridoxal phosphate [34]. Penicillin can form covalent linkages with hemoglobin [35]. Some patients with lead poisoning have a negatively charged hemoglobin component [36] which may arise because some of the heme groups have been substituted by zinc protoporphyrin [37].

In conclusion, this concise review has been concerned with structural modifications of hemoglobin owing to the presence of variable amounts of reactive compounds which are capable of forming covalent adducts. These modified hemoglobins can be viewed as reporter molecules because they not only provide clues to the diagnosis of such diverse diseases as diabetes, uremia and alcoholism, but also the extent of the metabolic derangement. More importantly, these well-defined chemical modifications on the hemoglobin molecule may serve as prototypes of similar modifications on other proteins that contribute to the pathogenesis of disease.

References

1. Allen, D. W., Schroeder, W. A., Balog, J.: Observations on the chromatographic heterogeneity of normal adult and fetal human hemoglobin. *J. Amer. Chem. Soc. 80*, 1628 (1958).
2. McDonald, M. J. Shapiro, R., Bleichman, M., Solway, J., Bunn, H. E.: Glycosylated minor components of human adult hemoglobin. *J. Biol. Chem. 253*, 2327 (1978).

3. Bookchin, R. M., Gallop, P. M.: Structure of hemoglobin A_{Ic}, nature of the N-terminal chain blocking group. *Biochem. Biophys. Res. Commun. 32*, 86 (1968).
4. Bunn, H. E., Haney, D. N., Gabbay, K. H., Gallop, P. M.: Further identification of the nature and linkage of the carbohydrate in hemoglobin A_{Ic}. *Biochem. Biophys. Res. Commun. 67*, 103 (1975).
5. Koenig, R. J., Blobstein, S. H., Cerami, A.: The structure of carbohydrate of hemoglobin A_{Ic}. *J. Biol. Chem. 252*, 2992 (1977).
6. Garrick, L. M., McDonald, M. J., Shapiro, R., Bleichman, M., McManus, M. J., Bunn, H. F.: Structural analysis of the minor human hemoglobin components: Hb A_{Ia1}, Hb A_{Ia2}, and Hb A_{Ib}. *Eur. J. Biochem. 106*, 353 (1980).
7. Shapiro, R., McManus, M. J., Zalut, C., Bunn, H. F.: Sites of nonenzymatic glycosylation of human hemoglobin A. *J. Biol. Chem. 255*, 3120 (1980).
8. Bunn, H. F., Haney, D. N., Kamin, S., Gabbay, K. H., Gallop, P. M.: The biosynthesis of human hemoglobin *in vivo*. *J. Clin. Invest. 57*, 1652 (1976).
9. Fitzgibbons, J. F., Koler, R. D., Jones, R. T.: Red cell age related changes of hemoglobins A_{Ia+b} and A_{Ic} in normal and diabetic subjects. *J. Clin. Invest. 58*, 820 (1976).
10. Fluckiger, R., Winterhalter, K. H.: *In vitro* synthesis of hemoglobin A_{Ic}. *FEBS Lett. 71*, 356 (1978).
11. Higgins, P. J., Bunn, H. F.: Kinetic analyses of the non-enzymatic glycosylation of hemoglobin. *J. Biol. Chem. 256*, 5204 (1981).
12. Higgins, P. J., Garlick, R. I., Bunn, H. F.: Glycosylated hemoglobin in human and animal red cells: role of glucose permeability. *Diabetes 31*, 743 (1982).
13. Bunn, H. F., Higgins, P. J.: Reaction of monosaccharides with proteins: possible evolutionary significance. *Science 213*, 222 (1981).
14. Bunn, H. F., Briehl, R. W.: The interaction of 2,3-diphosphoglycerate with various human hemoglobins. *J. Clin. Invest. 49*, 1008 (1970).
15. McDonald, M. J., Bleichman, M., Bunn, H. F., Noble, R.: Functional properties of the glycosylated minor components of human adult hemoglobin. *J. Biol. Chem. 254*, 702 (1979).
16. Fluckiger, R., Winterhalter, K. H.: Glycosylated hemoglobins. In: Labie, D., Poyart, C. Rosa, J. (eds): *Molecular interactions of hemoglobin. INSERM 70*, 319 (1977).
17. Arturson, G., Garby, L., Robert, M., Zaar, B.: Oxygen affinity of whole blood *in vivo* and under standard conditions in subjects with diabetes mellitus. *Scand. J. Clin. Lab. Invest. 34*, 19 (1974).
18. Ditzel, J., Standl, E.: The problem of tissue oxygenation in diabetes mellitus. *Acta Med. Scand., Suppl. 578*. p. 59 (1975).
19. Kilmartin, J. V., Rossi-Bernardi, L.: Inhibition of CO_2 combination and reduction of the Bohr effect in haemoglobin chemically modified at its α-amino groups. *Nature (Lond.) 222*, 1243 (1969).
20. Cerami, A., Manning, J. M.: Potassium cyanate as an inhibitor of the sickling of erythrocytes *in vitro*. *Proc. Natl. Acad. Sci. USA 68*, 1180 (1971).
21. Fluckiger, R., Harmon, W., Meier, W., Loo, S., Gabbay, K. H.: Hemoglobin carbamylation in uremia. *N. Engl. J. Med. 304*, 823 (1981).
22. Oimomi, M., Ishikawa, K., Kawasaki, T., Kubota, S., Yoshimura, Y., Baba, S.: Glycosylated haemoglobin in renal failure. *Diabetologia 21*, 163 (1981).
23. Stevens, V. J., Fantl, W. J., Newman, C. B., Sims, R. V., Cerami, A., Peterson, C. M.: Acetaldehyde adducts with hemoglobins. *J. Clin. Invest. 67*, 361 (1981).
24. Tsuboi, K. K., Thompson, D. J., Rush, E. M., Schwartz, H. C.: Acetaldehyde-dependent changes in hemoglobin and oxygen affinity of human erythrocytes. *Hemoglobin 5*, 241 (1981).
25. Hoberman, H. D., Chiodo, S. M.: Elevation of the hemoglobin A_I fraction in alcoholism. *Alcoholism: Clin. and Exp. Res. 6*, 260 (1982).
26. Marchis-Mouren, G., Lippman, F.: On the mechanism of acetylation of fetal and chicken hemoglobins. *Proc. Natl. Acad. Sci. USA 63*, 1147 (1965).

27. Granger, M., Tesser, G. I., Dejong, W. W., Bloemendal, H.: Model studies of enzymatic NH_2-terminal acetylation of proteins with des-N^1-acetyl- alpha-melanotropin as a substrate. *Proc. Natl. Acad. Sci. USA 73*, 3010 (1976).
28. Schroeder, W. A., Cua, J. T., Matsuda, G., Fenninger, W. D.: Hemoglobin F_1, an acetyl-containing hemoglobin. *Biochim. Biophys. Acta 63*, 532 (1962).
29. Moo-Penn, W. F., Bechtel, K. C., Schmidt, R. M., Johnson, M. H., Jue, D. L., Schmidt, D. E., Dunlap, W. M., Opella, S. J., Bonaventura, J., Bonaventura, C.: Hemoglobin Raleigh (1 Valine—Acetylalanine) Structural and functional characterization. *Biochemistry 16*, 4872 (1977).
30. Taketa, F., Attermeir, M. H., Mauk, A. G.: Acetylated hemoglobins in feline blood. *J. Biol. Chem. 247*, 33 (1972).
31. Klotz, I. M., Tam, J. W. D.: Acetylation of sickle cell hemoglobin by aspirin. *Proc. Natl. Acad. Sci. USA 70*, 1313 (1973).
32. Bridges, K. R., Schmidt, G. J., Jensen, M., Cerami, A., Bunn, H. F.: The acetylation of hemoglobin by aspirin: *in vivo* and *in vitro*. *J. Clin. Invest. 56*, 201 (1975).
33. Shamsuddin, M., Mason, R. G., Ritchey, J. M., Honig, G. R., Klotz, I. M.: Sites of acetylation of sickle cell hemoglobin by aspirin. *Proc. Natl. Acad. Sci. USA 71*, 4693 (1974).
34. Srivastava, S. K., Vanloon, C., Beutler, E.: Characterization of a previously unidentified hemoglobin fraction. *Biochim. Biophys. Acta 278*, 617 (1972).
35. Winterhalter, K. H.: Personal communication.
36. Charache, S., Weatherall, D. J.: Fast hemoglobin in lead poisoning. *Blood 28*, 377 (1966).
37. Lamola, A. A., Yamane, T.: Zinc protoporphyrin in the erythrocytes of patients with lead poisoning and iron deficiency anemia. *Science 186*, 936 (1974).

Haematologia 17 (2), pp. 187–198 (1984)

The Role of Transferrin in Lymphocyte Transformation

J. H. BROCK

University Department of Bacteriology and Immunology, Western Infirmary, Glasgow, G11 6NT, Scotland

(Received 11 January 1983; accepted 9 July 1983)

The ability of mouse lymphocytes to transform in a serum-free medium in response to to Concanavalin A was found to depend upon the presence of transferrin. Iron chelates were ineffective, as also were monoferric transferrin fragments, but both homologous and heterologous transferrins promoted transformation. Optimal transformation occurred with 10–50 μg/ml of transferrin, and at 30–70 per cent iron saturation. Transferrin was bound by the transforming cells, and subsequently released without degradation following the delivery of iron. Iron uptake preceded DNA synthesis. Iron chelates, despite their inability to promote transformation, could also deliver iron to lymphocytes. It is postulated that transferrin may fulfil functions additional to that of supplying iron.

Keywords: concanavalin A, iron, lymphocytes, transferrin, transformation.

Introduction

The iron-transport protein transferrin plays a key role in iron metabolism, since it is responsible for the delivery of iron to the bone marrow for the synthesis of haemoglobin. The interaction of transferrin with erythroid precursors has been studied extensively, and it is now known that transferrin delivers its iron to the cell by a mechanism involving the binding of transferrin to a specific membrane receptor, removal of iron from the protein and the release of iron-free (apo)transferrin to the circulation [1]. Although the majority of transferrin-bound iron is normally destined for erythrocytes, non-erythroid cells also have a small but definite iron requirement for the synthesis of iron-containing enzymes. Recently it has been found that transferrin is an essential component in the culture of a variety of non-erythroid cells in serum-free media [2–6], and it seems likely that this requirement is related to the need to acquire iron. This need is likely to be greatest when cells divide as an inadequate iron supply might impair cell division. During immune responses, lymphocytes undergo transformation and division, and consequently the reported impairment of cell-mediated immune responses in patients with severe iron deficiency [7–10] could be related to a suboptimal iron supply to transforming T-lymphocytes.

This study was therefore carried out to investigate whether an adequate iron supply was essential for lymphocyte transformation, and to determine the role of transferrin.

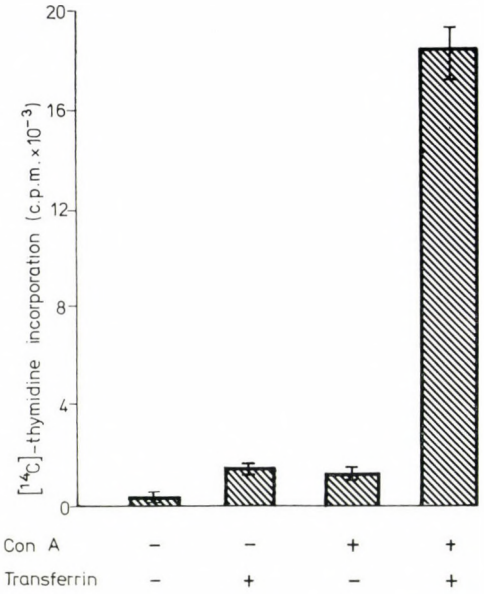


Fig. 1. ^{14}C-thymidine incorporation into mouse lymph node cells cultured for 72 h in serum-free medium in the presence or absence of Concanavalin A (1 μg/ml) and human transferrin (50 μg/ml; 30 per cent iron-saturated). Mean ± standard deviation (n = 3). Reproduced from [11] with permission

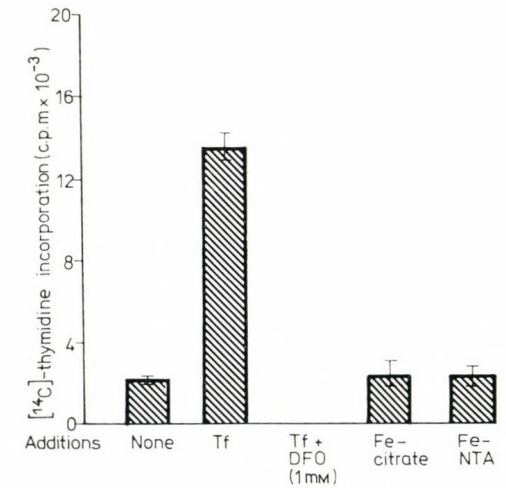


Fig. 2. ^{14}C-thymidine incorporation into mouse lymph node cells cultured for 72 h in serum-free medium in the presence of Concanavalin A (1 μg/ml) and various iron compounds. The iron concentration was 15 ng/ml in all cases, giving approximately 30 per cent saturation of transferrin (Tf). DFO = desferrioxamine (1 mM); NTA = nitrilotriacetate. Mean ± standard deviation (n = 3). Reproduced from [11] with permission

Experimental

Full details have been published elsewhere [11, 12] and will be summarized here. Mouse lymph-node cells were cultured in a serum-free medium consisting of RPMI 1640 (Flow Laboratories) supplemented with human serum albumin (1 mg/ml) and 2-mercaptoethanol (50 μM). Except where otherwise stated, human transferrin (Behringwerke) was added at a concentration of 50 μg/ml. The transferrin was supplied in the iron-free (apo) form and sufficient iron was therefore added (as the nitril otriacetate complex) to give 20 per cent saturation. In addition, the culture medium was found to contain 6 ng/ml of endogenous iron, which increased iron saturation of the added transferrin by a further 8 per cent. Transformation was induced by using Concanavalin A (1 μg/ml) as mitogen, and assessed by incorporation of ^{14}C-thymidine during a 4 h pulse after 3 days of incubation. Iron and transferrin uptake was assessed by using transferrin labelled with ^{59}Fe and ^{125}I, essentially as described by Esparza & Brock [13].

Results

Effect of iron and transferrin on lymphocyte transformation

In the absence of transferrin, lymphocytes transformed poorly in the serum-free medium, but addition of transferrin caused a marked improvement (Fig. 1). In the absence of mitogen, little transformation occurred (though even this low background level was improved by the presence of transferrin). Thus transferrin was not acting as a mitogen *per se*, but was required to permit expression of the response to Concanavalin A.

The above experiment did not determine whether transferrin itself, or its iron content, was the important factor. Lymphocytes were therefore incubated in media containing a similar quantity of iron added as citrate or nitrilotriacetate chelates (Fig. 2). Neither of these forms of iron produced any enhancement of transformation. Furthermore addition of 1 mM desferrioxamine, a microbial chelator with an affinity for iron greater than that of transferrin, abolished the enhancing effect of transferrin. Thus transferrin itself, as well as iron appeared to be necessary for transformation.

Uptake of iron

The above experiments were repeated using ^{59}Fe to label transferrin and the chelates, and iron uptake by the cells determined. Despite the inability of citrate and nitrilotriacetate chelates to enhance transformation, far more iron was taken up from these chelates than from transferrin (Table 1), while desferrioxamine-bound iron was not taken up. To eliminate the possibility that much of the chelate iron was bound extracellularly, cells were washed and resuspended at 0 °C in

Table 1

Uptake of iron from 30 per cent saturated transferrin and from low molecular weight chelates by mouse lymph node cells cultured in serum-free medium containing Concanavalin A (1 μg/ml)

Iron compound	Iron uptake (p mol/10^6 cells)	
	3 h	70 h
Transferrin	0.4	11.2
Nitrilotriacetate	4.3	46.1
Citrate	3.7	47.8
Ferrioxamine	0.5	0.9

The iron concentration was 0.015 μg/ml in each case. Ferrioxamine-iron uptake was performed in the presence of 1 mM desferrioxamine.

medium containing excess (1 mM) desferrioxamine, which should be capable of removing any extracellularly-bound iron. After 1 h it was found that desferrioxamine had removed only 14 per cent of the iron originally supplied by nitrilotriacetate and 17 per cent of that supplied by citrate. Furthermore, 11 per cent of

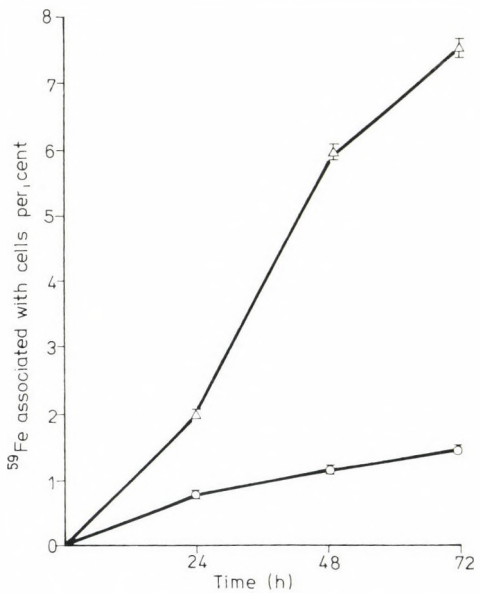


Fig. 3. Uptake of ^{59}Fe from 30 per cent saturated transferrin by mouse lymph node cells cultured in serum-free medium in the presence (△) or absence (○) of Concanavalin A (1 μg/ml) Vertical bars represent 2 standard deviations (n = 3). Reproduced from [12] with permission

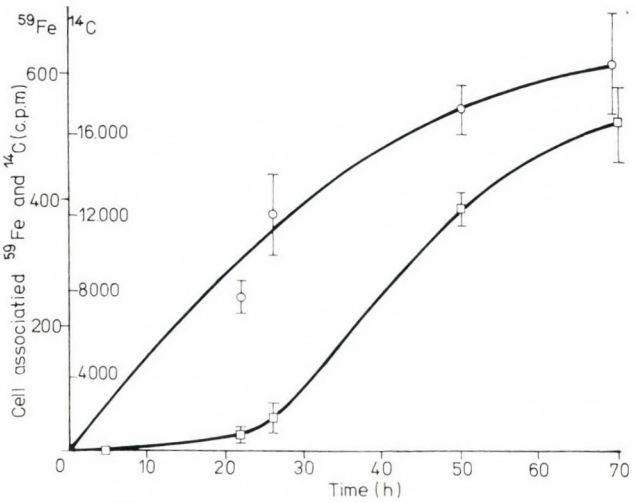


Fig. 4. Uptake of ^{59}Fe (○) and ^{14}C-thymidine (□) by mouse lymph node cells cultured in serum-free medium containing 50 μg/ml of 30 per cent saturated transferrin and 1 μg/ml Concanavalin A. The points on the ^{14}C-thymidine uptake curve represent the mid-point of 4 h pulse periods; ^{59}Fe-transferrin was present from zero time. Vertical bars represent 2 standard deviations (n = 3). Reproduced from [12] with permission

transferrin-supplied iron was also removed. Thus iron taken up from citrate or nitrilotriacetate chelates did not appear to be bound extracellularly to any appreciable extent, and it therefore seems unlikely that failure of the iron to enter the cell can explain the inability of these chelates to promote transformation. Uptake of iron from transferrin was greater in the presence of Concanavalin A than in controls without mitogen, indicating an increased need for iron during transformation (Fig. 3). Furthermore, iron uptake clearly preceded DNA synthesis (Fig. 4).

Specificity of the requirements for transferrin

To determine whether the use of a heterologous system (human transferrin with mouse lymphocytes) affected the response to transferrin, the ability of homologous (mouse) transferrin, and another heterologous protein, bovine transferrin, to enhance transformation was investigated. Both acted in a similar way as human transferrin (Fig. 5). However, monoferric half-molecules prepared by tryptic digestion of bovine transferrin [14] were ineffective in enhancing transformation despite the fact that they each still contained one intact iron-binding site. The specificity of the interaction is thus broad enough to permit interaction with heterologous transferrins, but does not permit cleavage of the molecule. These findings closely parallel earlier studies on the ability of transferrins and fragments to donate iron to reticulocytes [13, 15].

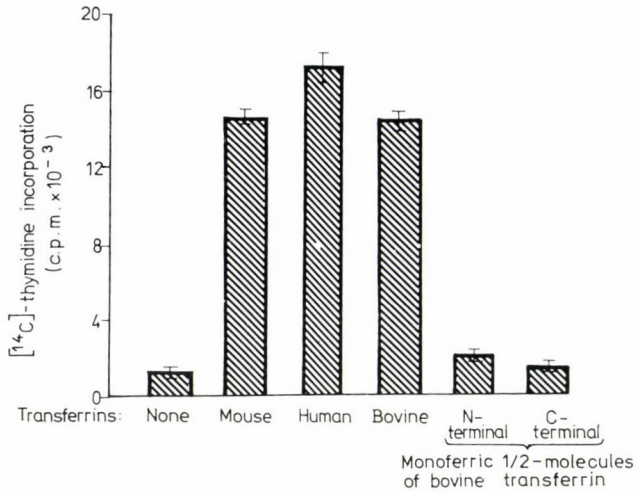


Fig. 5. ^{14}C-thymidine incorporation into mouse lymph node cells cultured for 72 h in serum-free medium in the presence of Concanavalin A (1 μg/ml) and transferrins from various species, and monoferric fragments of bovine transferrin. Transferrins and fragments were added at 50 μg/ml and 30 per cent iron saturation. Mean ± standard deviation (n = 3). Reproduced from [11] with permission

Effect of iron saturation and transferrin concentration

Incubation of mouse lymphocytes in media containing a constant amount of transferrin (50 μg/ml) but varying amounts of iron showed that maximum enhancement of lymphocyte transformation occurred when saturation was in the range of 30–70 per cent (Fig. 6). Due to the endogenous iron of the culture medium, it was not possible to test directly the effect of iron-free transferrin. To overcome this problem a further set of experiments was performed in which apotransferrin was added to culture medium containing varying amounts of desferrioxamine. Since desferrioxamine has a higher affinity for iron than transferrin, endogenous iron should be rendered unavailable to transferrin. It was found (Fig. 7) that once the desferrioxamine concentration exceeded that necessary to bind all endogenous iron, lymphocyte transformation was greatly reduced. It thus seems probable that apotransferrin has little or no ability to promote lymphocyte transformation.

When varying amounts of transferrin (20 per cent saturated) were added to culture media, it was found that optimal transformation occurred with 10–50 μg/ml, but even as little as 0.5 μg/ml markedly improved transformation over the level observed in the absence of transferrin (Fig. 8).

Transferrin binding and release by lymphocytes

When lymphocytes were incubated with ^{125}I, ^{59}Fe-labelled transferrin, ^{59}Fe was steadily accumulated by the cells as before, but binding of transferrin reached a maximum at 20 h and thereafter did not change appreciably (Fig. 9). The cells

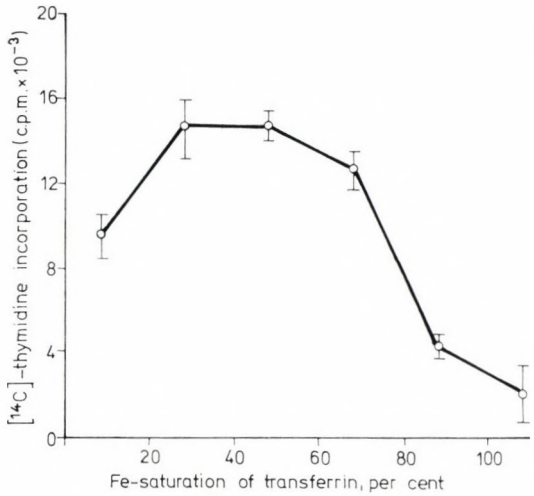


Fig. 6. ^{14}C-thymidine incorporation into mouse lymph node cells cultured for 72 h in serum-free medium in the presence of Concanavalin A (1 μg/ml) and 50 μg/ml transferrin, of varying iron saturation. Mean ± standard deviation (n = 3). Reproduced from [11] with permission

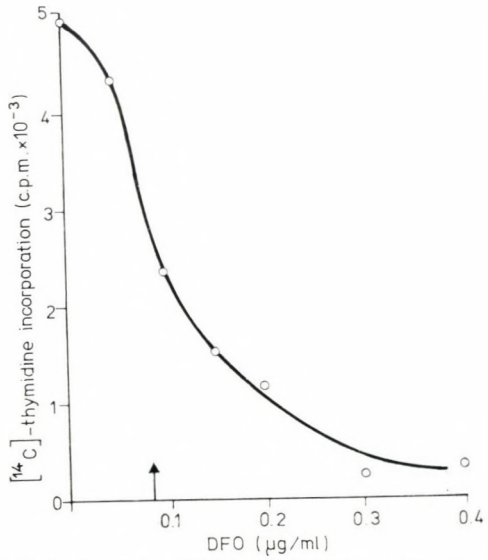


Fig. 7. The effect of desferrioxamine (DFO) on ^{14}C-thymidine incorporation into mouse lymph node cells cultured for 72 h in serum-free medium in the presence of Concanavalin A (1 μg/ml) and apotransferrin (50 μg/ml). The arrow represents the minimum amount of DFO required to bind the endogenous iron (6 ng/ml) present in the culture medium. Reproduced from [11] with permission

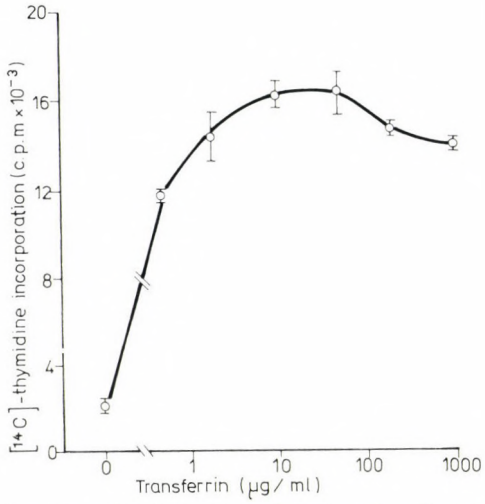


Fig. 8. ^{14}C-thymidine incorporation into mouse lymph node cells cultured for 72 h in serum-free medium in the presence of Concanavalin A (1 μg/ml) and various concentrations of transferrin (30 per cent iron saturated). Mean ± standard deviation (n = 3). Reproduced from [11] with permission

were therefore able to remove iron from transferrin, which was returned to the medium. To determine whether transferrin was degraded during the process of iron release, transformed cells were incubated for 4 h with doubly-labelled transferrin, then washed and 'chased' with unlabelled transferrin for 1 h. The cells and

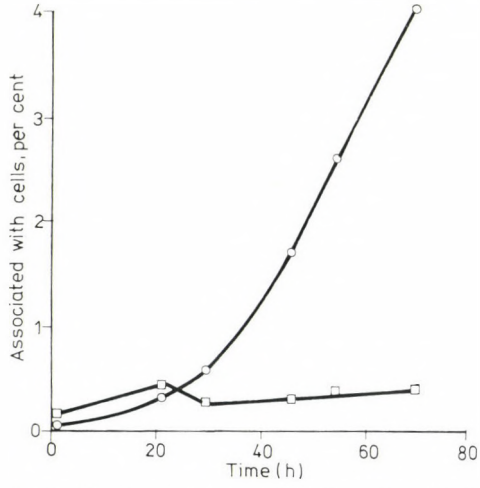


Fig. 9. Iron and transferrin binding by mouse lymph node cells incubated in serum-free medium in the presence of Concanavalin A (1 μg/ml) and ^{59}Fe, ^{125}I-transferrin (30 per cent iron saturated). (○) ^{59}Fe; (□) ^{125}I

Table 2

Distribution of ^{59}Fe and ^{125}I in cell pellet and supernatant fraction after incubation of mouse lymphoblasts with ^{59}Fe, ^{125}I-transferrin followed by a 'chase' of unlabelled transferrin

	Percentage	
	^{125}I	^{59}Fe
Cell pellet	47	95
Supernatant, TCA-insoluble fraction	44	1
Supernatant, TCA-soluble fraction	9	4

supernatant were separated by centrifugation, and the latter treated with an equal volume of 20 per cent (w/v) trichloroacetic acid, followed by a further centrifugation. It can be seen (Table 2) that 95 per cent of the ^{59}Fe, but less than half the ^{125}I activity was retained by the cells during the chase, and that most of the released ^{125}I was precipitated by trichloroacetic acid. This indicates that most of the transferrin which had delivered iron to the cells was subsequently released undegraded.

In a further experiment binding of ^{125}I-transferrin to transformed and untransformed lymphocytes was compared. The transformed cells (77 per cent blasts) bound 0.12 μg of transferrin per 10^6 cells, whereas untransformed small lymphocytes bound only 0.02 $\mu g/10^6$ cells.

Discussion

It has been shown in earlier studies [16, 17] that the transformation of human lymphocytes in serum-free medium in response to phytohaemagglutinin is enhanced by transferrin. The work reported here shows that transformation of mouse lymphocytes in response to Concanavalin A in serum-free medium is also transferrin-dependent. Both phytohaemagglutinin and Concanavalin A are T cell mitogens, but it has also been found that transferrin enhances transformation in response to the B-cell mitogen lipopolysaccharide [11].

The present study gives some information on the role of transferrin in lymphocyte transformation. The failure of low molecular weight iron chelates to enhance transformation despite their ability to provide the cells with iron indicates that iron alone cannot enhance transformation. Similar conclusions were recently drawn by Trowbridge and Lopez [18], who showed that blockage of the transferrin receptor on a tumour cell line by an anti-receptor monoclonal antibody depressed cell division even when extra iron was added to the culture medium. Conversely, the failure of transferrin to promote transformation when endogenous iron was rendered unavailable by desferrioxamine indicates that iron-free transferrin is also ineffective at enhancing lymphocyte transformation. Phillips and Azari [16] reported similar findings, though it is not clear how they avoided the problem of

endogenous iron in the culture medium, as this is not mentioned. On the other hand Dillner-Centerlind et al. [17] reported no difference in the enhancing activity of iron-free transferrin and 90 per cent saturated transferrin. These observations do not in fact contradict the present results which showed that neither apotransferrin nor 90 per cent saturated transferrin gave optimal transformation, which occurred at 30–70 per cent saturation. This observation was rather unexpected, but has been reproduced numerous times in this laboratory. Assuming that the fundamental role of transferrin in lymphocyte transformation is to supply iron one would expect optimal transformation to occur when transferrin is fully saturated, since studies with erythroid precursors have clearly shown that iron is more rapidly taken up from diferric than from monoferric transferrin [19]. The relative inefficiency of highly-saturated transferrin could be explained in several ways. One possibility, suggested by Iscove and Melchers [3] is that transferrin can sequester other heavy metal cations which are present in sufficient amounts to be toxic to the cells. This clearly cannot be the only mode of action of transferrin, since the monoferric fragments should then also enhance transformation, which was not the case. Increasing the iron saturation would, however, leave less binding capacity available for other cations (all of which are bound less avidly than iron, which they cannot therefore displace), so that the enhancing effect of iron-transferrin would be diminished by the presence of unbound toxic cations. A second possibility concerns the reports that lymphocyte transformation also requires transferrin-bound zinc [20], which would also be prejudiced at high iron saturations for similar reasons. Chesters & Will [21] have, however, questioned the ability of transferrin to bind zinc in serum, since it is bound with greater affinity by albumin. A further alternative explanation is that lymphocytes preferentially recognise transferrin containing only one iron atom. If true, this would suggest that lymphocytes possess a receptor distinct from that on erythroid precursors. Receptors on erythroid and non-erythroid cells appear, however, to be identical in molecular weight and immunological properties [22, 23]. Finally, it is possible that incorporation of iron from diferric transferrin occurs at a rate which exceeds the cell's ability to handle the metal, resulting in toxic effects. Support for this suggestion comes from the results obtained with different amounts of transferrin (Fig. 8); transformation in the presence of only 2 μg/ml of transferrin was only slightly less than with 50 μg/ml, yet the endogenous iron in the culture medium would be more than sufficient to convert all the former amount of transferrin into the diferric species. In this case, excessive iron uptake would be avoided due to the lower transferrin concentration. These data would tend to argue against a role for monoferric transferrin, or for chelation of toxic cations. Furthermore, lymphocytes, unlike other cells, do not synthesize ferritin in response to iron loading [24] and may therefore be less able to handle excess iron. Further work is currently in progress to examine these various possibilities.

Acquisition of tranferrin-bound iron occurred to a greater extent in transformed than in non-transformed cells, and preceded DNA synthesis. This may be related to the fact that the enzyme ribonucleotide reductase, which is involved in

DNA synthesis, requires iron [25]. Other studies have shown that iron-deprivation will arrest transformation in the G1 phase [26]. The greater uptake of iron by transforming cells could be related to a greater degree of transferrin binding, and several other workers have now shown that the number of transferrin receptors on lymphocytes increases greatly when transformation occurs [27, 28].

So far it has been assumed that the function of transferrin in lymphocyte transformation is to supply iron, without which transformation cannot proceed. Still, lymphocytes possess intracellular stores of ferritin-iron [29] from which, in the absence of transferrin, they should be able to mobilize iron. Furthermore, the failure of iron chelates to promote transformation despite their demonstrable ability to act as iron donors is also difficult to reconcile with the concept that the only function of transferrin is to supply iron. It therefore seems possible that transferrin may fulfil additional functions, for example, the binding of transferrin to its receptor may in itself provide a signal to the cell subsequent to that which is known to be given when mitogen or antigen is bound. This possibility is currently being investigated.

References

1. Morgan, E. H.: Transferrin; biochemistry, physiology and clinical significance. *Molec. Aspects Med. 4*, 3 (1981).
2. Rudland, P. S., Durbin, H., Clingan, D., Jimenez de Asuda, L.: Iron salts and transferrin are specifically required for cell division of cultured 3T6 cells. *Biochim. Biophys. Res. Comm. 75*, 556 (1977).
3. Iscove, N. N., Melchers, F.: Complete replacement of serum by albumin, transferrin and soybean lipid in cultures of lipopolysaccharide-reactive B-lymphocytes. *J. Exp. Med. 147*, 923 (1978).
4. Barnes, D., Sato, G.: Growth of a human mammary tumor cell line in a serum-free medium. *Nature 281*, 388 (1979).
5. Breitman, T. R., Collins, S. J., Keene, B. R.: Replacement of serum by insulin and transferrin supports growth and differentiation of human promyelocytic cell line HL-60. *Exp. Cell Res. 126*, 494 (1980).
6. Simms, E., Gadzar, A. F., Abrams, P. G., Minna, J. D.: Growth of human small cell (oat cell) carcinoma of the lung in serum-free growth factor-supplemented medium. *Cancer Res. 40*, 4356 (1980).
7. Joynson, D. H. M., Jacobs, A., Walker, D. M., Dolby, A. E.: Defect in cell mediated immunity in patients with iron-deficiency anaemia. *Lancet 2*, 1058 (1972).
8. Sawitsky, B., Kanter, R., Sawitsky, A.: Lymphocyte response to phytomitogens in iron deficiency. *Amer. J. Med. Sci. 272*, 153 (1976).
9. Srikantia, S. G., Prasad, J. S., Bhaskaram, C., Krishnamachari, K. A. V. R.: Anaemia and immune response. *Lancet 1*, 1307 (1976).
10. Kuvibidila, S. R., Baliga, B. S., Suskind, R. M.: Effects of iron deficiency anaemia on delayed cutaneous hypersensitivity in mice. *Amer. J. Clin. Nutr. 34*, 2635 (1981).
11. Brock, J. H.: The effect of iron and transferrin on the response of serum-free cultures of mouse lymphocytes to Concanavalin A and lipopolysaccharide. *Immunology 43*, 387 (1981).
12. Brock, J. H., Rankin, M. C.: Transferrin binding and iron uptake by mouse lymph-node cells during transformation in reponse to Concanavalin A. *Immunology 43*, 393 (1981).

13. Esparza, I., Brock, J. H.: The interaction of bovine transferrin and monoferric transferrin fragments with rabbit reticulocytes. *Biochim. Biophys. Acta 624*, 479 (1980).
14. Brock, J. H., Arzabe, F. R.: Cleavage of diferric bovine transferrin into two monoferric fragments. *FEBS Lett. 69*, 63 (1976).
15. Esparza, I., Brock, J. H.: The effect of trypsin digestion on the structure and iron-donating properties of transferrins from several species. *Biochim. Biophys. Acta 622*, 297 (1980).
16. Phillips, J. L., Azari, P. R.: Effect of iron transferrin on nucleic acid synthesis in phytohaemagglutinin-stimulated human lymphocytes. *Cell. Immunol. 15*, 94 (1975).
17. Dillner-Centerlind, M. L., Hammerström, S., Perlmann, P.: Transferrin can replace serum for *in vitro* growth of mitogen-stimulated T-lymphocytes. *Europ. J. Immunol. 9*, 942 (1979).
18. Trowbridge, I. S., Lopez, F.: Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth *in vitro*. *Proc. Natl. Acad. Sci. USA. 79*, 1175 (1982).
19. Huebers, H., Csiba, E., Josephson, B., Huebers, E., Finch, C.: Interaction of human diferric transferrin with reticulocytes. *Proc. Natl. Acad. Sci. USA 78*, 621 (1981).
20. Phillips, J. L.: Uptake of transferrin-bound zinc by human lymphocytes. *Cell. Immunol. 35*, 318 (1978).
21. Chesters, J. K., Will, M.: Zinc transport proteins in plasma. *Brit. J. Nutr. 46*, 111 (1981).
22. Enns, C. A., Shindelman, J. E., Tonik, S. E., Sussman, H. H.: Radioimmunochemical measurement of the transferrin receptor in human trophoblast and reticulocyte membranes with a specific anti-receptor antibody. *Proc. Natl. Acad. Sci. USA. 78*, 4222 (1981).
23. Frazier, J. L., Caskey, J. H., Yoffe, M., Seligman, P. A.: Studies of the transferrin receptor on both human reticulocytes and nucleated human cells in culture. Comparison of factors regulating receptor density. *J. clin. Invest. 69*, 853 (1982).
24. Lema, M. J., Sarcione, E. J.: A comparison of iron induction of rat lymphocyte ferritin synthesis *in vivo* and *in vitro*. *Comp. Biochem. Physiol. 69B*, 287 (1981).
25. Hoffbrand, A. V., Ganeshaguru, K., Hooton, J. W. L., Tattersall, M. H. N.: Effect of iron deficiency and desferrioxamine on DNA synthesis in human cells. *Brit. J. Haemat., 33*, 517 (1976).
26. Tormey, D. C., Mueller, G. C.: Biological effects of transferrin on human lymphocytes *in vitro*. *Exp. Cell Res. 74*, 220 (1972).
27. Larrick, J. W., Cresswell, P.: Modulation of cell surface iron transferrin receptors by cellular density and state of activation. *J. Supramolec. Struct. 11*, 579 (1979).
28. Galbraith, G. M. P., Goust, J. M., Mercurio, S. M., Galbraith, R. M.: Transferrin binding by mitogen-activated human peripheral blood lymphocytes. *Clin. Immunol. Immunopath. 16*, 387 (1980).
29. Summers, M., White, G., Jacobs, A.: Ferritin synthesis in lymphocytes, polymorphs and monocytes. *Brit. J. Haemat. 30*, 425 (1975).

Haematologia 17 (2), pp. 199–207 (1984

In Vitro Study of Ticlopidine, as a Model of Antisickling Action of Platelet Antiaggregant

M. SABLAYROLLES,[1] H. WAJCMAN,[1] J. P. CASTAIGNE,[2] D. LABIE[1]

Institut de Pathologie et Biologie Cellulairs et Moléculairs, INSERM U 15, 24, rue du Fg. St. Jacques, 75014 Paris[1] and Sanofi-Recherche, Paris[2], France

(Received 4 January 1983; accepted 15 February 1983)

The in vitro antisickling effect of ticlopidine may be explained by the action of the drug on the membrane. The drug molecule increases slightly the volume of the cell, decreases the mean intra-erythrocytic hemoglobin concentration and therefore affects the delay time for hemoglobin S polymerization.

Keywords: antisickling, drug, hemoglobin S, membrane, ticlopidine

*

At present, no specific efficient drug can be recommended for the treatment of sickle-cell disease. Nevertheless there is a permanent pressure for research in this field for at least two reasons, viz. (1) sickle-cell disease is a severe problem in many countries and (ii) the molecular pathology of the disease being well understood, any drug which could hinder the involved mechanism may be a step for further pharmacological studies.

In theory, an efficient therapy can act at any of the various levels of the pathway leading to sickling, the final goal of the drug being the inhibition of the gelation

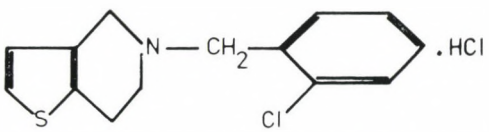


Fig. 1. Structural formula of ticlopidine, 5-(2 chlorobenzyl)-4,5,6,7 tetrahydrothieno (3,2,-c) pyridine hydrochloride

of hemoglobin S. This can be achieved either by inhibiting the mechanism of polymerization or by acting on one of the factors enhancing the polymerization.

Clinical trials performed in Ivory Coast have suggested some beneficial effects of ticlopidine (Fig. 1) in sickle-cell disease [1]. The drug is currently used as platelet antiaggregant. In this work we bring evidence for an in vitro effect on the red cell, and suggest a possible cellular mechanism.

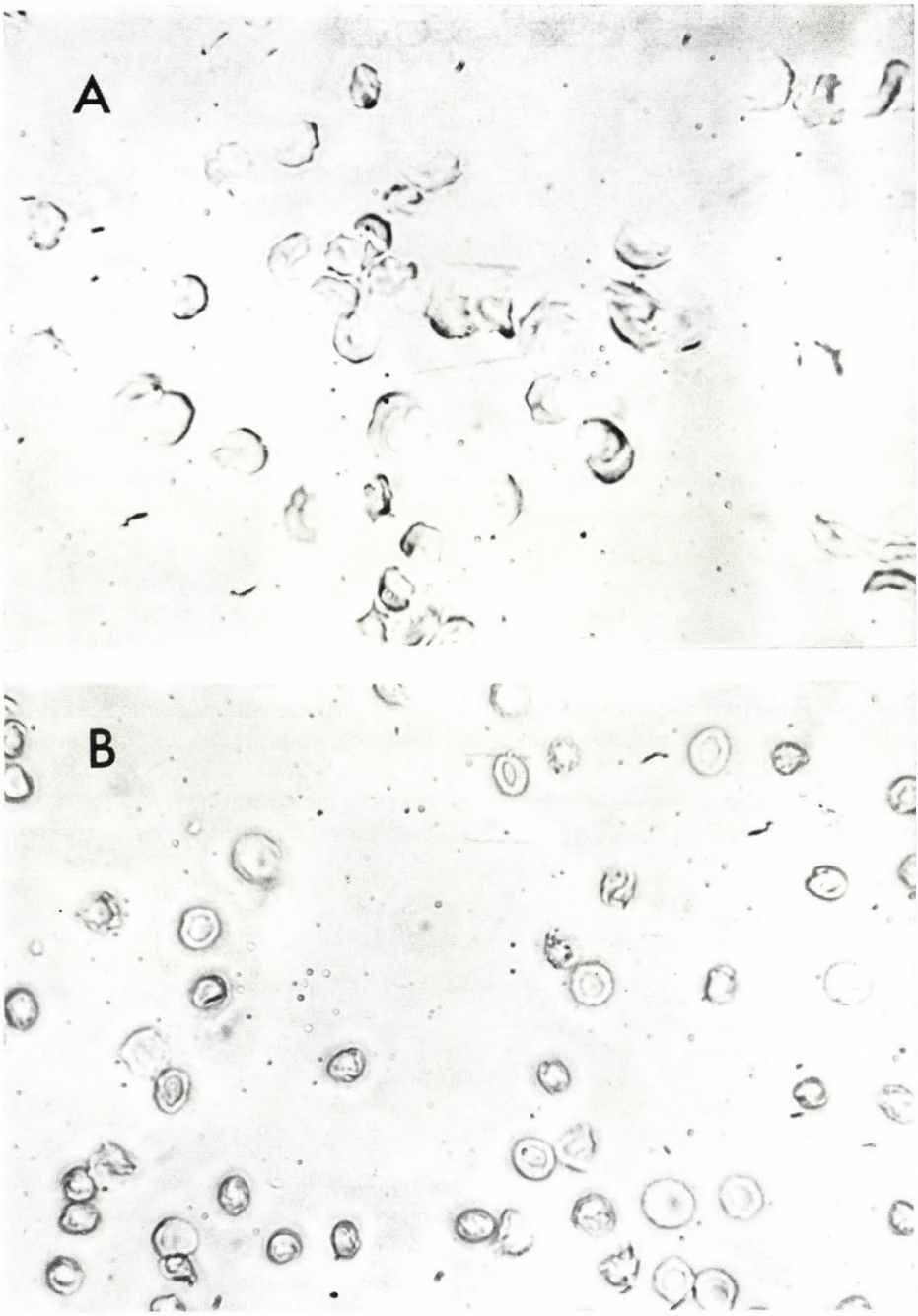


Fig. 2. Morphological changes of red blood cells from an A/S subject observed upon deoxygenation in presence of dithionite: A. untreated cells; B. erythrocytes preincubated with 1000 μM ticlopidine in phosphate buffer at 25 °C

Results and Discussion

In vitro effect of ticlopidine on the morphology of red blood cells

As shown in Figure 2, when cells containing hemoglobin S and suspended in phosphate buffer are preincubated with ticlopidine, much less highly deformed cells are observed upon deoxygenation in the treated than in the untreated sample. Using a drug concentration of 1000 μM, most of the cells are only spiculated, the number of cells of holly leaf shape being strongly reduced. Nevertheless, at this high concentration of the drug, which is much above the 0.1 μM reached in vivo, some other morphological alterations are also observed: hyperrefringent cells characteristic of reversible stomatocytes.

When the cells are preincubated at room temperature, the in vitro antisickling effect is clear at 1000 μM drug concentration. At the same temperature the effect, if any, is minimal at lower concentration (100 μM). In contrast, when the cells are preincubated at higher temperature (37 °C), a significant reduction of the number of highly deformed sickled cells is obtained even with the 100 μM concentration.

Binding of ticlopidine to the membrane of red blood cells

Using ^{14}C or ^{3}H labelled ticlopidine we have studied the binding of the drug to red blood cells.

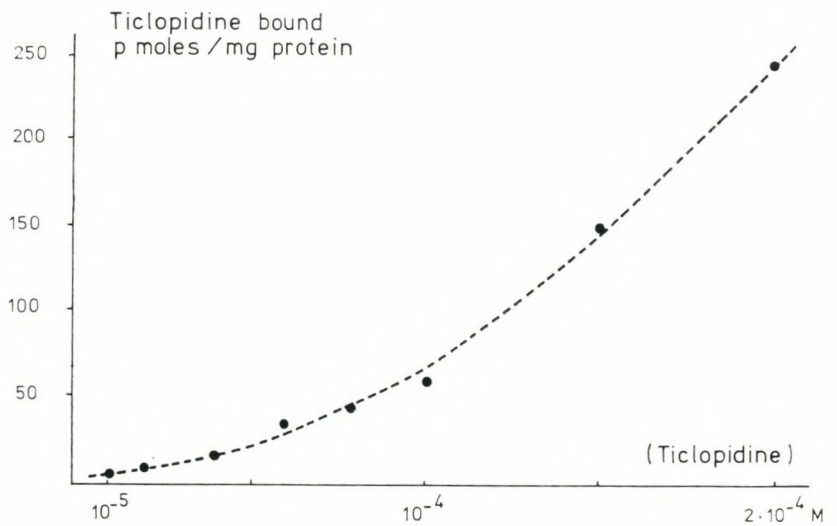


Fig. 3. Binding of ticlopidine to red blood cell ghosts. The membranes were incubated for 60 min at 37 °C in phosphate buffer with different amounts of ^{14}C labelled ticlopidine

When red blood cells were incubated in the presence of 1000 μM drug concentration for 1 h we found that ticlopidine was tightly bound to the membrane and that the quantity entering into the cell was negligible (0.1 μM).

The binding of ticlopidine to the ghosts was assayed with drug concentrations ranging from 0.01 μM to 1000 μM; the amount of ticlopidine bound to the membrane increased regularly without showing a plateau (Fig. 3). This binding may therefore be considered unsaturable in this range of concentration. In addition, the binding could not be reversed in buffered saline solutions, but partially in plasma.

The total amount of drug bound to the membrane, as well as the kinetics of the binding, increased with temperature, explaining the effect on cell morphology obtained with the 100 μM drug concentration at 37 °C.

Since the binding properties of erythrocyte ghosts were not modified after boiling, it is unlikely that specific protein receptors should be involved.

All these findings suggest that ticlopidine intercalates into the lipid bilayer of the membrane. The drug may therefore be classified into the group of antisickling agents acting through membrane modifications.

Changes in the properties of the red blood cells membrane

A large and heterogeneous series of drugs has been described as acting on the membrane and presenting some antisickling effect. Among those which could have a clinical interest, one of the first proposed was zinc [2] and more recently cetiedil [3]. Cetiedil is an analgesic with vasodilator properties and, as well as ticlopidine, has platelet anti-aggregant properties and is said to decrease blood viscosity.

It has been demonstrated by Brewer et al. [4] that zinc and cetiedil may act by membrane expansion, a calmodulin dependent mechanism being involved in this process. It has also been recognized that the antisickling effect of cetiedil is related to cations and water movements in the erythrocytes [5, 6].

We found that the action of ticlopidine, too, may be explained by membrane expansion.

The drug-induced alterations of the red blood cell membranes were first investigated by electron-spin resonance: the use of 16 nitroxide stearic acid to probe the hydrophobic core of the bilayer demonstrated a fluidizing effect [7]. This result was obtained even at a concentration of 0.1 μM which is 3 to 4 magnitudes below that leading to visible morphological changes.

Since membrane expansion increases the surface/volume ratio of the cell and results in a partial protection against lysis, the osmotic fragility test may be used as another approach to study the action of this family of drugs. When ticlopidine was added to the hypotonic solutions, the osmotic resistance of normal cells and of cells containing hemoglobin S were modified in a similar way: a protection against lysis was observed in the range of concentration from 5 to 500 μM with a maximum effect between 50 and 100 μM; in contrast, at concentrations above 500 μM the drug became hemolytic. Almost identical modifications of osmotic resistance were obtained when cetiedil was added instead of ticlopidine (Fig. 4).

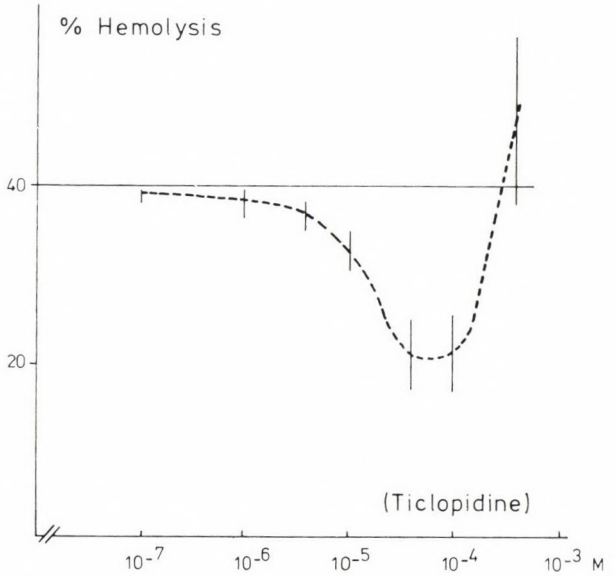


Fig. 4. Osmotic fragility test: the protection against lysis is shown by the dose–response curve obtained at 140 mOsm ionic strength which, with untreated normal cells, results in 40% lysis

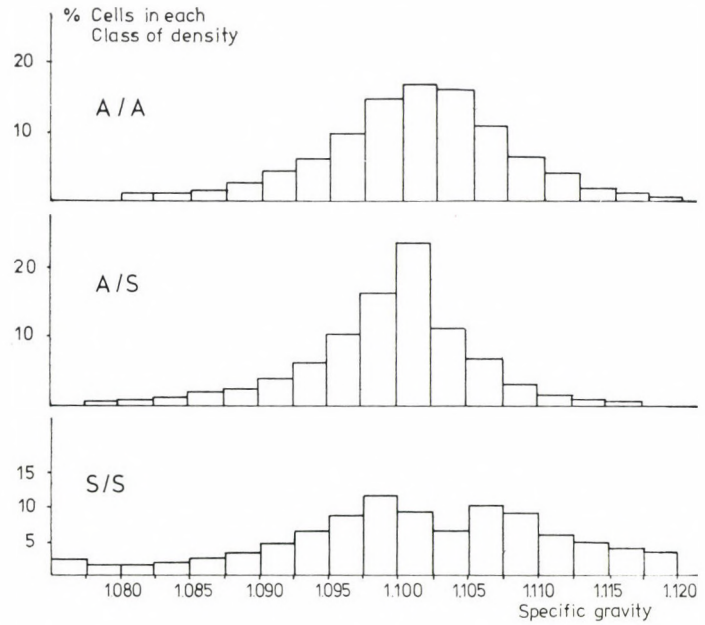


Fig. 5. Distribution of density of the red blood cell population. The histograms were derived from density profiles obtained using phthalate ester mixtures as specific gravity markers

The observed modifications of the physico-chemical properties of the membrane induced by ticlopidine or cetiedil, could be explained at least partially by the hypothesis that these hydrophobic molecules will disrupt the interactions of cholesterol, which stabilize the packing of the hydrocarbon chains of the phospholipids. Due to these structural rearrangements, the permeability to cations will change, resulting in cell swelling.

Modification of the size of red blood cells

Cell swelling can be appreciated in an indirect but accurate method by studying the density of the cells. The density profiles of the erythrocyte populations were determined in micro-hematocrit tubes using phthalate ester mixtures as specific gravity markers [8]. A Gaussian distribution of density was observed for normal or A/S cells with a maximum around a specific gravity of 1.100 g/ml. In contrast, the density of erythrocytes from sicklers reflected, as previously described in the literature [9], an heterogenous population spreaded over a broader range (Fig. 5).

Treatment of the cells by ticlopidine as well as by cetiedil resulted in a shift of the whole distribution towards the lower densities. All the cells including the younger ones at the top of the gradient and the denser ones at the bottom, showed a decrease of their specific gravity of about 0.002 to 0.004 g/ml. Such a change means that the volume of the cells was increased by 3 to 5 per cent and corresponded to a 1 to 2 g/dl difference in intracorpuscular hemoglobin concentration. This shift of density was independent of the ionic strength of the solution in which the

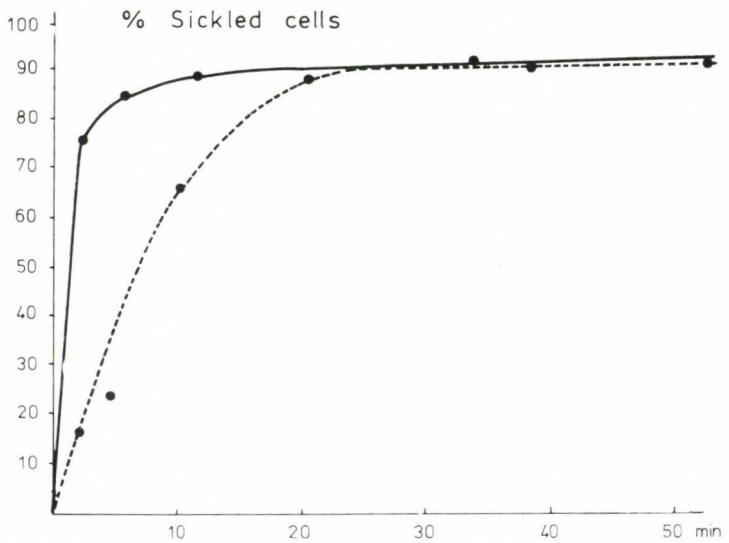


Fig. 6. Kinetic sickling of A/S cells

cells are suspended, its extent depended only on the drug concentration. Such a result may involve that a given drug concentration is responsible for a definite change in cation permeability and water gain.

Effect on hemoglobin S polymerization

Since the delay time for gelling is inversely proportional to the 30th power of the hemoglobin S concentration [10], any increase of the cell volume is expected to delay sickling.

In the first series of experiments the kinetics of sickling were measured by counting the number of sickled cells appearing after deoxygenation in the presence of dithionite. In such an experiment involving untreated A/S cells, half of the population had sickled within 2 min. After treatment with 1000 μM ticlopidine the same proportion of sickled cells was observed only after 8 to 10 min (Fig. 6). With cells

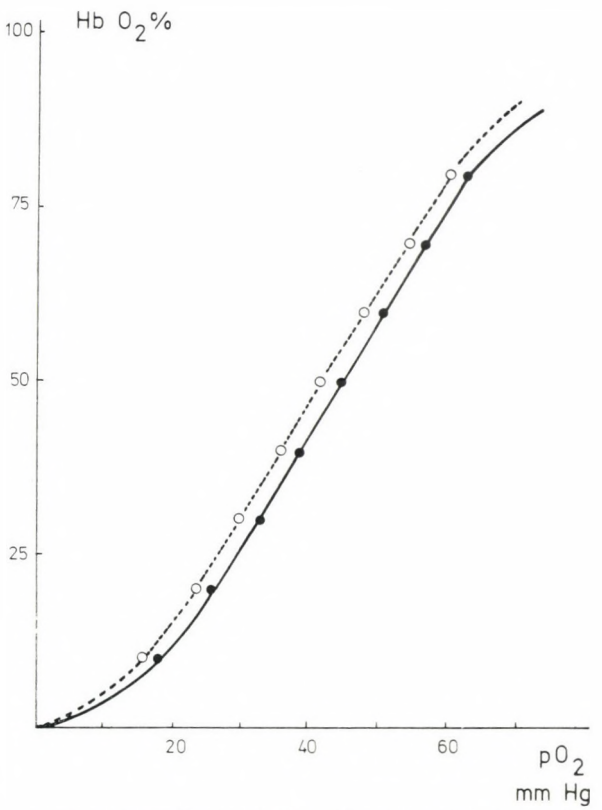


Fig. 7. Comparison of oxygen affinity of A/S cells, at 310 mOsm, in phosphate buffer without (●——●) and with 500 μM ticlopidine (○— —○)

containing only hemoglobin S, the kinetics were too rapid to distinguish any difference. In addition, other morphological changes resulting from secondary effects of the high concentration of the drug used in these experiments often hampered the accurate estimation of the true anti-sickling effect.

We found more convenient to study the inhibition of hemoglobin S polymerization by measuring the oxygen affinity of cells suspended in buffers of various ionic strengths. Since the volume of the cell is strongly dependent of the osmolality of the surrounding medium, it was possible to establish the variation of oxygen affinity as a function of the intra-erythrocytic hemoglobin concentration. In the case of normal cells, when changing the osmolality of the suspending buffer from 290 to 400 mOsm, the P 50 was almost unmodified. In contrast it is known [11] that the P 50 of cells containing hemoglobin S increases regularly with the hemoglobin concentration. After treatment of A/S cells by ticlopidine, at the low ionic strengths values, there was almost no difference in P 50 as compared to untreated cells. Around 300 mOsm the treated cells behaved like the untreated ones at lower ionic strengths (Fig. 7). A further increase in osmolality again reduced the difference in oxygen affinity.

It is therefore clear that, below some critical hemoglobin concentration, the increase of the cell volume induced by the drug, affected the time for nucleation. Once this concentration had been reached, the effect of the drug was no more observable.

Conclusions

Up to now ticlopidine has not been considered a true therapeutic agent against sickle crisis. The concentration of the drug used in these in vitro studies was not of the same magnitude as those reached in vivo. Moreover the metabolism of the compound and the concentration of active molecules obtained in the various tissues are only partially known.

Ticlopidine is neverthelss an interesting model of membrane expander, acting on sickling by cation permeability change and water gain. The molecule may be of some use for the potential design of other drugs, acting more specifically, which could then have a possible indication in a combined treatment.

References

1. Cabannes, R., Lonsdorfer, J., Sangare, A., Ondo, A., Plassart, P., Zohoun, I., Castaigne, J. P.: Clinical trials in sickle cell crisis with ticlopidine. *Abstracts Sixth Meeting, European and African Division, Int. Soc. Haematology, Athens, 1981*, p. 184.
2. Brewer, G. J., Oelshlegel, F.: Antisickling effects of zinc. *Biochem. Biophys. Res. Commun. 58*, 854 (1974).
3. Asakura, T., Ohnishi, S. T., Adachi, K., Ozguc, M., Hashimoto, K., Singer, M., Russel, M. O., Schwartz, E.: Effect of cetiedil on erythrocyte sickling: new type of anti-sickling agent that may affect erythrocyte membranes. *Proc. Nat. Acad. Sci. USA 77*, 2955 (1980).

4. Brewer, G. J., Bereza, U., Mizukami, I., Aster, J. C., Brewer, L. F.: Drug action hypothesis: how membrane expansion, calmodulin inhibition and sickle cell therapy relate. In: *The Red Cell: Fifth Ann Arbor Conf., 1981*, p 187.
5. Berkowitz, L. R., Orringer, E. P.: Effect of cetiedil, an *in vitro* anti-sickling agent, on erythrocyte membrane cation permeability. *J. Clin. Invest. 68*, 1215 (1981).
6. Schmidt, W. F., Asakura, T., Schwartz, E.: Effect of cetiedil on cation and water movements in erythrocytes. *J. Clin. Invest. 69*, 589 (1982).
7. Daveloose, D., Sablayrolles, M., Molle, D., Leterrier, F.: Interaction of ticlopidine with the erythrocyte membrane. *Biochem. Pharmacol. 31*, 3949 (1982).
8. Orringer, E. P., Roer, M. E. S., Parker, J. C.: Cell density profile as a measure of erythrocyte hydration: therapeutic alteration of salt and water content in normal and S/S red blood cells. *Blood Cells 6*, 345 (1980).
9. Fabry, M. E., Nagel, R. L.: Heterogeneity of red cells in the sickler: a characteristic with practical clinical and pathophysiological implications. *Blood Cells 8*, 9 (1982).
10. Hofrichter, J., Ross, P. D., Eaton, W. A.: Kinetics and mechanism of deoxyhemoglobin S gelation. *Proc. Nat. Acad. Sci. USA 71*, 4864 (1974).
11. May, A., Huehns, E. R.: The concentration dependence of the oxygen affinity of haemoglobin S. *Brit. J. Haemat. 30*, 317 (1975).

Haematologia 17 (2) pp. 209–226 (1984)

Histologic Criteria for Classification and Differential Diagnosis of Chronic Myeloproliferative Disorders

B. FRISCH,[1] R. BARTL,[2,3] R. BURKHARDT,[2,3] K. JÄGER[2]

Institute of Haematology, Ichilov Hospital, Tel-Aviv Municipal Governmental Medical Centre; Sackler School of Medicine, Tel-Aviv University, Weizman Street, Tel-Aviv, Israel[1]; Abteilung für Knochenmarksdiagnostik, Medizinische Klinik Innenstadt der Universität München, FRG[2]; Gesellschaft für Strahlen- und Umweltforschung mbH, Abteilung für Hämatomorphologie, Neuherberg bei München, FRG[3]

(Received 4 January 1983; accepted 12 February 1983)

The chronic myeloproliferative disorders (MPD) are well characterized clinical entities comprising polycythaemia vera (PV), idiopathic thrombocythaemia (IT) (also called megakaryocytic myelosis, mature type; MegM), chronic myeloid leukaemia (CML), and myelofibrosis/osteomyelosclerosis (MF/OMS). Three thousand one hundred and eight bone biopsies of 2629 patients with established MPD were examined to investigate the histologic features of MPD in a large material in order to identify criteria for the histologic classification and differential diagnosis of these disorders. Detailed histologic characteristics were defined for each of the disorders and the results showed that in the majority of cases MPD may be recognized and classified by the initial bone marrow histology. Utilizing the predominant proliferative cell(s) in the bone marrow, PV was categorized into 4 types: 1. the classic, tri-linear type; 2. a bi-linear type with hyperplasia of the erythroid and granulocytic lines; 3. a bi-linear type with hyperplasia of the erythroid and megakaryocytic cell lines and 4. a uni-linear type with isolated increased erythrocytic proliferation. CML showed 2 sub-divisions: 1. the granulocytic, uni-linear type and 2. the bi-linear type with proliferation of myeloid and megakaryocytic lines. The former had a tendency to evolve into blastic crisis, while the latter was prone to develop into MF/OMS. It was primarily uni-linear exhibiting increased megakaryocytes. In most cases, MF/OMS was shown (by means of follow-up biopsies) to arise out of those entities of MPD which had included megakaryocytic hyperplasia and to which the proliferation of fibroblasts was secondary. The conclusion is drawn that an initial bone marrow biopsy provides additional diagnostic and prognostic data in this group of haematologic malignancies.

Keywords: bone marrow biopsy, classification, leukaemia, myeloproliferative disorders

Introduction

The chronic MPD are haematologic neoplasias of clonal origin [1–4], differing from tumours in many other tissues in that they infiltrate one of the largest organs in the body, the bone marrow, diffusely and extensively rather than forming localized tumour masses. In addition there is progressive encroachment on areas normally not occupied by red haematopoietic marrow [5] and in many cases a considerable tumour burden will have accumulated before the malignancy is diagnosed. Moreover, there are no unequivocal histologic characteristics which distinguish the neoplastic cells in MPD from their normal counterparts in the

bone marrow. Consequently though unaffected precursors may co-exist with the progeny of the abnormal clone, they cannot usually be recognized as such histologically. Nevertheless, the overall histologic and histotopographic pattern is invariably altered and thereby permits reliable interpretation and diagnosis.

The aims of this study were to 1. provide precise characterization of the bone marrow histology in MPD; 2. to differentiate these features from other secondary hyperplasias in the bone marrow and 3. to follow the disease progression and the evolution of metamorphosis and thereby clarify the interrelationships between the entities comprising the MPD. This report deals exclusively with bone marrow histology. Other parameters and their clinical and haematologic, biochemical and cytogenetic correlation have been dealt with extensively by others [6–11] and in previous articles and reviews [12–14].

Materials and Methods

Two thousand and seventy-eight patients diagnosed by established criteria [8–10] as suffering from one of the MPD were biopsied before the administration of specific therapy. Of these, 311 patients had sequential biopsies during the course of the disease (follow-up periods of 1 to 10 years). Biopsies of 551 patients who had already been treated for one of the MPD were also taken, making a total of 3108 biopsies. Additional 915 biopsies of 810 patients diagnosed as having secondary erythrocytosis, haemolytic conditions, leukaemoid reactions, hepatic disease, thrombocytopenia and fibrosis of the marrow due to causes other than MPD, were studied for comparison. Normal bone marrow histology and its variations were derived from bone marrow biopsies of 158 individuals without evidence of disease. Informed consent was obtained in all cases, after the procedure had been fully explained. The biopsies were taken from the anterior or posterior iliac crests under local anaesthesia [11]. All biopsies were fixed, dehydrated and embedded in synthetic resin without prior decalcification and cut and stained as previously described [11, 14]. The structural and histomorphometric variables investigated in each biopsy were utilized for multivariate data analyses by selected BMDP computer programs [13].

Results

The initial distribution of the patients in the clinical entities was as follows: PV 620; IT 200; CML 645; MF/OMS 613. The male to female ratio was 0.8 : 1; the median age at the time of the first biopsy was 60 years, with a range of 13 to 89 years. When considered according to the predominant proliferative cell lines, the haematopoietic clone(s) involved in the clinical entities is (are) shown in Table 1. The distribution of the patients according to these entities and their transformations are also given in Table 1. The normal bone and bone marrow

Table 1

Histologic classification of MPD according to the proliferative cell line(s)

Clinical entities	Proliferative cell line(s) in marrow histology			Percentage in each clinical entity	Median survival[1]	Predominant metamorphosis
PV (620)	—	—	—[2]	2	—	0
	ERY	—	—	3	115	0
	ERY	GRAN	—	5	96	0
	ERY	—	MEG	44	82	MF/OMS
	ERY	GRAN	MEG	46	75	MF/OMS
IT (200)	—	—	MEG		61	MF/OMS
CML (645)	—	GRAN	MEG	45	25	MF/OMS
	—	GRAN	—	55	18	Blastic crisis

[1] Survival time (months) from the time of biopsy to death or date of last contact
[2] Normal haematopoietic tissue

is composed of the parenchyme: consisting of the precursors of erythrocytes, granulocytes, monocytes and platelets 40 vol%; fat cells 28 vol%; within a stroma containing sinusoids 4 vol%; as well as the following cellular elements per mm^2: mast cells 2, macrophages 16, plasma cells 21, lymphoid cells 15; trabecular bone 26 vol%. These values were taken as baselines in the evaluation of increases and decreases in the MPD.

PV. On the basis of the bone marrow findings, PV was divided into 4 subtypes: 1. the classic tri-linear type, in which there was hyperplasia of the erythro-, megakaryo- and granulocytic cell lines; 2. bi-linear: erythro- and megakaryocytic, without hyperplasia of the granulocytic cell line in the biopsy; 3. bi-linear: erythro-granulocytic, in which there was no increase of megakaryocytes; 4. uni-linear, in which only the erythroid cell line exhibited hyperplasia in the bone marrow histology. These are illustrated in Figure 1. Exceptions did, however, occur in which the bone marrow histology appeared within normal limits (Fig. 1*a*) or could not be assigned to any of the groups (2 per cent of all cases). Such patients were usually classified by a sequential biopsy performed later during the course of the disease. Bone marrow biopsies in PV were characteristic as the overall cellularity was increased with a corresponding decrease in fat cells. In the classic type there was hyperplasia of the erythroid, myeloid and megakaryocytic precursors mostly in their normal topographic locations (Fig. 1*b*): erythroid in the para-sinusoidal zones, granulocytic in para-trabecular spaces and perivascular regions, and megakaryocytes in the inter-trabecular areas. The megakaryocytes increased to 5 times or more their normal number, from 8 to over 40/mm^2 of bone marrow, in which case they were also in the para-trabecular regions (heterotopia). In addition, many showed extreme hypertrophy, others polymorphism and pyknosis. There were numerous immature forms with large round to oval or

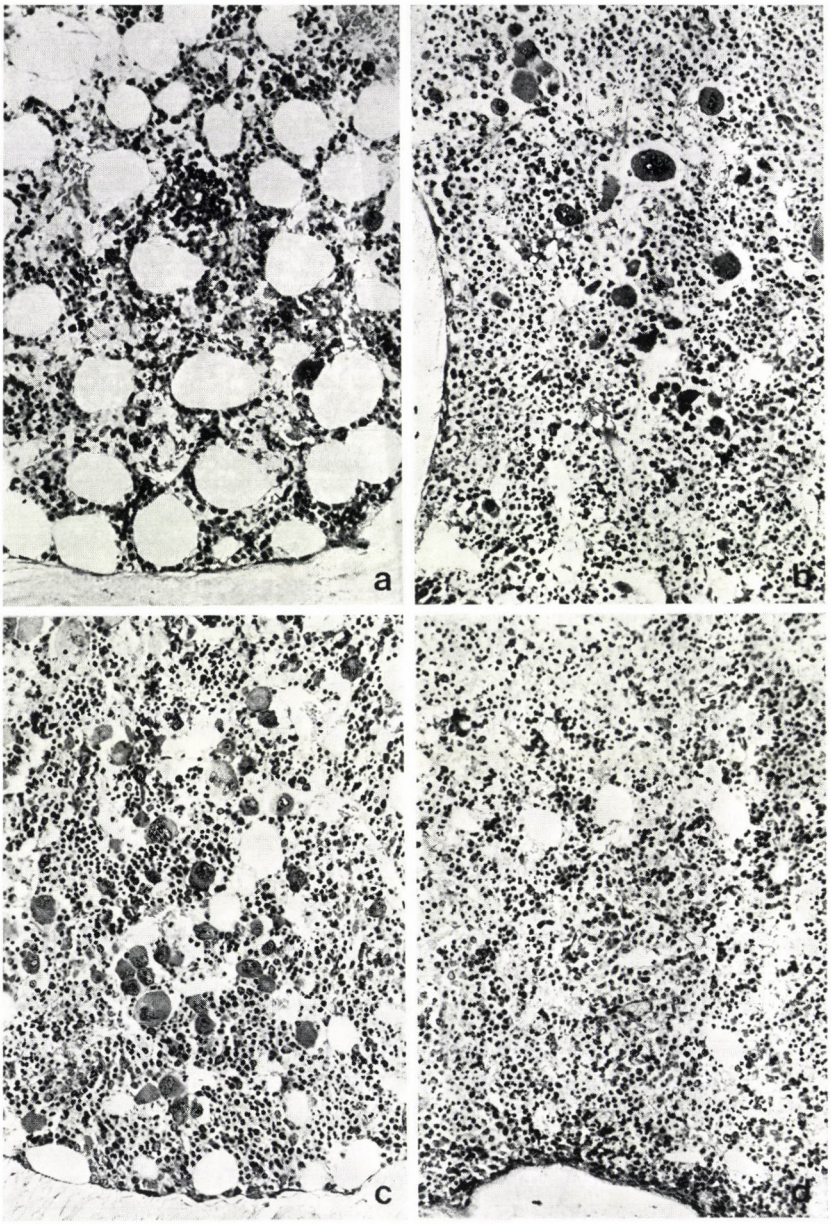


Fig. 1. Sections of bone marrow biopsies of patients with PV, stained with Giemsa, × 120. *a.* normal distribution and quantity of haematopoietic tissue; *b.* trilinear proliferation; *c.* predominant proliferation of erythro- and megakaryocytic cell lines; *d.* predominant proliferation of erythro- and granulocytic cell lines

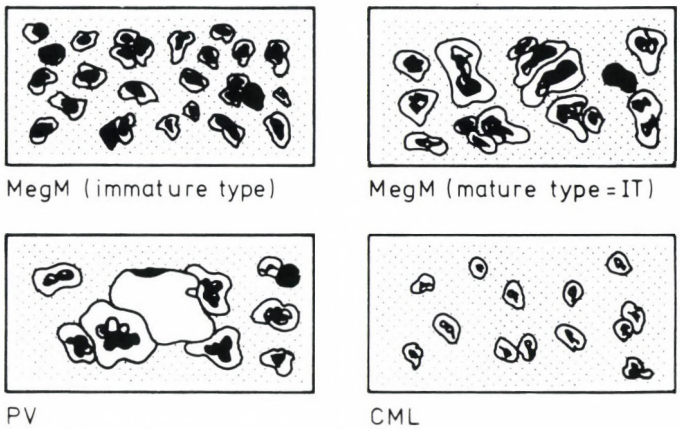


Fig. 2. Schematic representation of megakaryocytes in chronic MPD

indented nuclei and prominent nucleoli, hypertrophic megakaryocytes with bizarre nuclear configurations, others with variable cytoplasmic density (indicating a variable content of cytoplasmic organelles), as well as nuclear masses devoid of cytoplasm (Fig. 2). Emperipolesis was frequent in the large megakaryocytes but also in the smaller ones. In all types of PV there was an increase in all bone marrow blood vessels and the normal 4 vol% occupied by the sinusoids rose to an average of 7 vol%. Whether this represented a genuine hyperplasia or a hyperaemia of previously collapsed and therefore unidentified sinuses, is not known. Complete depletion of iron stores was found in 89 per cent of the cases. The increase in reticulin fibres was variable, mostly only slight to moderate and generally near the vessels. All cases showed rarefaction of the cancellous bone. Increased numbers of lymphocytes and plasma cells occurred regularly, lymphoid nodules or aggregates were seen in 15 per cent of the cases. Macrophages with nuclear debris were not prominent. Eosinophils and basophils were increased in number in some cases.

In PV type 2, the granulocytic cell line was not markedly increased (Fig. 1*c*) so that the para-trabecular regions were also occupied by the other two lines. This type of PV exhibited the same features of abnormal megakaryocytic proliferation as the classic type. PV type 3 was characterized by hyperplasia of erythroid and myeloid elements, without participation of the megakaryocytes (Fig. 1*d*); while in the 4th type, the smallest group, there was an isolated hyperplasia of erythroid percursors. As indicated in Table 1, progression of the disease depended on the predominant proliferative cell(s) with evolution to MF/OMS only in the first and second types in which the megakaryocytes had been involved in the hyperplasia. Metamorphosis of PV to terminal blastic crisis was observed in 7 patients, and to other entities in 41 cases; CML 19; IT 16; non-Hodgkin's lymphoma 8; and multiple myeloma 7 patients.

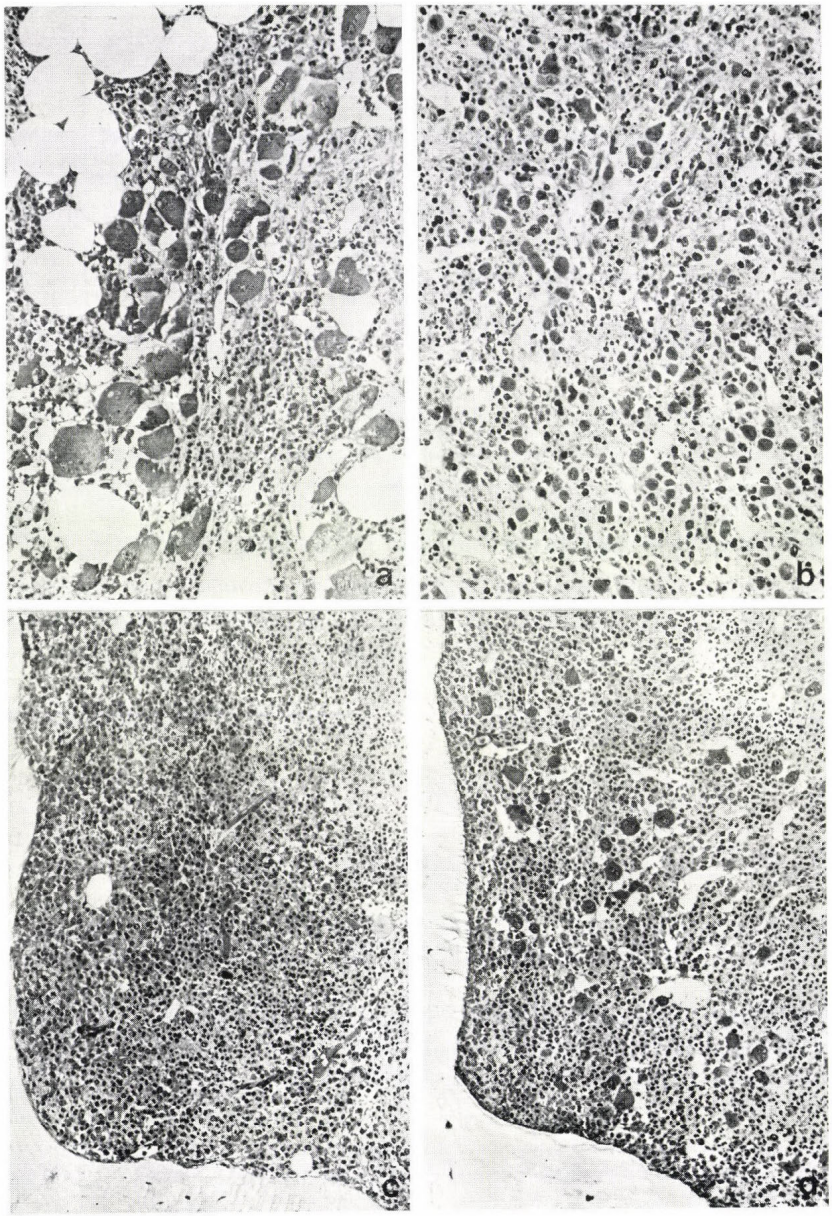


Fig. 3. Sections of bone marrow biopsies: *a.* in IT; *b.* in megakaryocytic myelosis, immature type; *c.* in CML granulocytic type; *d.* in CML mixed type; stained with Giemsa, × 10

In secondary erythrocytosis, there was a hyperplasia of the erythroid series. The other histologic features listed above were either absent or very mild. There was no marked polymorphism, clustering or pyknosis of megakaryocytes, a moderate reduction in fat cells, no depletion of iron stores which generally were normal or even increased, or increase in reticulin. In some acquired haemolytic conditions and hepatic diseases there was considerable erythroid hyperplasia with a predominance of immature precursors, in contrast to PV type 4 in which the erythroid islands were conspicuous due to the numerous normoblasts. The histologic differentiation between the classic tri-linear PV and the transition to the hypercellular phase of MF with little fibrosis may be difficult. In the latter, there is some preservation of fat cells, incomplete iron depletion, and more numerous lymphocytes, plasma cells and mast cells. In refractory anaemia, megakaryocytic hyperplasia and polymorphism are absent and the erythroid hyperplasia affects mainly the erythro- and pro-erythroblasts many of which contain cytoplasmic iron. There is an increased amount of iron in the stromal cells and numerous macrophages containing haemosiderin and nuclear debris are found. In preleukaemia the cellularity may be increased but not dense, fat cells are preserved, immature precursors of all three cell lines may predominate, the megakaryocytes in particular tend to be small and rarely clustered, and the monocytes may be numerous. Rarefaction of the cancellous bone, associated with chronic erythroid hyperplasia [15] is frequent.

IT (megakaryocytic myelosis, mature type). At first presentation there may be normo-, hypo- or hypercellularity with some reduction of fat cells (Fig. 3*a*). The characteristic feature is the hyperplasia of megakaryocytes, resulting in numerous clusters of often contiguous polymorphic cells, ranging in size from micro to gigantic forms (Fig. 2); with relatively small single nuclei or highly convoluted nuclear masses and pyknotic cells (Fig. 3*a*). Accumulation of interstitial platelets may be found as they are shed not only into sinuses but also into the surrounding stroma. In some cases red and white cell precursors were decreased while in others increased in number. Some iron was usually demonstrable in stromal cells. Coarse fibrosis was first localized in the vicinity of clusters of polymorphous megakaryocytes; and, as shown in sequential biopsies, this entity was particularly prone to develop into MF/OMS (n = 85). In other cases a gradual transition to the immature (promegakaryocytic) form occurred (Fig. 3*b*) and this was accompanied by a decrease in the platelet counts in peripheral blood. There was usually little difficulty in distinguishing the bone marrow histology in IT from that in other conditions in which an increase in the number of megakaryocytes may occur: after haemorrhages, in haemolytic states, as a reaction to the presence of a (non-haematologic) malignancy and in many other conditions. The most important single factor is the absence of marked polymorphism [16] of large clusters and of pyknotic forms of megakaryocytes. In idiopathic thrombocytopenic purpura the hyperplasia of megakaryocytes consists mainly of young cells, and this form must be distinguished from thrombocytopenia with decreased megakaryocytes [16], as well as the immature form of MegM for which a bone biopsy is required.

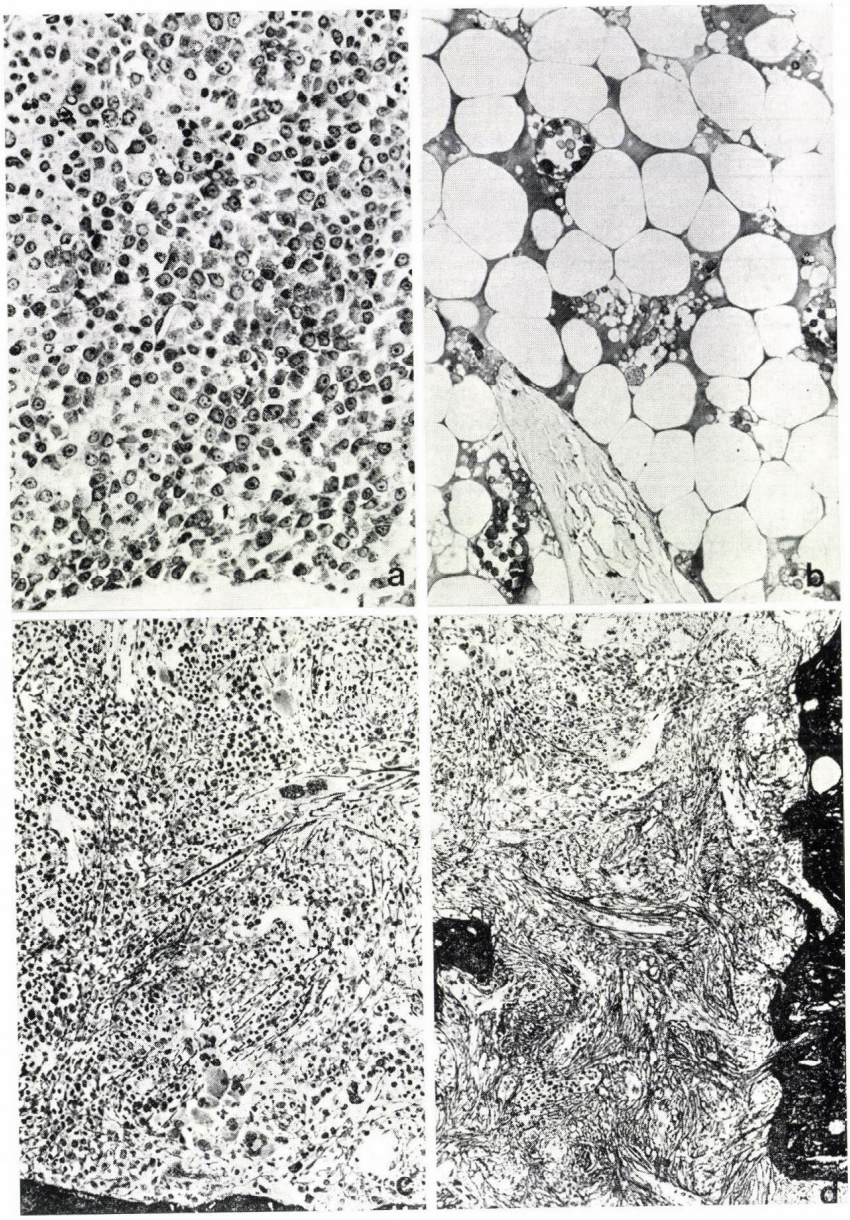


Fig. 4. Sections of bone marrow biopsies: *a.* blastic crisis in CML; *b.* post-therapeutic hypoplasia in CML, both stained with Giemsa, × 160; *c.* MF hypercellular phase; *d.* MF obliterative phase (both Gomori's reticulin stain, × 120)

CML. The marrow in CML is generally densely packed with almost complete absence of fat cells (Fig. 3*c*). The histologic picture was dominated either by a uni-linear proliferation of the granulocytic series, or a bi-linear hyperplasia of both myeloid and megakaryocytic elements (Fig. 3*d*). Both types were characterized by periosteal seams and peri-vascular cuffs of granulopoiesis, with progressive maturation proceeding outward so that the central inter-trabecular area contained numerous band and polymorphonuclear leukocytes. The proportion of immature to mature granulocytes is, however, usually higher in the marrow than in the peripheral blood. Some degree of asynchronous maturation is present, as evidenced by early cytoplasmic granulation and by hypo-segmentation of the nucleus (the Pelger–Huet anomaly) which also points to nuclear aberrations. Macrophages were increased and frequently contained crystalloid inclusions (especially in the bi-linear type of CML with eosinophilic predominance), or occasionally resembled Gaucher cells. There was infiltration with plasma and mast cells and lymphocytes; red cell precursors showed an arrest of maturation. The cancellous bone was rarefied, generally without evidence of osteoclastic remodelling, though occasionally localized circumscribed osteolysis was found. Blood vessels, notably arterial capillaries, were increased in number. In the bi-linear type, in addition to the features described above, there was hyperplasia (Fig. 2), polymorphism and heterotopia of megakaryocytes (Fig. 3*d*) with some degree of focal or diffuse fibrosis usually in the vicinity of megakaryocytes. This type revealed a tendency to develop into MF/OMS, in contrast to the uni-linear type in which transformation (accelerated phase, metamorphosis) to a blastic crisis occurred in a high percentage of the cases (Table 1). In sequential biopsies this event was heralded by broadening para-trabecular seams of immature myeloid precursors (Fig. 4*a*). Both types often showed an increase in eosinophils, ranging from mild to marked, while an increase in basophils was noted especially during transformation to blastic crisis [17]. Histologically the distinction between CML and a leukaemoid reaction may present difficulties. In the latter case the fat cells are usually not drastically reduced, the overall cellularity is less dense, macrophages with crystalloid inclusions are rare, lymphocytic infiltration is minimal, while reactive, especially peri-vascular plasmacytosis is pronounced. Eosinophilia and basophilia are slight or absent, the increase in megakaryocytes is moderate and they do not display the morphologic anomalies noted above. There is no increase in reticulin; erythropoiesis may appear relatively reduced and show maturation arrest; while granulocytic hyperplasia is peri-vascular and inter-trabecular rather than endosteal. There is little or no osteopenia except in elderly patients, as myelogenous osteodysplasia is mostly observed with very hyperplastic bone marrows [18] and as a bone resorbing factor is produced by the neoplastic myeloid leukaemia cells [19]. With very high peripheral blood leukocyte counts, the immature myeloid precursors may dominate the picture which then resembles that seen in promyelocytic leukaemia. Likewise, differentiation of a blastic crisis from an acute leukaemia, or eosinophilic CML from the hypereosinophilic syndrome [19] may not be possible on purely morphologic grounds without a knowl-

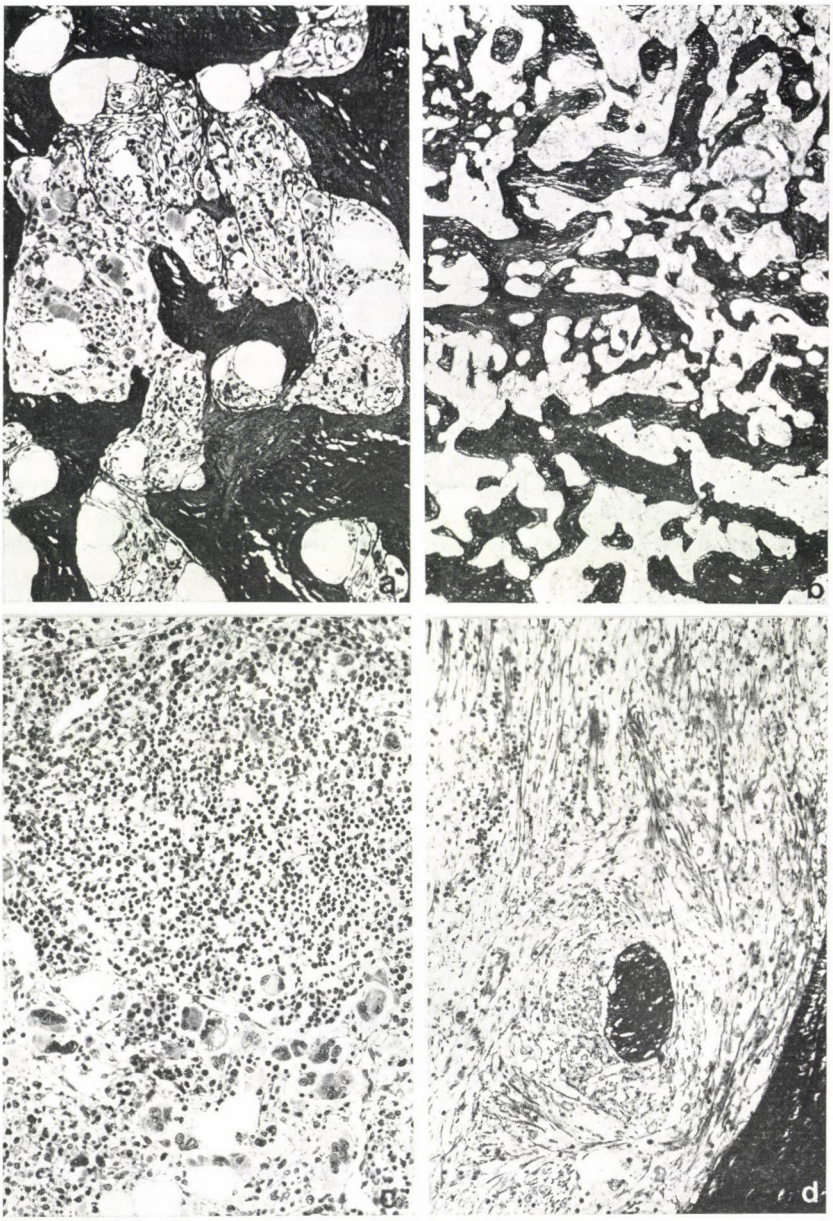


Fig. 5. Sections of bone marrow biopsies: *a*. in OMS, × 160; and *b*. in OMS, × 60, both Gomori's reticulin stain; *c*. in IT with incipient immunocytoma, stained with Giemsa, × 160; *d*. fibrosing myelitis, Gomori's reticulin stain, × 160

edge of the patient's history and other clinical data. A high basophil count in the peripheral blood is indicative of CML. After therapy, a hypocellular, even aplastic phase may ensue (Fig. 4*b*), and a bone biopsy is required to distinguish this from a dry tap caused by an increased fibrosis of the marrow.

MF/OMS. Three major kinds of bone marrow histology have been described in MF: 1. hypercellular; 2. partially fibrotic and 3. obliterative. In the first (Fig. 4*c*), there is hyperplasia of the three cell lines, and the clusters of polymorphic megakaryocytes are particularly striking. Fibrosis is incipient or minimal. The second shows a patchy cellularity alternating with fibrosis, and in the third, the marrow cavities are obliterated by an almost acellular sclerosis (Fig. 4*d*). These pictures, however, are not sharply delineated, so that there is some overlap between them; also, they are not necessarily sequential. Indeed they may be present simultaneously in the marrow in the same biopsy and in different parts of the skeleton, as shown by bi-lateral biopsies. Consequently a more accurate division in MF might be 1. haematopoietic hyperplasia and 2. haematopoietic hypoplasia. Both are accompanied by variable amounts and type of fibrosis, which is not necessarily uniform throughout the marrow. Extension of haematopoietic tissue into normally non-active areas also occur. In early MF the haematopoietic hyperplasia is accompanied by a reduction of fat cells and an increase in the number of blood vessels, especially capillaries and fibroblasts. There are clusters of polymorphic megakaryocytes, interstitially deposited platelets [12], foci of oedema and gelatinous degeneration, infiltration with lymphocytes, plasma and mast cells. Mast cells in particular were far more frequently observed than has previously been appreciated. When sections stained with Giemsa or toluidine blue were examined at high magnification under oil, many cells at first identified as plump or elongated fibroblasts were found to contain red, metachromatic granules. In 14 per cent of the cases, nodular lymphoid aggregates were found. Macrophages containing haemosiderin may be prominent and, especially in cases which developed from CML, they had crystalloid inclusions. Near the blood vessels, the networks of reticulin fibres merged with bundles of collagen causing a disruption of the sinusoidal architecture and sclerosis of the sinus walls. Haematopoietic tissue was progressively reduced, until eventually only a few, mainly intra-sinusoidal, islands of haematopoietic precursors remained, though groups of megakaryocytes with many pyknotic forms were still present. It should, however, be noted that intra-sinusoidal haematopoietic tissue was often found even in the cellular phases of MF/OMS. In MF there may be rarefaction of the cancellous bone, without evidence of increased osteoclastic remodelling. In OMS, the inter-trabecular connective tissue is frequently loose, oedematous and contains fewer collagenous fibres while more fat cells are preserved than in MF (Fig. 5*a*), but haematopoiesis is drastically reduced in established OMS. Appositional new bone produced by osteoblasts lining the trabeculae, as well as the formation of irregular spicules of woven bone (endophytes) contribute to the progressive diminution of the marrow spaces (Fig. 5*b*). In sequential biopsies, some patients with an initially hypercellular marrow developed the fibrotic,

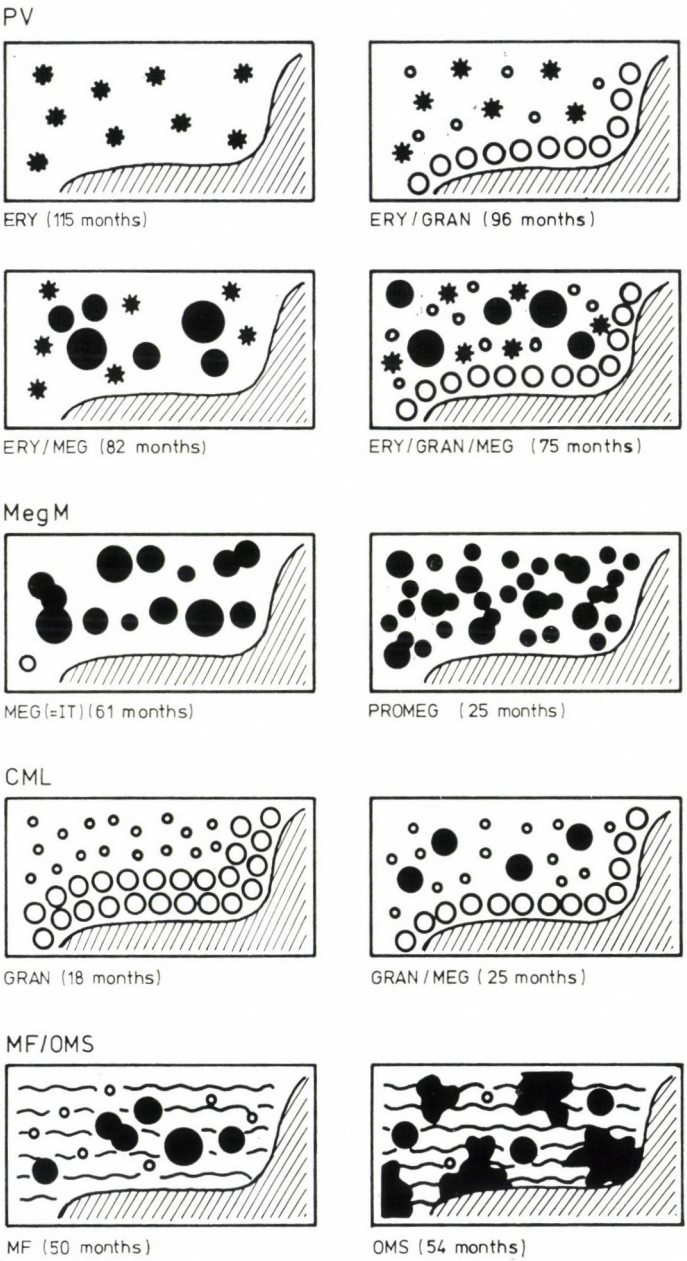


Fig. 6. Schematic representation of predominant cell line(s), bone marrow architecture and median survivals in chronic MPD

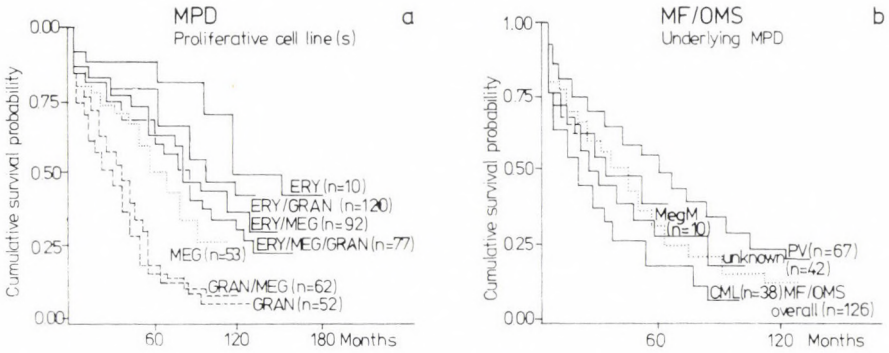


Fig. 7. Cumulative survival curves in MPD: *a.* according to proliferative cell line(s); *b.* in MF/OMS according to underlying MPD

obliterative type of MF while others who presented without a decrease in marrow fat – that is, less obvious proliferative activity – showed a more indolent course and evolved into OMS. In MF/OMS transformation to acute leukaemia occurred in about 5 per cent of the cases. Development of CLL or immunocytoma was also observed (Fig. 5*c*) sometimes earlier in the bone biopsy than it was apparent clinically. Fibrosis may be encountered in the bone marrow, in addition to MPD, in numerous other conditions. These include the lymphoproliferative disorders, Hodgkin disease, metastatic carcinoma, primary hyperparathyroidism, and myelitis due to various kinds of injury and toxic agents. In the latter, the bone marrow was characterized by a loose network of fibres, together with oedema and infiltration with lymphocytes, plasma and mast cells, an increase in macrophages and a decrease in haematopoietic tissue including the megakaryocytes (Fig. 5*d*). When fibrosis was secondary to metastatic carcinoma, the neoplastic cells were found and in lymphoproliferative disorders the cells of the particular lymphoma were recognized. The correlation between predominant cell line, histologic pattern and median survival in chronic MPD is presented in Figure 6, and the cumulative survival curves are given in Figure 7.

Discussion

All the MPD are characterized by anomalies in growth which result in a steadily increasing number of specific haematologic cells in the absence of an appropriate stimulus [20, 21]. This increase in cell number is generally due to excessive production and not to alterations in the life span of the cells, though the rate of cell division may be lower than normal [22]. Although originating from a single clone the initially limited but finally unchecked replication and related maturation disturbances give rise to a progeny which eventually overwhelms the normal haematopoietic marrow. As a consequence, the proliferation

patterns in the bone marrow are altered from the start and the results presented here show that in the majority of cases this abnormal growth may be recognized and its type, distribution and related stromal reaction contribute valuable information for differential diagnosis and prognosis in the individual patient in MPD.

There is still controversy in the literature concerning the value of bone marrow biopsy in PV as well as in other entities of the MPD [23]. Some authors believe that a bone marrow biopsy may not permit differentiation between MPD and preleukaemia and refractory anaemia [24]. In a comprehensive and widely used textbook of haematology PV is listed as one of the conditions in which a bone biopsy is of little or no diagnostic value [10a]. Our results clearly contradict these contentions. Indeed the bone marrow picture in PV has been described in previous publications [12, 25, 26], and our findings confirm and extend these observations. In a large series only very few pre-treatment patients with clinically established PV (7 of 281) had a comparatively normal bone marrow [27]. It should, however, be borne in mind that especially in the older age groups [28] this may represent an increase in a previously hypocellular area in particular in the sub-cortical regions in iliac crest biopsy. In all our cases, patients with initially inconclusive bone marrow pictures displayed a proliferation pattern indicative of PV in subsequent biopsies. The proposed sub-classification of PV into 4 histologic subtypes was shown to have prognostic significance and may be tested in future prospective investigations.

Previous studies of staging, classification and of prognostic features in CML have not included bone marrow biopsies or failed to recognize their potential value [24, 29, 30] both for differential diagnosis and for predicting the likelihood of blastic transformation or development of MF. Other investigations into the evolution of the disease process in CML have also proposed a bi-linear histologic classification without, however, a statistical evaluation of its prognostic significance [31]. Since the MPD originate in pluripotent stem cells, progression from one cell type to another, in other words an altered or additional morphologic expression, is an intrinsic component of the disease process [32, 33]. Moreover, the possibility of chromosomal aberrations rises with expansion of the total tumour mass and the number of mitoses since new abnormal clones may originate a sub-population of the primary expanding abnormal clone. And these sequential sublines may have a proliferative advantage. This may be one explanation for the phenotypic conversions in the MPD. Others include increased susceptibility due to dysfunction of the immune system, inherent instability of the pluripotent stem cells, or primary multiple neoplasms [34, 35]. Therapy-induced secondary malignancies must also be considered in treated patients. The transformations have in common an increase in immature precursors, but the impairment in maturation and the lack of, or unresponsiveness to, control mechanism apparently cannot be overcome so that a sustained and ineffective stimulus to proliferation is given by the demand for functioning cells leading to continued bone marrow hyperplasia. It is of interest that the erythrocytic, granulocytic, megakaryocytic and lymphocytic cell lines have all been identified as belonging to abnormal clones

in the MPD [3], but no entity has yet been described with proliferation of the osteoclast, though it is now thought also to be derived from the haematopoietic pluripotent stem cell [36]. In some studies with glucose-6-phosphate dehydrogenase, the erythrocytic, granulocytic and megakaryocytic lines were shown to be involved in the neoplastic clone in individual cases [37] but not the monocytic one from which the osteoclast is presumed to arise.

The MPD are characterized by a variability of the stromal reactions which accompany them [38]. It is well established in pathology that all tumours have two basic components: the constituent neoplastic cells, the parenchyma; and the connective tissue and blood vessels: the stroma, which is secondary, reactive, non-neoplastic, and varies with the type of tumour that evokes it. The same holds true for the neoplastic entities in the bone marrow in MPD, and probably also the lymphoproliferative disorders [39–41].

Recent work has identified some of the mechanisms involved in the production of fibrosis in the bone marrow and lends support to conclusions previously drawn from histologic observations [12–14]. Fibroblast stimulating activities have been isolated from megakaryocytes and platelets, precisely the cells with which the development of fibrosis in MPD is associated [2]. Immunologic mechanisms may also participate, including immune complexes [42] and the activation of the complement system [43]. This stimulatory activity may be combined with the inhibition of collagenases by platelet factor 4 [9a], thus aggravating the fibrosis. The progressive fibrous distortion of the bone marrow undoubtedly has an adverse affect on the micro-environment necessary for the normal induction of haematopoiesis and the production of blood cells, as well as permitting egress of immature cells into the circulation, possibly from sites of intravascular haematopoiesis. In this report, the classification of MPD as outlined by Damashek in 1951 [44] has been utilized, but the variant, transitional and intermediate forms have not been considered. A large material has accrued in the intervening 3 decades and it has become increasingly evident that this classification is no longer adequate. As recently pointed out by Galton [45], CML alone includes 5 clearly distinguishable clinical entities: 1. CML, the common variant; 2. atypical CML; 3. chronic myelo-monocytic leukaemia (CMML); 4. neutrophilic leukaemia and 5. juvenile CML. In addition, the clinical entity of chronic monocytic leukaemia [46] is also lacking in Damashek's classification, and so are the myelo-dysplastic syndromes, though these are characterized by anomalies of proliferation, they belong to the myeloid series, and are chronic. There is also a certain lack of clarity in current subdivisions: CMML, for example, is listed as a variant of CML (as noted above) as well as one of the myelodysplastic syndromes in the recent proposals for their classification into 5 subgroups [47]; and also in previous studies [48, 49]. The diagnostic criteria proposed, however, do not include bone biopsies and histology. Other retrospective studies [50] have shown that three major morphologic subgroups emerged in the 'smouldering leukaemias': 1. refractory anaemia with excess of blasts; 2. chronic myelomonocytic leukaemia; 3. erythraemic myelosis, though several cases displayed transitional features.

Our own experience with bone marrow biopsies of a large series of patients with myeloproliferative disorders indicates that further histologic sub-divisions may be made in the recognized entities of PV, IT, CML and MF/OMS [51, 52], in addition to those discussed in this report. Whether such groupings have clinical and prognostic significance is still under investigation and these findings will be published separately, together with a proposal for a more comprehensive classification of the chronic myeloproliferative disorders.

In conclusion, the results presented here show that bone marrow histology is relevant to patient management as the proliferative cell type, its maturity and the degree of stromal reaction are reliable indicators for differential diagnosis, transformation, metamorphosis and prognosis in MPD.

*

The authors are indebted to all colleagues who referred patients or sent biopsies. The skilful assistance of the technical staff of the laboratory, especially of Mrs. W. Sommerfeld and Mr. H. Vondracek, is gratefully acknowledged. Thanks are due to Mrs. G. Huber and Mrs. I. Pascher for secretarial help. This work was supported by the Gesellschaft für Strahlen- und Umweltforschung mbH, Munich.

References

1. Boggs, D. R.: Clonal origin of leukaemia: Site of origin in the stem cell hierarchy and the significance of chromosomal changes. *Blood Cells 7*, 205 (1981).
2. Castro-Malaspina, H., Gay, R. E., Jhanwar, S. C., Hamilton, J. A., Chiarieri, D. R., Meyers, P. A., Gay, S., Moore, M. A. S.: Characteristics of bone marrow fibroblast colony-forming cells (CFU-F) and their progeny in patients with myeloproliferative disorders. *Blood 59*, 1046 (1982).
3. Fialkow, P. J., Faguet, G. B., Jacobson, R. J., Vaidya, K., Murphy, S.: Evidence that essential thrombocythemia is a clonal disorder with origin in a multipotent stem cell. *Blood 58*, 916 (1982).
4. Clare, N., Elson, D., Manhoff, L.: Cytogenetic studies of peripheral myeloblasts and bone marrow fibroblasts in acute myelofibrosis. *Amer. J. Clin. Path. 77*, 762 (1982).
5. Kurnick, J. E., Mahmood, T., Napoli, N., Block, M. H.: Extension of myeloid tissue into the lower extremities in polycythemia. *Amer. J. Clin. Path. 74*, 427 (1980).
6. McBrine, P. A., Miller, A., Zimelman, A. P., Koft, R. S.: Polycythemia vera with myelofibrosis and myeloid metaplasia. Acute hepatic failure following splenectomy. *Amer. J. Clin. Path. 74*, 693 (1980).
7. Zucker-Franklin, D., Greaves, M. F., Grossi, C. E., Marmont, A. M.: *Atlas of Blood Cells: Function and Pathology*. Lea & Febiger, Philadelphia 1981.
8. Newcom, S. R., Kadin, M. E. (eds): *Hematologic Malignancies in the Adult*. Addison-Wesley, California 1982.
9. Hoffbrand, A. V. (ed.): *Recent Advances in Haematology*. Churchill Livingstone, Edinburgh 1982.

9a. Moore, M. A. S.: Idem: Pathogenesis of MF. p. 136.

10. Wintrobe, M. M., Lee, G. R., Boggs, D. R., Bithell, T. C., Foerster, J., Athens, J. W., Lukens J. N. (eds), *Clinical Hematology*. Lea & Febiger, Philadelphia 1981.

10a. Idem p. 68.

11. Catovsky, D. (ed.): *Methods in Haematology. The Leukemic Cell*. Churchill Livingstone, Edinburgh 1981, p. 49.

12. Burkhardt, R., Frisch, B., Bartl, R.: Bone biopsy in haematological disorders. *J. Clin. Path. 35*, 257 (1982).
13. Bartl, R., Frisch, B., Burkhardt, R.: *Bone Marrow Biopsies Revisited. A New Dimension for Haematologic Malignancies.* Karger, Basel 1982.
14. Frisch, B., Bartl, R., Burkhardt, R.: Bone marrow biopsy in clinical medicine: An overview. *Haematologia 15*, 245 (1982).
15. Weinstein, R. S., Hutson, M. S., Ide, L. F., Lutcher, C. L.: Chronic erythroid hyperplasia and accelarated bone turnover. *Clin. Res. 29*, 864 (1981).
16. Stoll, D. B., Blum, S., Pasquale, D., Murphy, S.: Thrombocytopenia with decreased megakaryocytes. Evaluation and prognosis. *Ann. Intern. Med. 94*, 170 (1981).
17. Denburg, J. A., Wilson, W. E. C., Bienenstock, J.: Basophil production in myeloproliferative disorders: Increases during acute blastic transformation of chronic myeloid leukemia. *Blood 60*, 113 (1982).
18. Burkhardt, R.: Myelogene Osteopathien. In: *Handbuch der inneren Medizin. Klinische Osteologie.* F. Kuhlencordt, H. Bartelheimer (eds). Springer, Berlin–Heidelberg–New York 1980. Bd.6/I, p. 1057.
19. Gerwitz, G., Viguez, A., Stewart, A., Hoffman, B.: Production of a bone resorbing factor by myeloid leukemia cells. *Blood 58*, Suppl. 1, 138 (1981).
20. Gilbert, H. S.: The spectrum of myeloproliferative disorders. *Med. Clin. N. Amer. 57*, 355 (1973).
21. Laszlo, J.: Myeloproliferative disorders (MPD): Myelofibrosis, myelosclerosis, extramedullary haematopoiesis, undifferentiated MPD, and haemorrhagic thrombocythemia. *Semin. Hemat. 12*, 409 (1975).
22. Stoll, C., Oberling, F., Roth, M. D.: Sister chromatoid exchange and growth kinetics in chronic myeloid leukemia. *Cancer Res. 42*, 3240 (1982).
23. Burkhardt, R., Bartl, R.: Histobioptische Differentialdiagnose und Prognose der myeloproliferativen Störungen. *Beitr. Onkol. 13*, 201 (1982).
24. Spiers, A. S. D.: Annotation. Metamorphosis of chronic granulocytic leukaemia: Diagnosis, classification and management. *Brit. J. Haemat. 41*, 1 (1979).
25. Vykoupil, K. F., Thiele, J., Stangel, W., Krmpotic, E., Georgii, A.: Polycythemia vera. I. Histopathology, ultrastructure and cytogenetics of the bone marrow in comparison with secondary polycythemia. *Virchows Arch. A. Path. Anat. 389*, 307 (1980).
26. Krause, J. R.: *Bone Marrow Biopsy.* Churchill Livingstone, Edinburgh 1981.
27. Ellis, J. T., Peterson, P.: The bone marrow in polycythemia vera. *Pathol. A. 14*, 383 (1979).
28. Williams, L., Udupa, K. B., Lipschitz, D. A.: Age and erythropoiesis. *Clin. Res. 29*, 846 (1981).
29. Tura, S., Baccarani, M., and the Italian cooperative study group on chronic myeloid leukaemia: Staging of chronic myeloid leukaemia. *Brit. J. Haemat. 47*, 105 (1981).
30. Gomez, G. A., Sokal, J. E., Ealsh, D.: Prognostic factors at diagnosis of chronic myelocytic leukaemia. *Cancer 47*, 2470 (1982).
31. Georgii, A., Vykoupil, K. F., Thiele, J.: Chronic megakaryocytic granulocytic myelosis, CMGM. A subtype of chronic myeloid leukaemia. *Virchows Arch. A. Path. Anat. 389*, 253 (1980).
32. Alimena, G., Dallapiccola, B., Gastaldi, R., Mandelli, F., Brandt, L., Mitelman, F., Nilsson, P. G.: Chromosomal, morphological and clinical correlations in blastic crisis of chronic myeloid leukaemia. A study of 69 cases. *Scand. J. Haemat. 28*, 103 (1982).
33. Williams, W. C., Weiss, G. B.: Megakaryoblastic transformation of chronic myelogenous leukaemia. *Cancer 49*, 921 (1982).
34. Manoharan A., Catovsky, D., Clein, P., Traub, H. E., Costello, C., O'Brien, M., Boralossa, H., Galton, D. A. G.: Simultaneous or spontaneous occurrence of lympho and myeloproliferative disorders: a report of 4 cases. *Brit. J. Haemat. 48*, 111 (1981).
35. Papayannis, A. G., Nikiforakis, E., Anagnostou-Keramida, D.: Development of chronic lymphocytic leukaemia in a patient with polycythaemia vera. *Scand. J. Haemat. 29*, 65 (1982).

36. Chambers, T. J.: Cellular basis of bone resorption. *Clin. Orthop. 151*, 283 (1980).
37. Gaetani, G. F., Ferraris, A. M., Galiano, S., Giuntini, P., Canepa, L., d'Urso, M.: Primary thrombocythemia: Clonal origin of platelets, erythrocytes, and granulocytes in A Gd^B/Gd Mediterranean subject. *Blood 59*, 76 (1982).
38. Editorial: Myelofibrosis. *Lancet 1*, 127 (1980).
39. Duhamel, G., Stachowiak, J.: Bone marrow fibrosis in malignant hemopathies and cancers. Histological study of 2786 biopsies. *Sem. Hôp. 57*, 111 (1982).
40. Newcom, S. R., O'Rourke, L.: Potentiation of fibroblast growth by nodular sclerosing Hodgkin's disease cell cultures. *Blood 60*, 228 (1982).
41. Karcher, D. S., Pearson, C. E., Butler, W. M., Hurwitz, M. A., Cassel, P. F.: Giant lymph node hyperplasia involving the thymus with associated nephrotic syndrome and myelofibrosis. *Amer. J. Clin. Path. 77*, 100 (1982).
42. Cagilaris Cappio, F., Vigliani, R., Novarino, A., Camussi, G., Campana, D., Gavosto, F.: Idiopathic myelofibrosis: a possible role for immune-complexes in the pathogenesis of bone marrow fibrosis. *Brit. J. Haemat. 49*, 17 (1981).
43. Gordon, B. R., Coleman, M., Kohen, P., Day, N. K.: Immunologic abnormalities in myelofibrosis with activation of the complement system. *Blood 58*, 904 (1981).
44. Dameshek, W.: Some speculations on the myeloproliferative syndromes. *Blood 2*, 372 (1951).
45. Galton, D. A. G.: The chronic leukaemias. In: *Recent Advances in Haematology*. A. V. Hoffbrand (ed.). Churchill Livingstone, Edinburgh–London 1982, Chapter 9.
46. Bearman, R. M., Kjeldsberg, C. R., Pangalis, G. A., Rappaport, H.: Chronic monocytic leukaemia in adults. *Cancer 48*, 2239 (1981).
47. Bennet, J. M., Catovsky, D., Daniel, M. T., Flandrin, M. T., Galton, D. A. G., Gralnich, H. R., Sultan, C., the French–American–British (FAB) Co-operative Group: Proposals for the classification of the myelodysplastic syndromes. *Brit. J. Haemat. 51*, 189 (1982).
48. Economopoulos, T., Stathakis, N., Maragoyannis, Z., Gardikas, E., Dervenoulas, L.: Myelodysplastic syndrome. Clinical and prognostic significance of monocyte count, degree of blastic infiltration, and ring sideroblasts. *Acta Haemat. 65*, 97 (1981).
49. Nowell, P. C.: Cytogenetics of preleukemia. *Cancer Genet. Cytogenet. 3*, 265 (1982).
50. Joseph, A. S., Cinkotal, K. I., Hunt, L., Geary, C. G.: Natural history of smouldering leukaemia. *Brit. J. Cancer 46*, 160 (1982).
51. Burkhardt, R., Bartl, R., Beil, E., Demmler, K., Hofmann, E., Irrgang, G., Kronseder, A., Langegger, H., Saar, U., Ulrich, M., Wiemann, H.: Myelofibrosis–osteomyelosclerosis syndrome. Review of literature and histomorphology. In: *Advances in the Biosciences. Vol. 16*. Pergamon Press–Vieweg, Braunschweig 1975, p. 9.
52. Burkhardt, R.: *Farbatlas der klinischen Histopathologie von Knochenmark und Knochen*. Springer, Berlin–Heidelberg–New York 1970.

Haematologia 17 (2), pp. 227—246 (1984)

Lymphoproliferative Disorders in the Bone Marrow: Histologic Criteria for Classification and Staging

R. BARTL,[1, 2] B. FRISCH,[3] G. HOFFMANN-FEZER,[4] R. BURKHARDT[1, 2]

Abteilung für Knochenmarks diagnostik, Med. Klinik Innenstadt, University of Munich, Ziemssenstr. 1a, D-8000 München 2, FRG[1]; Department of Haematologic Morphology, Institute of Haematology, Gesellschaft für Strahlen- und Umweltforschung mbH, Munich, FRG[2]; Institute of Haematology, Ichilov Hospital, Tel-Aviv Municipal Governmental Medical Centre; Sackler School of Medicine, Tel-Aviv University, Israel[3]; Department of Immunology, Institute of Haematology, Gesellschaft für Strahlen- und Umweltforschung mbH, Munich, FRG[4]

(Received 4 January 1983; accepted 15 February 1983)

Malignant lymphoproliferative disorders (LPD) were investigated in bone marrow biopsies of 1565 untreated patients. Marrow involvement was found in 90 per cent of multiple myeloma (MM), 70 per cent of non-Hodgkin lymphomas (NHL) and 10 per cent of Hodgkin disease (HD). In MM and NHL there were five major entities according to the predominant proliferative cell type: 1. plasmacytic, 2. lymphocytic, 3. hairy cell, 4. immunocytic and 5. centrocytic. On the basis of bone marrow histology they were classified into 3 distinct sub-types each of which had an independent prognostic significance. The mode of spread of all the LPD fell into one of six architectural patterns which together with the tumour cell burden (the quantity of infiltration in the biopsy) also had a significant predictive value. Consequently, bone marrow biopsy is a valuable diagnostic tool for histologic classification and clinical staging of any patient with LPD.

Keywords: bone marrow histology, classification, lymphoproliferative disorders staging

Introduction

The current widespread application of bone marrow biopsy in the evaluation of patients with LPD originally sprang from the realization of the necessity of assessing the progression of disease (i.e. staging), before the administration of specific therapy [1, 2]. It soon became evident that additional information of value in arriving at management decisions could be derived from examination of bone marrow biopsies. For example, a bone marrow biopsy may be diagnostic in a patient without a readily accessible lymph node [3]; it may aid the classification of a lymphoma when divergent histologies are found at other sites at initial staging evaluation [4]; it may help to indicate the extent of tumour cell burden [5]; and it may provide information on the functional state of the bone marrow [6, 7]. Since the results of bone marrow examinations in the malignant lymphomas published so far ranged from 16 per cent to 75 per cent positivity in the cases investigated [8, 9], a study was undertaken with the aims 1. to establish the incidence and to classify the bone marrow histopathology according to the criteria of the Kiel classification for lymph node histology, and 2. to analyse the growth patterns and the extent of infiltration of the bone marrow as criteria for staging, and as predictive factors of prognostic significance.

Materials and Methods

One-thousand five hundred and sixty-five patients diagnosed by the established criteria [10–12] as suffering from one of the malignant lymphoproliferative disorders (LPD) were biopsied for initial evaluation and staging before the administration of specific therapy. The criteria of the Kiel classification for lymph node histology were applied to the bone marrow and the distribution of patients with positive biopsies was as follows: multiple myeloma (MM) 220, non-Hodgkin lymphomas (NHL or ML) 462, and Hodgkin disease (HD) 69. Two hundred and eleven patients had sequential biopsies taken during follow-up periods of 1 to 10 years. Normal bone marrow values were derived from biopsies of 158 individuals without evidence of disease. All biopsies were taken under local anaesthesia from the iliac crest either by means of the Burkhardt drill or the Jamshidi needle [13, 14]. The biopsies were fixed, dehydrated and embedded in methacrylate without prior decalcification, and cut and stained as described previously [15]. In 68 cases immunohistologic and/or histochemical studies (PAP or FITC methods) were performed on fresh frozen cryostat sections of one half of the longitudinally halved biopsy cores [16, 17]. Smears of aspirates taken at the same time in 410 patients, and imprints of the biopsies in 312 cases were stained by routine methods. The histologic and histomorphometric variables investigated in each biopsy were used for multivariate data analysis by selected BMPD computer programs [18, 19].

Results

The overall frequency of bone marrow involvement in untreated patients with LPD is shown in Table 1. There were 5 major entities according to the pre-

Table 1

Frequency of bone marrow involvement in LPD (at time of initial diagnosis)

Histologic type		Patient No.	Positive biopsy, %
MM	plasmacytic	159	92
	plasmablastic	86	86
ML	lymphocytic	163	99
	hairy cell	117	95
	immunocytic	132	85
	centrocytic	40	71
	centroblastic/cytic	105	20
	'blastic' (sarcoma type)	116	25
HD[1]	nodular sclerosis	218	4
	lymphocyte predominance	86	8
	mixed cellularity	204	9
	lymphocyte depletion	69	22

[1] lymph node histology

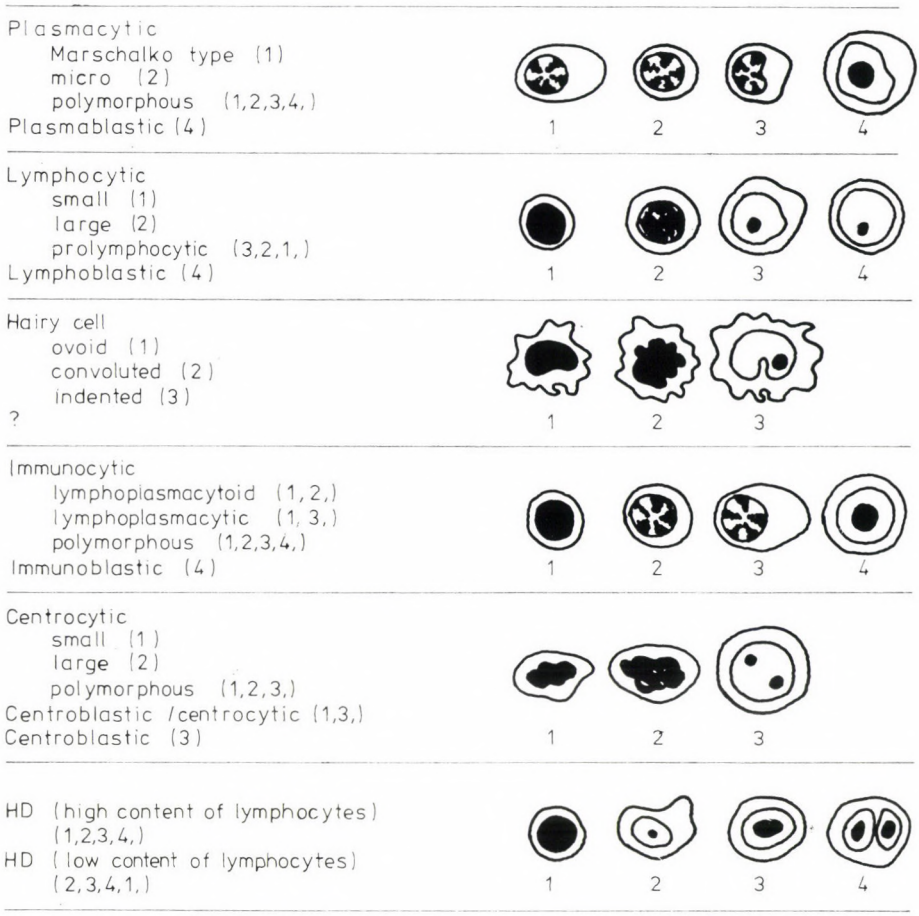


Fig. 1. Cytologic characteristics of LPD-types in bone marrow

dominant proliferative cell type: 1. plasmacytic, 2. lymphocytic, 3. hairy cell, 4. immunocytic and 5. centrocytic. Each of these was subclassified according to the bone marrow histology and the results are presented in Table 2 and Figure 1. The mode of spread of all malignant lymphomas fell into one of six architectural patterns in the bone marrow (called growth pattern) and these are shown in Table 3 and Figure 2. The amount of infiltration in the biopsy, that is the tumour cell burden (vol%), was estimated in the initial bone marrow biopsy of all untreated patients. Three cutoff points were utilized; less than 20 vol% of the marrow cavity occupied by the infiltration, 20 to 50 vol%, and more than 50 vol%. The results, according to the histologic type of malignant lymphoma, are presented in Table 4.

Table 2

Classification of LPD by bone marrow histology (at time of initial diagnosis)

	Histologic types and subtypes	Patients in each type, No.	%	Median survival[1]
MM	plasmacytic	149		32
	Marschalko		75	44
	micro		11	20
	polymorphous		14	16
	plasmablastic	71		8
ML	lymphocytic	134		41
	small		70	46
	large		25	27
	prolymphocytic		5	—
	hairy cell	87		21
	ovoid		47	56
	convoluted		37	12
	indented		16	5
	immunocytic	90		44
	lymphoplasmacytoid		49	74
	lymphoplasmacytic		46	25
	polymorphous		5	12
	centrocytic	20		25
	small		60	36
	large		25	15
	polymorphous		15	—
	centroblastic/cytic	15		50
	'blastic' (sarcoma type)	24		5
HD	high content of lymphocytes	26		58
	low content of lymphocytes	43		5

[1] Survival time (months) from the time of biopsy to death or date of last contact

Classification of MM by bone marrow histology

On the basis of the cytologic features of the plasma cells in the bone marrow histology, multiple myeloma was divided into two broad groups: 1. plasmacytic, consisting primarily of mature type plasma cells, and 2. plasmablastic consisting mainly of immature type plasma cells (Figs 1 and 3).

MM plasmacytic. Although a spectrum of plasma cells was usually present in any one biopsy, they could be divided into three main subtypes: 1. predominance of mature Marschalko type plasma cells, 2. predominance of small 'micro' type plasma cells, and 3. polymorphous plasma cells. These types are illustrated in Figs 1 and 4 and Table 2. Type 1 comprised mainly plasma cells indistinguishable from normals with excentric, cartwheel nuclei, perinuclear 'hof' and basophilic cytoplasm; only a small minority had nucleoli. Type 2 comprised mainly

Table 3

Frequency of bone marrow patterns and their prognostic relevance in LPD (at time of initial diagnosis)

	Histologic type	Patient no.	Interstitial	Interstitial/nodular	Nodular	Paratrabecular	Patchy, focal	Packed marrow
MM	plasmacytic	149	40 (36)	39 (29)	—	—	—	21 (16)
	plasmablastic	71	20 (21)	50 (10)	—	—	—	30 (5)
ML	lymphocytic	134	42 (35)	32 (107)	—	—	—	26 (28)
	hairy cell	87	—	—	—	—	75 (26)	25 (8)
	immunocytic	90	6	33 (52)	41 (72)	—	—	20 (17)
	centrocytic	20	—	—	—	60 (28)	—	40 (18)
	centroblastic/cytic	15	—	—	100 (50)	—	—	—
	'blastic' (sarcoma type)	24	—	—	—	—	—	100 (5)
HD	high content of lymphocytes		—	—	—	—	80 (60)	20 (42)
	low content of lymphocytes		—	—	—	—	62 (7)	38 (4)

First column = percentage in each histologic type
( ) = median survival time (months) from time of biopsy to death or date of last contact

small to micro plasma cells, which had little cytoplasm; and type 3 was characterized by a range of plasma cells from Marschalko type to micro, and many had somewhat ovoid nuclei with slight to moderate indentations or notches. The infiltrations in all cases had a fine reticulin network, and there was an increase in fat cells, reduction in haematopoiesis, with maturation arrest of erythropoiesis. Iron content varied from absent to overload, and the majority of biopsies displayed osteoclastic resorption and pronounced rarefaction of trabeculae. Complete marrow replacement by myeloma cells occurred in 13 per cent, the rest had variable amounts of residual normal tissues. MM in the bone marrow was characterized by an interstitial infiltration as well as dense aggregates of plasma cells in paratrabecular and perivascular regions, which were not seen in reactive plasmacytosis. In addition, 42 per cent of the cases had intertrabecular nodules, 'myelomas'. The prognostic value of the initial plasma cell burden is shown in Table 4. Progression of the disease was slow and accompanied by anaemia, infections and bone related problems. In myeloma patients of the micro plasma cell type, the frequency of a leukaemic blood picture was higher than in those of the two other types, corresponding to plasma cell leukaemia and thus indicating a more unfavourable prognosis.

Differential diagnosis: In smears and imprints the relative numbers of plasma cells, multinuclearity, polymorphism and Russell bodies were not reliable

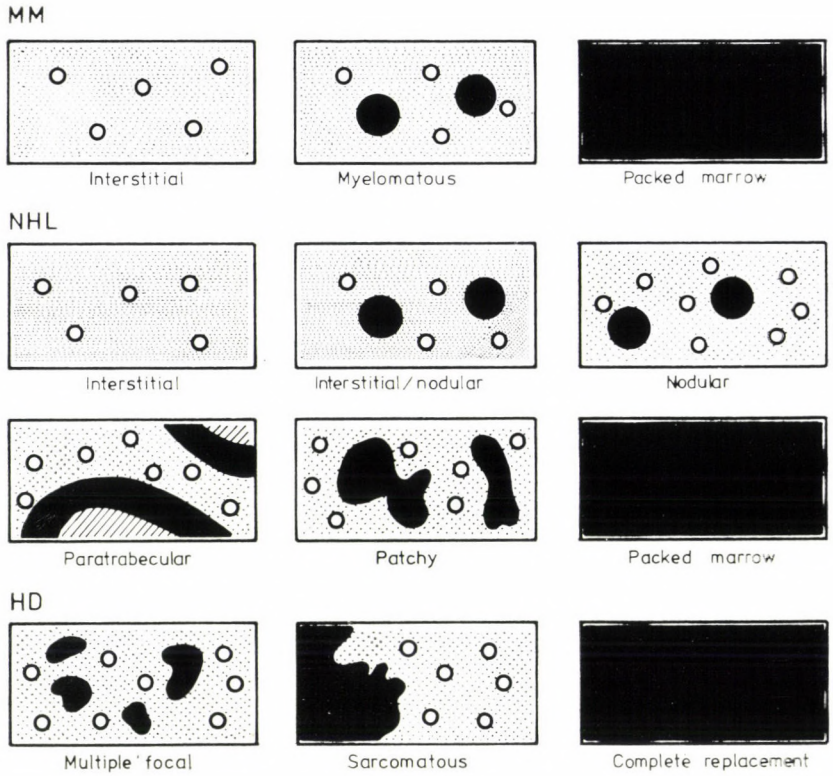


Fig. 2. Growth patterns of LPD in bone marrow

Table 4

Staging of cumulative LPD by bone marrow histology (at time of inital diagnosis)

	Histologic type	Patient no.	Infiltration volume in the biopsy (vol%)[1] Stage I (< 20)		Stage II (20–50)		Stage III (< 50)		Survival statistics[2]
MM	plasmacytic	149	40	(36)	39	(29)	21	(16)	++
ML	lymphocytic	134	17	(78)	44	(38)	39	(31)	++
	hairy cell	87	7	(29)	44	(20)	49	(15)	+
	immunocytic	90	43	(75)	30	(57)	27	(18)	++
	centrocytic	20	25	—	40	(30)	35	(15)	+

[1] = volume percentage of the whole biopsy core
[2] = Breslow- and Mantel-Cox test; + = < 0.05, ++ = < 0.01
First column = percentage in each histologic type
( ) = median survival time (months) from time of biopsy to death or date of last contact
— = number of patients too low for survival statistics

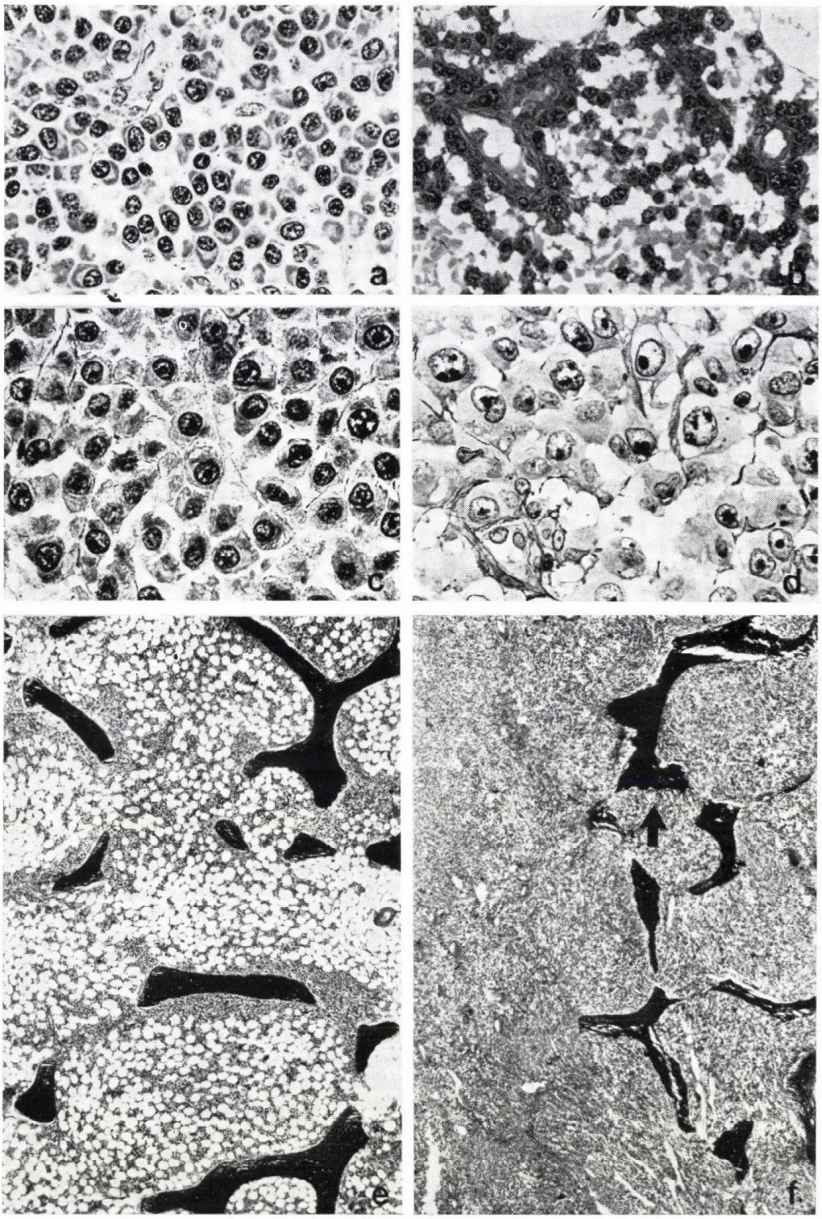


Fig. 3. Bone marrow histology in MM: *a*. plasmacytic, Marschalko type. Giemsa, × 360; *b*. plasmacytic, micro type. Giemsa, × 360; *c*. plasmacytic, polymorphous type. Gomori, × 400; *d*. plasmablastic, Gomori, × 400; *e*. interstitial growth pattern plus dense paratrabecular seams of myeloma cells. Gomori, × 40; *f*. 'packed marrow' pattern with osteoclastic bone resorption (arrow) and osteolytic lesion. Gomori, × 40

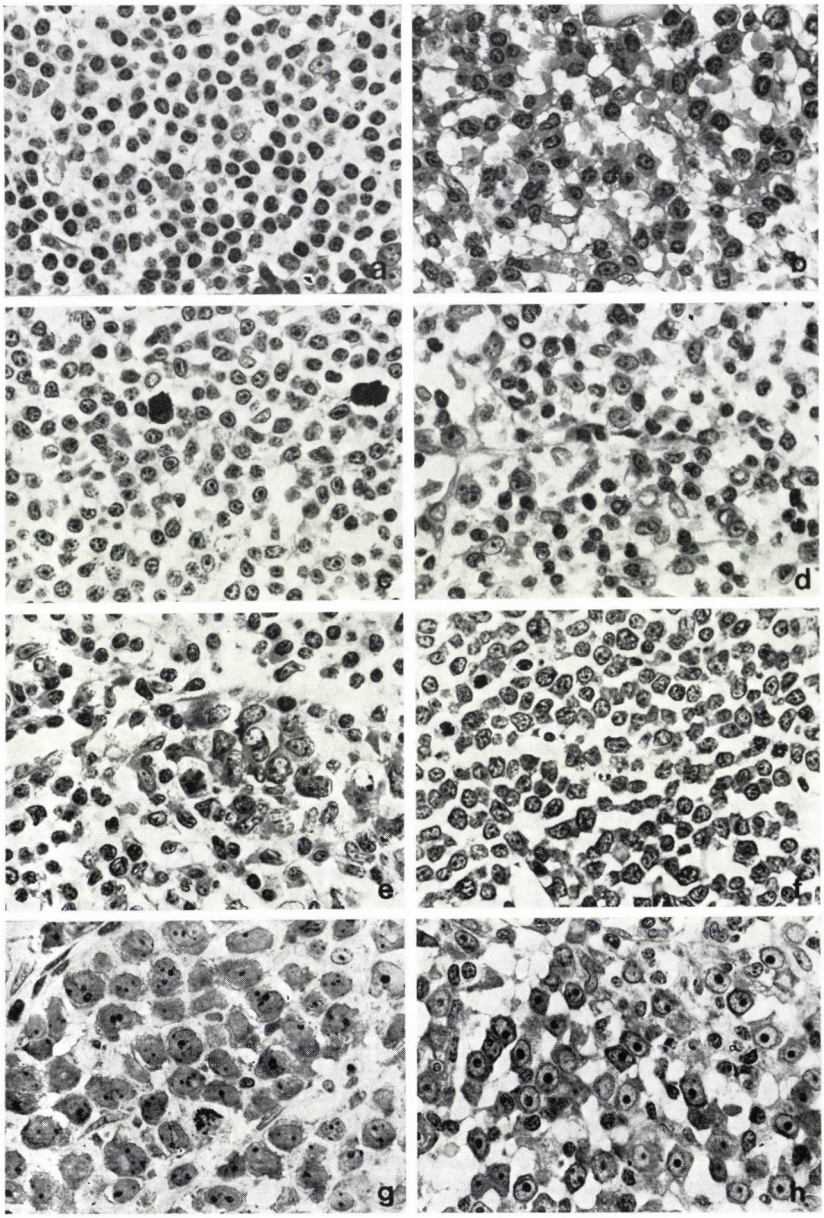


Fig. 4. Histologic types of ML in the bone marrow (Kiel classification) Giemsa, × 360; *a*. lymphocytic; *b*. hairy cell; *c*. immunocytic; *d*. centrocytic; *e*. centroblastic/centrocytic; *f*. lymphoblastic; *g*. centroblastic; *h*. immunoblastic

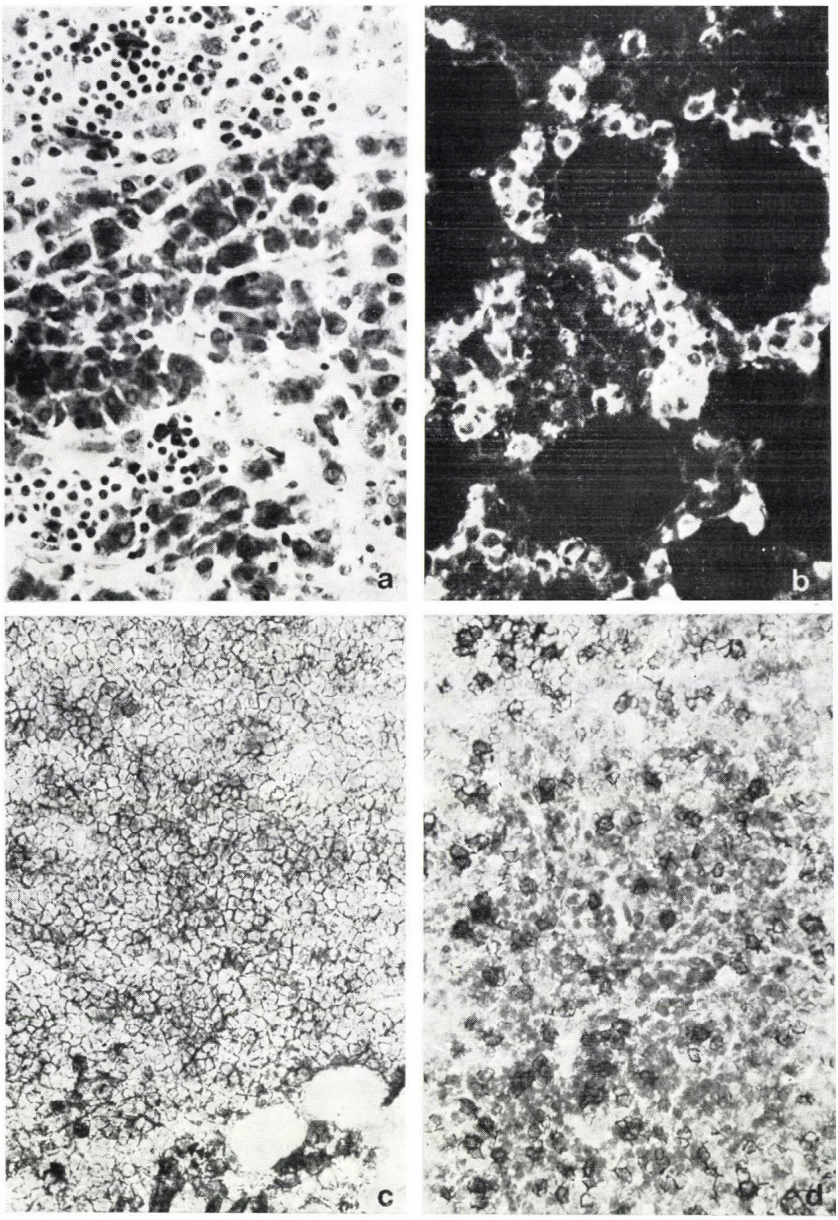


Fig. 5. Histology on bone marrow cryostat sections: *a*. histochemistry: MM, acid phosphatase, × 250; *b*. immunohistology: early stage of MM-IgA type, FITC, anti-IgA, × 250; *c*. immunohistochemistry: CLL-B cell type, PAP, anti-IgM, × 250; *d*. immunohistochemistry: CLL-B cell type, PAP, anti-T × 250

criteria for malignancy; only nucleo-cytoplasmic asynchrony was indicative of MM. The histologic growth pattern proved to be the most reliable characteristic for distinguishing between reactive and malignant plasmacytosis, except in very early cases of MM in which immunohistology was required to establish monoclonality of the plasma cells (Fig. 5).

MM plasmablastic. The myeloma cells were large, polymorphic, often multinuclear, with prominent central nucleoli, moderate to large amounts of cytoplasm, and showed numerous mitotic figures. Mature plasma cells, lymphocytes and immunoblasts were scattered among the plasmablasts. Fat and haematopoiesis were scanty, as the most frequent growth pattern was the packed marrow type (Table 3). The rapid downhill course (Table 2) was aggravated by renal insufficiency and hypercalcaemia.

Classification of NHL by bone marrow histology

Using the criteria of the Kiel classification derived from lymph node histology, the bone marrow biopsies were classified as follows (Figs 1 and 4).

ML lymphocytic (CLL). The bone marrow involvement was characterized by infiltration with typical small lymphocytes (indistinguishable from normal cells) showing three growth patterns with prognostic significance: 1. interstitial, 2. interstitial and nodular, and 3. packed marrow (Table 3). Haematopoietic tissue and fat were reduced as the tumour cell burden increased and this constituted a reliable prognostic factor in all cases (Table 4). Nodules with follicular centres were present in 25 per cent of the biopsies. There was some variation in lymphocyte size and the number of nucleolated cells; a higher proportion of either indicated a poorer prognosis (Table 2). In six cases the lymphoid cells were large with nucleoli, moderate amounts of cytoplasm and a positive acid phosphatase reaction: ML prolymphocytic [20]. The bone marrow showed extensive replacement with a corresponding reduction in normal elements. The prognosis was poor (Table 2).

Differential diagnosis: The distinction between benign lymphoid nodules and lymphomatous infiltration cannot always be made on the basis of morphology alone [21]. Benign lymphoid hyperplasia was found in 8 per cent of our 30 000 biopsies, with a higher incidence in the older age groups, 36 per cent in patients over 70 years [22]. The only reliable distinction between monoclonal and reactive lymphoid infiltrates is by means of immunohistology on cryostat sections of bone marrow biopsies [23, 24].

ML hairy cell. Bone marrow involvement varied from patchy to complete replacement by hairy cells: lymphoid cells with abundant cytoplasm frequently showing lateral extensions and rod-like inclusion bodies (in 45 per cent of the cases). The infiltrations also contained plasma cells, lymphocytes, mast cells and extravasated erythrocytes within a network of reticulin. When examined under high power, the hairy cell nuclei displayed a wide morphologic spectrum with

three main configurations, one of which usually predominated in each biopsy and had a predictive value: type I (in 47 per cent of the cases) with small ovoid nuclei; type II (37 per cent) medium sized convoluted nuclei, and type III (16 per cent) large indented nuclei, usually with a single prominent nucleolus (Table 2, Fig. 1). There were three infiltration patterns: 1. small multiple patches, 2. large confluent areas, and 3. complete replacement. The greater the tumour cell burden, the shorter the survival (Table 2). A high incidence of inclusion bodies in any of the subtypes indicated a poor prognosis. Splenectomy significantly prolonged survival in patients displaying ovoid and convoluted types.

Differential diagnosis: This includes ML lymphocytic, ML centrocytic, ML centroblastic/centrocytic, and proliferations of monocytes and histiocytes. When evaluating sections of undecalcified and plastic embedded biopsies, the distinction usually did not present difficulties.

ML immunocytic. The bone marrow was generally hypercellular with a marked reduction in fat cells. The specific infiltration comprised mainly small lymphocytes with variable numbers of mature plasma cells, plasmacytoid cells and mast cells. Nucleolated lymphoid cells were rare, but most cases had some lymphoid cells with cytoplasmic or nuclear PAS-positive inclusions. Three subtypes with significant prognostic differences were distinguished: 1. the lymphoplasmacytoid (49 per cent) showing mainly a nodular growth pattern and splenomegaly; 2. the lymphoplasmacytic (46 per cent) with numerous plasma and mast cells and a combined interstitial and nodular bone marrow pattern and lymphadenopathy; 3. the polymorphous subtype (5 per cent) composed of lymphocytes, plasma cells, centrocytes, centroblasts and immunoblasts and exhibiting the packed marrow pattern with lymphadenopathy, splenomegaly and pancytopenia. In all types the extent of infiltration in the biopsy correlated with the survival time (Table 4). There was a fine reticulin fibrosis in areas of infiltration, and haematopoietic precursors were found in the infiltrations as well as in the areas between them. Clinically 89 per cent of the patients had IgM paraproteinaemia, the disease thus corresponding to Waldenström's macroglobulinaemia.

Differential diagnosis: In cases in which the intranuclear or cytoplasmic inclusions of PAS-positive material were not immediately obvious, the picture often resembled that of ML lymphocytic. However, mast cells are not numerous in the latter type.

ML centrocytic. The infiltration consisted of small to medium sized lymphoid cells whose nuclei were cleaved and possessed narrow rims of cytoplasm. About 90 per cent of the biopsies demonstrated a paratrabecular growth pattern. There was moderate fibrosis within the infiltrations, the fibres radiating out from the trabeculae. Between these paratrabecular seams the marrow spaces were occupied by fat cells and residual haematopoietic precursors. The size and polymorphism of the infiltrating cells were used for subtyping: small cleaved (46 per cent), large cleaved (43 per cent), and polymorphous (11 per cent). The subtype classification and the extent of infiltration in the biopsy both showed significant correlations with survival (Tables 2 and 4).

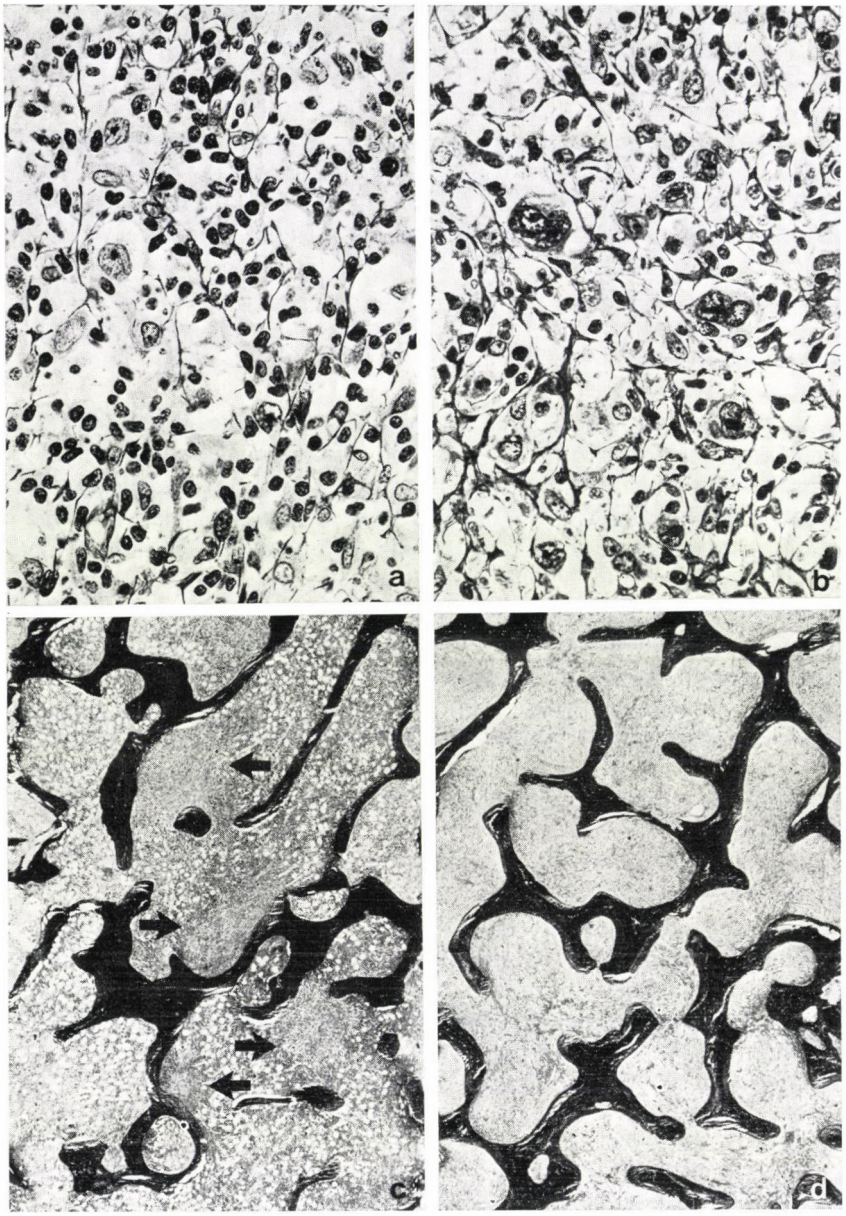


Fig. 6. Bone marrow histology in HD: *a.* high content of lymphocytes with few Hodgkin cells. Gomori, × 400; *b.* low content of lymphocytes with many Hodgkin and RS cells. Gomori, × 400; *c.* multiple focal infiltration (arrows) of the bone marrow. Gomori, × 40; *d.* total replacement of the bone marrow. Gomori, × 40

Differential diagnosis: The greatest difficulty was encountered in distinguishing ML centrocytic from lymphoblastic and monocytic leukaemias. In contrast, the delineation of ML centrocytic from CLL and immunocytoma was easy, as neither of these malignancies consisted of a 'pure population' of proliferating cells like ML centrocytic.

ML centroblastic/centrocytic. The pattern of bone marrow involvement was strictly nodular, and many of these nodules were follicles with germinal centres consisting of centrocytes, lymphocytes and centroblasts within a fine reticular network. The bone marrow between the nodules appeared normal in structure and cellular composition. Subtyping of this lymphoma was not possible. With 50 months, it had the longest median survival of all the lymphomas with bone marrow involvement.

Differential diagnosis presented difficulties only in patients with minimal bone marrow involvement when nodular lymphoid hyperplasia had to be taken into consideration.

ML lymphoblastic (only sarcoma type, [16]), *ML centroblastic* [8], and *ML immunoblastic* [5]. The few patients in each of these groups showed similar clinical and histologic features. All had a packed marrow pattern, with a very unfavourable course (median survival of 5 months for all three).

Classification of HD by bone marrow histology

The recognized diagnostic criteria [25] were used. The initial diagnosis required Reed–Sternberg (RS) cells within a granulomatous stroma, while atypical mononuclear cells within a suitable cellular environment were taken as evidence of bone marrow involvement when HD had already been diagnosed. Biopsies with epithelioid cell granulomas, foci of fibrosis, or lymphoid nodules were designated as negative. The pattern of bone marrow involvement was either focal (69 per cent) or diffuse with complete replacement (31 per cent) (Fig. 6). The foci were generally multiple, partly confluent and 1 to 4 mm in size. RS cells were found in 62 per cent and mononuclear Hodgkin cells in 95 per cent of the involved cases. The granulomatous tissue showed marked variations in fibrosis, vasculature and cellular composition both in the same biopsy and from patient to patient. Only the degree of lymphocytic infiltration proved to be a significant and reliable predictive factor; a low content of lymphocytes indicating a much shorter life expectancy than a high content (median survivals of 5 and 54 months, respectively) (Table 2, Figs 1 and 6).

Differential diagnosis: Other conditions such as angioimmunoblastic lymphadenopathy, malignant histiocytosis and systemic mastocytosis may present pictures similar to that of HD in the bone marrow, though generally without the typical RS or Hodgkin cells.

Discussion

Classification of LPD by bone marrow histology

Though the modern trend is towards an immune-based classification which takes into consideration the functional capabilities of the cell lines involved, morphology remains the bed-rock of histopathology, and in many cases the differentiation between B and T lymphocytes may be made on that basis alone, as well as distinctions between the various descendants of the B cell which constitute the majority of the malignant lymphomas [26]. In morphologic classification of the NHL, the histologic assessment may involve estimation of the relative proportions of several cell types as well as the identification of the predominant one according to which it is classified. In the B cell lymphomas such a cellular composition may be regarded as reflecting the diverse structural forms of a single neoplastic clone. As pointed out by Taylor et al. [27], each neoplasm thus derived always includes cells in the four different stages of the developmental pathway (lymphocytes, follicular centre cells, immunoblasts and plasma cells), but their relative proportions differ in the various entities. This view has received increasing support from immunologic studies: such as the detection of circulating monoclonal B lymphocytes in the blood of patients with myeloma or lymphomas [28–30], and the demonstration of monoclonal cytoplasmic immunoglobulins in leukaemic B lymphocytes from patients with CLL [31].

The present results show that histologic classification on the basis of bone marrow histology is feasible, reproducible and has prognostic significance. The criteria of the Kiel classification could readily be applied to bone marrow sections and no difficulty was encountered in their recognition. This classification does not encompass separation of the NHL by B and T cell origin, however (as seen in the NHL considered in this study), it has not been proved that this had any therapeutic or prognostic consequences in the chronic LPD. The survival curves of the patients closely paralleled those of comparable groups reported by the Kiel Lymphoma Study Group [3].

Sub-type classification of LPD in the bone marrow

The morphologic diversity which the members of a single B cell clone may assume helps to explain the mixed populations of cells seen in the bone marrow infiltrations which enabled sub-type recognition each of the chronic LPD [32, 33]. The question still remains whether, at least in some cases, there may be reactive cells (polyclonal lymphocytes and plasma cells, suppressor and helper cells) together with the neoplastic clone [34]. The question is currently under investigation by means of immunologic and marker studies.

The developmental sequences for the maturation of the B lymphocytic series, deduced originally from the morphologic types present in lymph node follicles, are to some extent still speculative. For example, an electron micro-

scopic study of the structural changes undergone by lymphocytes during transformation in vitro, did not confirm a pathway involving the so-called cleaved cell [35]; acid hydrolases used as markers of mature cells in the chronic LPD are also present in some lymphoblasts and prolymphocytes [36]; and studies examining the link between immunoglobulin secretion and the developmental stage of the neoplastic B cell, have demonstrated that centrocytes may precede or follow the centroblasts in the maturation of B cells [37]. These observations indicate that the strict categorization of cells to specific steps in the maturation sequence may be somewhat arbitrary. Thus, the variable mixtures of cells in the sub-types noted in chronic LPD may account for the different responses to therapy of superficially similar LPDs; or the simultaneous occurrence of HCL and MM indicating divergent differentiation from a common B cell precursor [38]; or the evolution of Sezary syndrome in the course of HCL [39]. Moreover, since 'blasts' are normally present in chronic LPD, a 'transformation' such as Richter's in CLL or B-immunoblastic sarcoma in MM points to a shift in predominance of one cell type already present over another [40, 41]. Of particular interest would be to identify the factor(s) which lead to a proliferation advantage for the 'blasts' thus tipping the balance in their favour. Another intriguing problem is posed by the termination of CLL in acute myeloid leukaemia in untreated patients [42]. Whether this represents a change of phenotype, the emergence of a new clone, or an unrelated second malignancy is not known. Hybrid leukaemic cells in CLL have been described with T lymphocytic and myelo-monocytic features [43]. Furthermore, the simultaneous presentation of acute myelo-monocytic leukaemia and MM has recently been reported [44].

Extent of marrow involvement – total tumour mass

Estimation of tumour cell burden (total tumour mass) is one of the aims of staging [45]. Our results show that in the chronic LPD the volume percentage in the bone marrow reflects the tumour cell burden and is a reliable prognostic indicator. It should, however, be borne in mind that lymphoid cells may be produced in the marrow, may circulate through it, and may 'home' to it: the cell population in the bone marrow is not a static one [46, 47]. Nevertheless, there also appears at least in MM to be some connection between tumour cell burden and circulating neoplastic cells because the incidence of the latter rises as the tumour cell burden increases, possibly due to occupation and replacement of the marrow cavities by the slowly cumulative LPD [30].

Growth pattern of LPD in the bone marrow

The results presented in this report have confirmed and extended previous observations [9, 48–56] on the mode of spread of the MM, NHL and HD in the bone marrow, as well as on the prognostic significance of the different patterns [3, 32, 57–63] in a large series of patients. In evaluating the growth patterns

of the lymphoid malignancies in the bone marrow a number of considerations is pertinent. These include: 1. the inherent behavioural tendency of the cells involved to aggregate or congregate, as various embryonic cells will reaggregate after artificial disaggregation in vitro; 2. the property to form a strict architectural pattern as in their natural habitat, the lymph node; 3. some topographic influence or chemotactic attraction to specific areas. The first and second are closely linked, though not identical. The nodular lymphoma pattern falls into this category, it may be said to mimic a lymph node structure; indeed it is only quantitatively harmful as shown by its morphologic equivalent the so-called benign lymphoid hyperplasia in the bone marrow. In this respect it might be called 'lymphoma' in the strict sense of the word and, as shown by Rappaport [64], it has the most favourable prognosis. The diffuse growth pattern (where the same type of lymphoma is concerned) would then indicate that the cells lack a corresponding characteristic but whether this implies a separate sub-set or a different stage of development, cannot be answered at present. Why the centrocytic lymphomas should show a paratrabecular localization is equally unclear, especially if they really are the equivalent of the follicular centre cells, as there is nothing in the lymph node corresponding to the trabeculae of cancellous bone. Perhaps an association with the paratrabecular sinus is involved. This, however, is normally the granulocytic generation zone. The red and white blood cells and platelet precursors all have preferred topographic localizations, but there is no known predilection site for lymphopoiesis in the human bone marrow. Another factor may also play a role in all bone marrow involvements showing focal lesions. It has been shown in vitro that a cluster of cells which has settled in a comparatively non-resistant environment will tend to assume a spherical shape and expand symmetrically outwards [65]. This is seen in many metastases of solid tumours to the bone marrow and may also be operative in NHL and HD. Such lesions may also grow by accretion, the addition of cells from the surroundings, or outgrowths of some particular cellular component(s) from within. But whatever the reason, chronic LPD in the marrow exhibits characteristic modes of spread which contribute to its classification and prognostic evaluation.

Our results clearly demonstrate that at first presentation a high percentage of patients with LPD have positive bone marrow biopsies (MM 90 per cent, NHL 70 per cent, HD 10 per cent) indicating systemic disease. Indeed this fact may be an important reason for establishing bone marrow biopsy as an initial diagnostic and staging procedure in all patients with suspected LPD. We have further shown that the widely practiced classification systems based on lymph node histology (Rappaport [64]), Lukes [12], Lennert [11] are reproducible in the bone marrow and have prognostic significance in MM, NHL and in HD. The growth patterns in the bone marrow contribute additional and independent prognostic information and should be included in any classification system of LPD in the bone marrow. Therefore we conclude that bone marrow biopsy is a useful diagnostic tool for histologic classification and clinical staging of any patient with LPD.

*

The authors are greatly indebted to all colleagues who referred patients or sent biopsies. The skilful assistance of the technical staff of the laboratory, especially of Mrs. W. Sommerfeld and Mr. B. Buchenrieder, is gratefully acknowledged. Thanks are also due to Mrs. I. Weltmeier, Mrs. G. Huber and Mrs. I. Pascher. This work was supported by the Gesellschaft für Strahlen- und Umweltforschung mbH, Munich.

References

1. Come, S. E., Chabner, B. A.: Staging in non-Hodgkin's lymphoma: Approach, results and relationship to histopathology. In: *Clinics in Haematology*. Canellos (ed.) Saunders, Philadelphia 1979, vol. 8, p. 645.
2. Chabner, B. A., Fischer, F. J., Young, R. C., De Vita, V. T.: Staging of non-Hodgkin's lymphomas. In: *Hodgkin's and Non-Hodgkin's Lymphomas*. Coltman, Golomb (eds). Grune & Stratton, New York 1980, p. 193.
3. Bartl, R., Frisch, B., Burkhardt, R., Kettner, G., Fateh-Moghadam, A., Sund, M.: Assessment of bone marrow histology in the malignant lymphomas (non-Hodgkin's). Correlation with clinical factors. *Brit. J. Haemat. 51*, 511 (1982).
4. Fischer, R. I., Jones, R. B., DeVita, V. T., Simon, R. M., Garvin, A. J., Berard, C. W., Young, R. C.: Natural history of malignant lymphomas with divergent histologies at staging evaluation. *Cancer 47*, 2022 (1981).
5. Bartl, R., Burkhardt, R., Frisch, B., Hill, W.: Extent of bone marrow infiltration in the bone marrow biopsy and prognostic factor in haematologic malignancies. In: *Proc. XI. Triennal World Congress of WASP, Jerusalem 1981.*
6. Burkhardt, R., Zettl, R., Bartl, R.: Significance of non-specific changes of the bone marrow tissues from the bioptic viewpoint. *Bibl. Haemat. 45*, Karger, Basel 1978, p. 38.
7. Velde, J.te, Ottolander, J. G. den, Spaander, P. J., Berg, C. van den, Hartgrink-Groeneveld, C. A.: The bone marrow in Hodgkin's disease: the non-involved marrow. *Histopathology 2*, 31 (1978).
8. Coller, B. S., Chabner, B. A., Gralnick, H. R.: Frequencies and patterns of bone marrow involvement in non-Hodgkin's lymphomas: observation on the value of bilateral biopsies. *Amer. J. Haemat. 3*, 105 (1979).
9. Brunning, R. D., McKenna, R. W.: Bone marrow manifestations of malignant lymphoma and lymphoma-like conditions. In: *Pathology Annual*. Sommer, Rosen (eds). Appleton-Century-Crofts, New York 1979, part I, p. 1.
10. Paredes, J. M., Mitchell, B. S.: Multiple myeloma. Current concepts in diagnosis and management. *Med. Clin. N. Amer. 64*, 729 (1980).
11. Lennert, K., Stein, H.: *Histopathology of Non-Hodgkin's Lymphomas (based on the Kiel Classification)*. Springer, Berlin 1981.
12. Lukes, R. J., Butler, J. J.: The pathology and nomenclature of Hodgkin's disease. *Cancer Res. 26*, 1063 (1966).
13. Burkhardt, R.: *Bone Marrow and Bone Tissue. Colour Atlas of Clinical Histopathology*. Springer, Berlin 1971.
14. Jamshidi, K., Swaim, W. R.: Bone marrow biopsy with unaltered architecture: a new biopsy device. *J. Lab. Clin. Med. 77*, 335 (1971).
15. Burkhardt, R.: Bone marrow histology. In: *Methods in Haematology. The Leukemic Cell*. D. Catovsky (ed.). Churchill Livingston, Edinburgh 1981, p. 49.
16. Bartl, R., Burkhardt, R., Vondracek, H., Sommerfeld, W., Hagemeister, E.: Rationelle Beckenkamm-Biopsie. Längsteilung der Proben zur Anwendung von mehreren Präparationsverfahren ohne Materialverlust. *Klin. Wschr. 56*, 545 (1978).
17. Hoffmann-Fezer, G., Thierfelder, S., Pielsticker, K., Rodt, H.: Immunohistochemical demonstration of cell surface antigens on tissue sections of lymphomas. *Leuk. Res. 3*, 297 (1979).

18. Hennig, A.: Kritische Betrachtungen zur Volumen- und Oberflächenmessung in der Mikroskopie. *Zeiss Werkzeitschr. 30*, 78 (1958).
19. Dixon, W. J., Brown, M. B.: *BMDP-79 Biomedical Computer Programs p-series. System, Program and Statistical Development.* University of California Press, Berkeley 1979.
20. Galton, D. A. G., Goldman, J. M., Wiltshaw, E., Catovsky, D., Henry, K., Goldenberg, G. J.: Prolymphocytic leukaemia. *Brit. Haemat. 27*, 7 (1974).
21. Rywlin, A. M., Ortega, R. S., Dominguez, C. J.: Lymphoid nodules of bone marrow: normal and abnormal. *Blood 43*, 389 (1974).
22. Bartl, R., Burkhardt, R., Schlag, R., Hill, W.: Nodular lymphoid hyperplasia and malignant lymphoma in the bone marrow biopsy in advanced age. In: *Proc. 6th Meet. Int. Soc. Haematology, Athens 1981.*
23. Pizzolo, G., Chilosio, M., Cetto, G. L., Fiore-Sonati, L., Janossy, G.: Immuno-histological analysis of bone marrow involvement in lymphoproliferative disorders. *Brit. J. Haemat. 50*, 95 (1982).
24. Wood, G. S., Warnke, R. A.: The immunologic phenotyping of bone marrow biopsies and aspirates: Frozen section techniques. *Blood 59*, 913 (1982).
25. Lukes, N. J.: Criteria for involvement of lymph node, bone marrow, spleen and liver in Hodgkin's disease. *Cancer Res. 31*, 1755 (1971).
26. Wright, D. H.: The identification and classification of non-Hodgkin's lymphoma: A review. *Diagn. Histopath.* 73 (1982).
27. Taylor, C. R., Parker, J. W., Pattengale, P. K., Lukes, R. J.: Malignant lymphomas: an exercise in immunopathology. Leukaemia and non-Hodgkin-lymphoma. In: *Medical Oncology. Research and Education.* D. G. Growther (ed.). Pergamon Press, Oxford 1979, vol. 7, p. 125.
28. Mellstedt, H., Pettersson, D., Holm, G.: Monoclonal B-lymphocytes in peripheral blood of patients with plasma cell myeloma. *Scand. J. Haemat. 16*, 112 (1976).
29. Ault, K. A.: Detection of small numbers of monoclonal B lymphocytes in the blood of patients with lymphoma. *New Engl. J. Med. 300*, 1401 (1979).
30. Pettersson, D., Mellstedt, H., Holm, G., Björkholm, M.: Monoclonal blood lymphocytes in benign monoclonal gammopathy and multiple myeloma, in relation to clinical stage. *Scand. J. Haemat. 27*, 287 (1981).
31. Han, T., Ozer, H., Bloon, M., Sagawa, K., Minowada, J.: The presence of monoclonal cytoplasmic immunoglobulins in leukaemic B cell from patients with chronic lymphocytic leukaemia. *Blood 69*, 435 (1982).
32. Bartl, R., Frisch, B., Burkhardt, R.: *Bone Marrow Biopsies Revisited. A New Dimension for Haematologic Malignancies.* Karger, Basel 1982.
33. Bartl, R., Frisch, B., Hill, W., Burkhardt, R., Sommerfeld, W., Sund, M.: Bone marrow histology in hairy cell leukaemia: Identification of sub-types and their prognostic significance. *Amer. J. Clin. Path. 79*, 531 (1983)
34. Taylor, C. R.: *Immunopathology of Hodgkin's Disease.* In: *Malignant Lymphoproliferative Diseases*, van den Tweel et al. (eds). University Press, Leiden 1980, p. 399.
35. Dardick, I., Setterfield, G., Hall, R., Bladon, T., Little, J., Kaplan, G.: Nuclear alterations during lymphocyte transformation. Relationship to the heterogeneous morphologic presentations of non-Hodgkin's lymphomas. *Amer. J. Path. 103*, 10 (1981).
36. Grossi, E. E., Zicca, A., Leprini, A., Cadoni, A., Pilstoia, V., Ferrarini, M.: Acid hydrolases as markers of maturation in B-cell chronic lymphocytic leukaemia. *Blood 60*, 220 (1982).
37. Hannam-Harris, Gordon, J., Wright, D. H., Smith, J. L.: Correlation between Ig-synthesis patterns and lymphoma classification. *Brit. J. Cancer 46*, 167 (1982).
38. Catovsky, D., Costello, C., Loukopoulos, D., Fessas, P. R., Poxley, J. M., Traub, N. E., Mills, M. J., O'Brien, M.: Hairy cell leukaemia and myelomatosis; Change association or clinical manifestations of the same B-cell disease spectrum. *Blood 57*, 758 (1981).
39. Zucker-Franklin, D., Amorosi, E. L., Ritz, N. D.: Evolution of Sézary syndrome in the course of hairy cell leukaemia. *Blood 59*, 1181 (1982).

40. Harousseau, J. L., Flandrin, G., Tricot, G., Brouet, J. C., Seligmann, M., Bernard, J.: Malignant lymphoma supervening in chronic lymphocytic leukaemia and related disorders. Richter's syndrome: A study of 25 cases. *Cancer 48*, 1302 (1981).
41. Falini, B., De Solas, I., Levine, A. M., Parker, J. W., Lukes, R. J., Taylor, C. R.: Emergence of B-immunoblastic sarcoma in patients with multiple myeloma: A clinicopathologic study of 10 cases. *Blood 59*, 923 (1982).
42. Stern, N., Shemesh, J., Ramot, B.: Chronic lymphatic leukaemia terminating in acute myeloid leukaemia: Review of the literature. *Cancer 47*, 1849 (1981).
43. Schlimok, G., Thiel, E., Rieber, E. P., Huhn, D., Feucht, H., Lohmeyer, J., Riethmüller, G.: Chronic leukaemia with a hybrid surface phenotype (T lymphocytic/myelomonocytic): Leukemic cells displaying natural killer activity and antibody-dependent cellular cytoxicity. *Blood 59*, 1157 (1982).
44. Parapia, L., Abbott, C. R., Masters, G., Roberts, B. E.: Simultaneous presentation of acute myelomonocytic leukaemia and multiple myeloma. *Acta Haemat. 68*, 153 (1982).
45. Jaksic, B., Vitale, B.: Total tumour mass score (TTM): a new parameter in chronic lymphocytic leukaemia. *Brit. J. Haemat. 49*, 404 (1981).
46. Patinkin, D., Grover, N. B., Yoffey, J. M.: The lymphocyte production pathway in bone marrow: Possible significance of the size spectrum of lymphocytes and their precursors. *Brit. J. Haemat. 41*, 309 (1979).
47. Sousa, M. de: *Lymphocyte Circulation: Experimental and Clinical Aspects*. Wiley, Chichester 1981.
48. Canale, D. D., Collins, R. D.: Use of bone marrow particle sections in the diagnosis of multiple myeloma. *Amer. J. Clin. Path. 61*, 383 (1974).
49. Bartl, R., Burkhardt, R., Gierster, P., Sandel, P., Fateh-Moghadam, A.: Significance of bone marrow biopsy in the multiple myeloma. In: *Recent Progress in Cell Biology: Leukocytes and Platelets*. Ulutin (ed.), Karger, Basel 1978.
50. Georgii, A.: Classification of non-Hodgkin's lymphomas from biopsies of the bone marrow with special emphasis to their spread. In: *Leukaemia and Non-Hodgkin's Lymphoma*. D. G. Growther (ed.). Pergamon Press, Oxford 1979, p. 179.
51. Foucar, K., McKenna, R. W., Frizzera, G., Brunning, R. D.: Incidence and patterns of bone marrow and blood involvement by lymphoma in relationship to the Lukes–Collins classification. *Blood 54*, 1417 (1979).
52. Foucar, K., McKenna, R. W., Frizzera, G., Brunning, R. D.: Bone marrow and blood involvement by lymphoma in relationship to the Lukes–Collins classification. *Cancer 49*, 888 (1982).
53. Baroni, C. D., Manente, L., Occhionero, M., Marzullo, A., Mandelli, F., Biagini, C.: Involvement of the bone marrow by non-Hodgkin's lymphomas: Incidence, histology and pathologic correlations. *Tumori 67*, 191 (1981).
54. Bartl, R., Burkhardt, R., Lengsfeld, H., Huhn, D.: Die Bedeutung der histologischen Knochenmarksbeurteilung bei Morbus Hodgkin. *Klin. Wschr. 54*, 1061 (1976).
55. O'Carroll, D. I., McKenna, R. W., Brunning, R. D.: Bone marrow manifestations of Hodgkin's disease. *Cancer 38*, 1717 (1976).
56. Colby, T. V., Hoppe, R. T., Warnke, R. A.: Hodgkin's disease at autopsy: 1972–1977. *Cancer 47*, 1852 (1981).
57. Wutke, K., Várbiró, M., Rüdiger, K. D., Kelényi, G.: Cytological and histological classification of multiple myeloma. *Haematologia 14*, 315 (1981).
58. Rozman, C., Hernandez-Nieto, L., Montserrat, E., Brugues, R.: Prognostic significance of bone marrow patterns in chronic lymphocytic leukaemia. *Brit. J. Haemat. 47*, 529 (1981).
59. Damber, L., Lenner, P., Lundgren, E.: The impact of growth pattern on survival in non-Hodgkin's lymphomas classified according to Lukes and Collins. *Path. Res. Pract. 174*, 42 (1982).
60. Burkhardt, R., Frisch, B., Bartl, R.: Bone biopsy in haematological disorders. *J. Clin. Path. 35*, 257 (1982).

61. Bartl, R., Frisch, B., Burkhardt, R., Hoffmann-Fezer, G., Demmler, K., Sund, M.: Assessment of marrow trephine in relation to staging in chronic lymphocytic leukaemia. *Brit. J. Haemat. 51*, 1 (1982).
62. Bartl, R., Frisch, B., Burkhardt, R., Huhn, D., Pappenberger, R.: Assessment of bone marrow histology in Hodgkin's disease: Correlation with clinical factors. *Brit. J. Haemat. 51*, 345 (1982).
63. Bartl, R., Frisch, B., Burkhardt, R., Fateh-Moghadem, A., Gierster, P., Sund, M., Kettner, G.: Bone marrow histology in myeloma: Its importance in diagnosis, prognosis, classification and staging. *Brit. J. Haemat. 51*, 371 (1982).
64. Rappaport, H.: Tumors of the hematopoietic system. In: *Atlas of Tumor Pathology*. Armed Forces Institute of Pathology, Washington 1966, vol. 8. Section 3.
65. Schwartz, R. S., Greenberg, P. L.: Stromal colony-stimulating activity production and myeloid colony forming cells in human hemopoietic and non-hemopoietic bone marrow. *Blood 57*, 771 (1981).

Haematologia 17 (2), pp. 247–257 (1984)

Immunohistological Analysis of Bone Marrow Involvement in Lymphoproliferative Disorders. The Use of Cryostat Sections from Trephine Biopsies*

G. PIZZOLO,[1] M. CHILOSI,[1] L. FIORE-DONATI,[1] G. JANOSSY[2]

Cattedra di Ematologia and Istituto di Anatomia Patologica, University of Verona, 37134 Verona, Italy[1]; Department of Immunology, Royal Free Hospital, School of Medicine, London, UK[2]

(Received 5 December 1982; accepted 10 January 1983)

Cryostat sections from frozen samples of human bone marrow trephine biopsies were used for immunohistochemical analysis. Immunoperoxidase and immunofluorescence techniques as well as enzyme histochemistry were applied to these sections in order to characterize bone marrow infiltrates in lymphoproliferative diseases. Various conventional antisera and monoclonal antibodies and several enzymes were able to identify different normal and malignant cell populations. This technology provides a new tool to be used in combination with classical morphology for studying bone marrow involvement during lymphoid malignancies.

Keywords: bone marrow, cryostat sections, histochemistry, immunofluorescence, immunoperoxidase

*

Recently developed immunohistological techniques have been applied by several authors to the study of normal and pathologic lymphoid tissues. These techniques included the use of immunofluorescence (IF) [1, 2], immunoperoxidase (IP) [3] or immuno-alkaline phosphatase [4], as well as enzyme histochemistry [5]. These methods distinguish different cell populations and tissue components by the virtue of antibody selectivity, a useful adjunct to classical morphology. A number of points have emerged when these techniques were used: *(a)* cryostat sections of frozen samples are the material of choice to detect cell membrane antigens using either conventional antisera or monoclonal antibodies; *(b)* both IF and IP (and more recently immuno-alkaline phosphatase) are suitable for tissue immunostaining; *(c)* cryostat sections also represent the material of choice to detect certain enzymes which do not survive after embedding procedures (e.g. ATPase, 5'nucleotidase and DAP IV). These methods can be applied to any tissue and the same principles apply for analysing bone marrow (BM).

In order to evaluate BM involvement in non-Hodgkin's lymphoma by IF and enzyme histochemistry the procedure for obtaining cryostat sections of unfixed non-decalcified trephine biopsies has been standardized in our and other laboratories [6–8]. In this paper we report now our experience with the use of BM cryostat sections for the immunohistological and histochemical analysis of BM involvement during lymphoproliferative diseases.

* Supported in part by a grant (No. 81.01817.04) from the Consiglio Nazionale delle Ricerche, Italy.

Materials and Methods

Bone marrow trephine biopsies were obtained with a 11-gauge Jamshidi needle in local anaesthesia from patients mainly as part of staging procedures for Hodgkin's and non-Hodgkin lymphomas. BM samples were cut with a razor blade so that two cylinders (generally 2 and 1 cm long) were obtained. The 2 cm long one was conventionally fixed and embedded in paraffin after decalcification whereas the 1 cm long one was soaked for 1–2 h in cold (4 °C) gum sucrose solution* or Histocon medium**. The soaked BM samples were then put on a cork, covered with a drop of O.C.T. (Ames), snap-frozen in liquid nitrogen and cut on a cryostat. Sections were stuck on albuminized glass slides and air dried for 2–4 h at room temperature before fixation. Various fixatives were tested for immuno-histochemistry. These included cold ethanol, acetone, chloroform-acetone (1 : 1), and 10 per cent buffered formalin. Fixation time was 10 min for all the fixatives tested. In each case myeloperoxidase and haematoxylin counterstaining were performed. Depending on the histological picture of the sample, various immunohistological and enzyme-histochemical methods were applied in order to answer specific diagnostic questions, as previously described [6, 7]. The studied enzymes and reagents are listed in Table 1.

Table 1

Enzyme histochemical reactions on bone marrow

Enzyme	Reagents	Marker for
Peroxidase	DAB, H_2O_2	myeloid cells
Chloracetate esterase	Naphthol AS-D chloroacetate	myeloid cells
Nonspecific esterase	Naphthylacetate HPR	monocytes and macrophages
Alkaline phosphatase	Naphthol AS-BI phosphate, HNF	fibroblastic cells, capillary endothelium
Acid phosphatase	Naphthol AS-BI phosphate, HPR	macrophages
ATPase	ATP, lead nitrate	B lymphocytes
5'nucleotidase	AMP, lead nitrate	B lymphocytes, dendritic cells
Dipeptidyl-amino-peptidase	gly-pro-methoxy naphthylamide (DAP–IV)	T lymphocytes, sinus lining cells

* 1 g gum acacia + 30 g sucrose dissolved in 100 ml distilled water

** Registered name of a fluid of non-specified composition (Polysciences, Inc., Warrington, PA)

Immunostaining

The conventional antisera and monoclonal antibodies used in our study are listed in Table 2.

Table 2

Conventional antisera and monoclonal antibodies for the immuno-histological study of bone marrow cryostat sections

Antisera	
anti-human IgM	from Nordic and Dakopatt
anti-human IgD	
anti-human IgA	
anti-human IgG	
anti-human κ	
anti-human λ	
Monoclonal antibodies	
2D1	ref. [2]
UCHT1	ref. [13]
anti-Leu-1	from Becton Dickinson
anti-Leu-2	
anti-Leu-3	
anti-HLA-DR	
OKT3	from Ortho
OKT4	
OKT8	

IF staining was carried out in 38 cases in which a lymphoid infiltrate, either nodular or diffuse, suggested a bone marrow involvement by non-Hodgkin lymphoma as judged by morphological examination.

Six specimens from patients with Hodgkin's disease were also investigated. IF staining was performed as previously described [1, 6]. Briefly, the sections were covered with monoclonal antibodies (0.1–0.2 μg in 10–20 μl fluid) or diluted antisera (1 : 20–1 : 40), incubated for 30 min and washed in buffered saline (PBS; 5–10 min). Antisera to human Ig isotypes were directly conjugated to fluorochromes fluorescein isothiocyanate (FITC, green) or tetra-ethyl rhodamine isothiocyanate (TRITC, red) and used in direct IF in various combinations (e.g. anti-k TRITC/anti-λ FITC). Monoclonal antibodies were used in indirect IF using a goat anti-mouse FITC or TRITC as second layer (from Nordic). Each step was followed by a quick washing (5–10 min) in PBS. After the final washing the sections were mounted with a drop of glycerol and coverslip and examined under a Leitz Dialux microscope equipped for epifluorescence. In order to prevent the fading of FITC, 0.2 per cent o-phenylene diamine was added to the mounting medium [9].

Thirty-five samples studied with IF were also investigated with the IP staining method on serial sections. Indirect IP and the peroxidase-antiperoxidase (PAP) methods described by Stein et al. [3] were used.

Prior to staining for IP the endogenous myeloperoxidase was inhibited with methanol-H_2O_2 solution or with 0.1 per cent phenylhydrazine in PBS for 30 min [10]. The sections were then incubated with unlabelled monoclonal antibodies and antisera followed by peroxidase conjugated rabbit-anti-mouse serum (Dakopatt) when monoclonal antibodies were used as first layer. Swine-anti-rabbit serum followed by PAP (Dakopatt) was used as second layer when rabbit antisera to human Ig isotypes were applied as first layers. Following each incubation the sections were gently washed in PBS and finally stained for peroxidase using 3,3 diaminobenzidine. A darkening copper sulphate solution was used as final step [11] before haematoxylin counterstaining (when needed) and mounting.

Results

Preparation of sections and fixation

Preliminary experiments showed that specimens directly frozen without any previous embedding were difficult to cut and suitable sections could not be obtained in a reproducible manner. In contrast, sections of good morphological quality could be prepared after soaking the samples in either of the embedding media.

As a fixative ethanol gave the best results when used for IF and some of the enzyme-histochemical reactions, e.g. peroxidase and DAP-IV. Buffered formalin was used for the other enzymes. Chloroform-acetone was the fixative of choice for IP staining especially when the PAP method was applied.

Histochemistry

All tested enzymes could successfully be demonstrated on cryostat sections as described previously [7]. The use of cryostat sections was particularly important with the enzymes DAP IV, ATPase and 5'nucleotidase which are inhibited or destroyed by paraffin embedding [5]. A list of cells characterized by different enzymes used as markers on bone marrow sections is given in Table 1. An example is shown in Figure 1.

Immunofluorescence

Using IF all tested antigens could be detected on BM cryostat sections without difficulty. In particular, surface Ig could be demonstrated on B cell infiltrates in non-Hodgkin's lymphoma cases even when cells expressed low levels of membrane associated Ig (e.g. cases of CLL, Figs 2 and 3). A monoclonal light chain expression (k or λ restriction) was evident in most of these cases. However, in some of

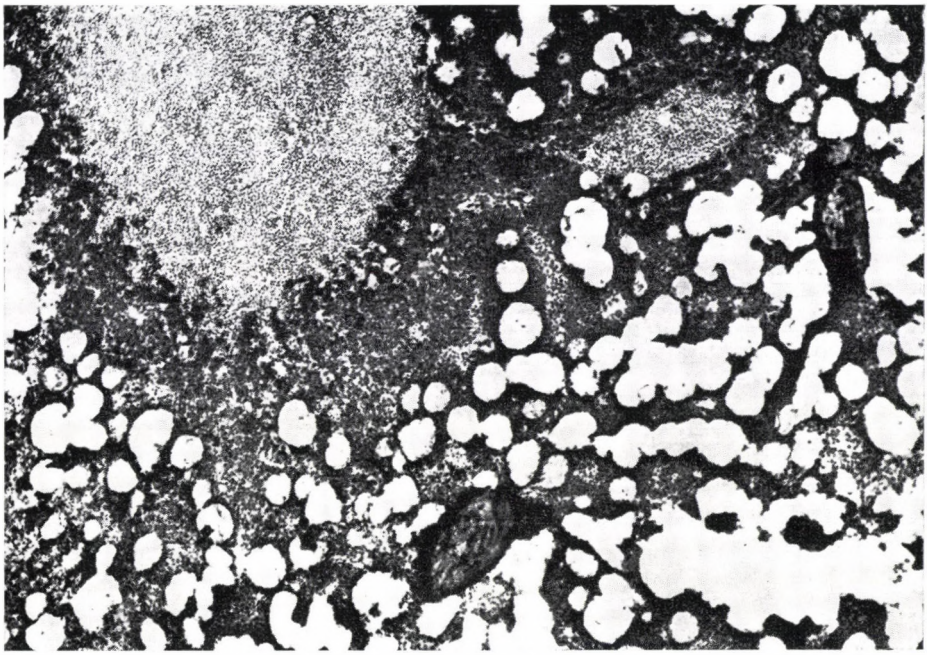


Fig. 1. Bone marrow cryostat section obtained during staging procedure of non-Hodgkin's malignant lymphoma. Two lymphoid nodules of different size can be distinguished from the peroxidase reactive haemopoietic tissue. Peroxidase histochemical staining and haematoxylin counterstaining. × 70

the tissue samples, especially when serum hypergammaglobulinemia was present, it was difficult to demonstrate a clear specific staining for surface Ig. This apparent lack of specificity was probably due to intercellular Ig deposits which are also stained by anti-Ig antisera and therefore masked the surface Ig. In these cases we were able to elute this 'floating' Ig background by washing the sections, prior to staining, in acetate buffer as described by Wood and Travers [12]. On the other hand, membrane positivity was always clear and specific when monoclonal antibodies against non-Ig products were used. Lymphoid cells, both normal and malignant, were clearly stained by 2D1 and B-cells were HLA-DR positive, whereas T-cells and their subsets were detected by the various anti-T monoclonal reagents (Table 2). When compared to staining with anti-Ig in the IP system, immunostaining for heavy and light immunoglobulin chains was better evaluable with direct IF, especially when antibodies conjugated to different fluorochromes were used in combination (e.g. anti-k and anti-λ). IF staining was therefore generally preferred for the characterization of neoplastic non-Hodgkin's lymphoma cells in involved BM samples and in the analysis of lymphoid nodules seen in BM during staging procedures.

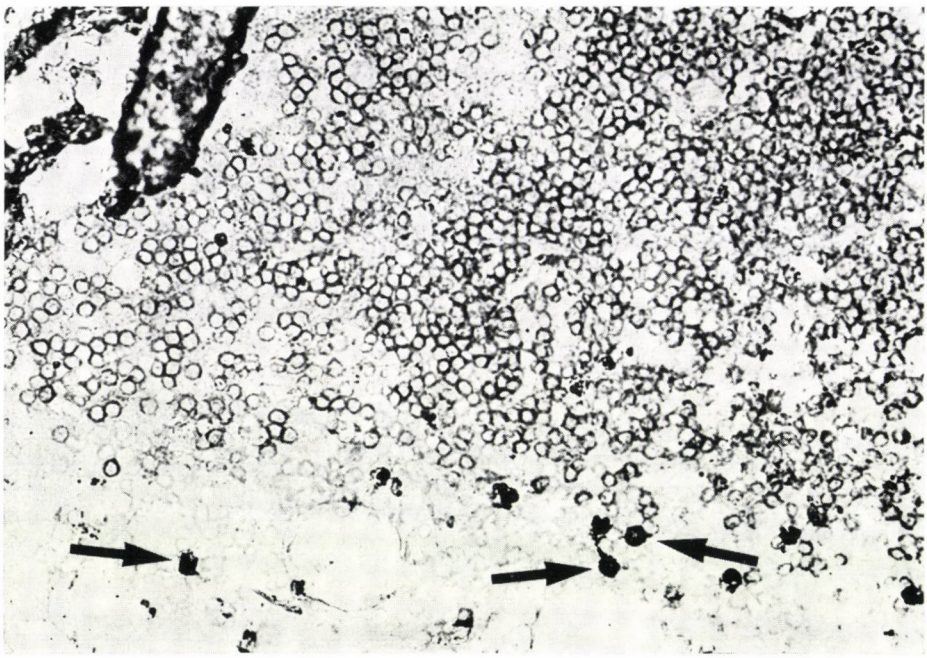


Fig. 2. Cryostat section of bone marrow biopsy involved by non-Hodgkin's lymphoma. A lymphoid nodule is immunostained with the PAP method showing a light chain monoclonality (λ^+). A strong peroxidase (non-immune) reaction is also evident in the eosinophils (arrows). PAP method, $\times 250$

In these cases the combination k/λ and IgM/UCHT1 (OKT3-like) or IgM/Leu-1 were mostly useful to establish B-cell monoclonality and the relationship between B and T cells (Fig. 5).

Immunoperoxidase

Owing to the presence of endogenous peroxidase on myeloid cells an inhibition of this enzyme was always mandatory before IP staining. The only exception was the heavy lymphoid infiltration which completely obliterated the haemopoietic tissue. As inhibition with methanol-H_2O_2 mixture was deleterious for most membrane antigens, 0.1 per cent phenylhydrazine in PBS was used. This compound did not affect the cell-antigen expression. In such conditions phenylhydrazine completely inhibited the endogenous peroxidase of neutrophilic cells. Only eosinophilic myeloid cells retained their strong peroxidase reaction. Nevertheless this staining was characteristic and it could be easily distinguished from specific immunostaining

Fig. 3. Cryostat section of a bone marrow biopsy diffusely infiltrated by neoplastic cells. In this case of chronic lymphocytic leukaemia most lymphocytes show surface staining for IgM. PAP method, × 250

(Figs 2 and 6). Using the PAP method on chloroform-acetone fixed cryostat sections heavy and light chains were generally demonstrable on B cell membranes (Figs 2 and 3). When monoclonal antibodies were applied as first reagents, followed by peroxidase conjugated anti-mouse antibody, excellent immunostaining was obtained (Figs 4 and 6). This technique was particularly informative to evaluate the distribution of T lymphocytes and T-cell subsets in relationship to the normal and pathological BM microenvironment. Many T lymphocytes were demonstrated, for example, in the specimens obtained from six patients with their BM affected by Hodgkin's disease. T lymphocytes in these cases were observed within the neoplastic nodules and scattered throughout the residual non-affected tissue. They mainly expressed the helper/inducer phenotype as defined by monoclonal antibodies [14] and corresponding to a similar T-cell subset distribution pattern observed in other tissues involved in Hodgkin's disease [15]. A variable number of T lymphocytes (mainly expressing the helper/inducer phenotype) were associated to neoplastic B lymphoid cells in various cases with non-Hodgkin's lymphoma and CLL. In CLL these lymphocytes were both scattered and grouped in small clusters (Fig. 4).

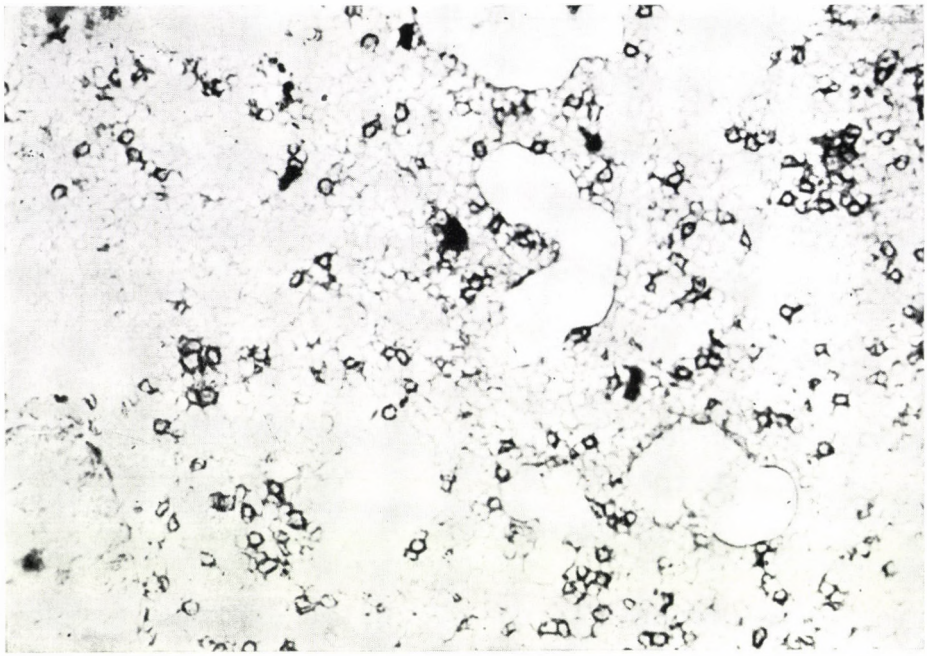


Fig. 4. A cryostat section immunostained with anti-T monoclonal antibody UCHT1 (OKT3-like). In the same case also shown in Fig. 3 numerous scattered and grouped T cells are demonstrated among the neoplastic B lymphocytes. Indirect immunoperoxidase method, × 250

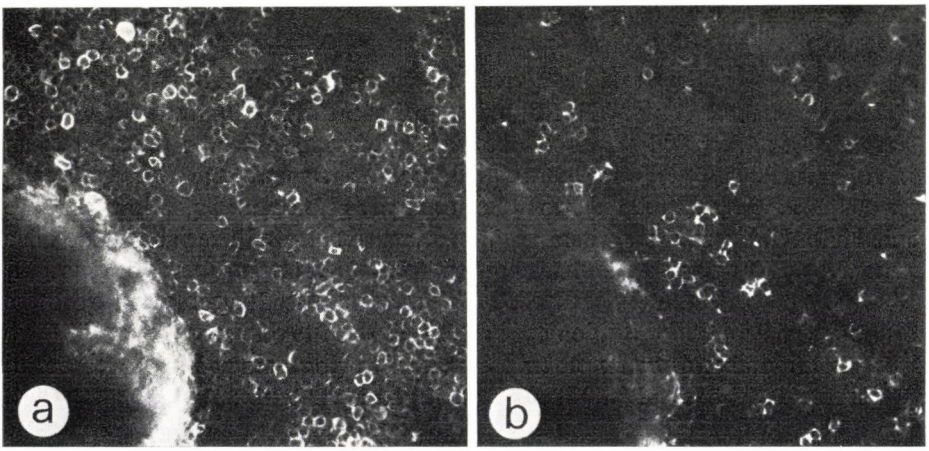


Fig. 5. Bone marrow cryostat section from the same case of Figs 3 and 4, double stained by immunofluorescence with anti-IgM-TRITC and UCHT1 (OKT3-like)-FITC. *a* and *b* are from the same field photographed with TRITC filter (anti-IgM) and FITC filter (UCHT1) respectively. B lymphocytes (IgM^+) and T-cells, stained with immunoperoxidase in different section (as shown in Figs 3 and 4), can here simultaneously be detected and evaluated on the same field of the same section. × 320

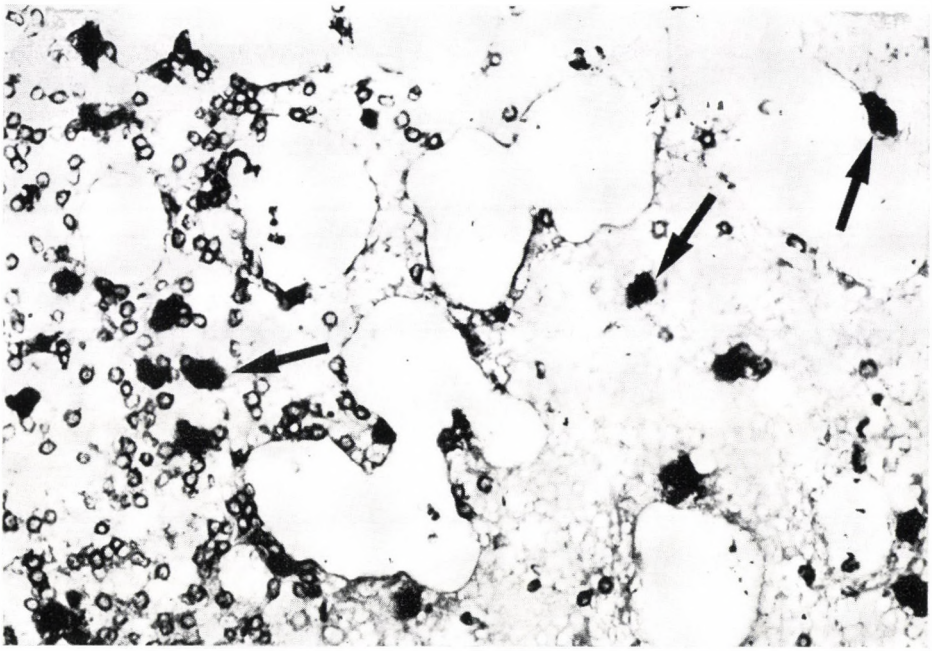


Fig. 6. Cryostat section of a bone marrow biopsy involved by Hodgkin's disease, immunostained with UCHT1 monoclonal antibody. Many T cells are scattered within a neoplastic area (left) whereas a few are located in residual haemopoietic tissue. Strong residual endogenous peroxidase is evident in some eosinophils which is not inhibited by phenylhydrazine (arrows). Indirect immunoperoxidase method, × 250

Discussion

The main point emerging from our study is that cryostat sections can easily be obtained from BM frozen samples and that they are suitable for immunohistological and histochemical analysis. Until recently immunological and cytochemical marker studies were only feasible on cell suspensions or smears obtained from BM aspirates. Although these studies give clinically useful informations, especially in the typing of leukaemia, they are not useful for the 'in situ' characterization of BM cell populations or the evaluation of cell–cell interactions.

The combined application of different techniques such as IF, IP and enzyme histochemistry on serial cryostat sections of the same sample allow a thorough analysis of the haemopoietic tissue and provide important information for the diagnosis and the study of different diseases. These techniques are not alternative to other histological methods, such as methacrylate embedding [16], but rather a complementary method. Thus valuable immunological and histochemical markers can be assessed together with excellent morphology.

Although both IP and IF could successfully be applied to BM cryostat sections in order to characterize different cell populations, a number of considerations emerged from our study. The main advantage of IF seems to be related to the possibility of using combinations of reagents labelled with different fluorochromes. This allows a simultaneous detection of different membrane antigens which are able to characterize different cell populations (e.g. T-cells and B-cells) (Figs 5a and b). The IP technique seems the method of choice in order to evaluate the immunohistological data in relationship to the BM morphology (Figs 2, 3, 4 and 6). The problem of endogenous peroxidase, which can interfere with specific IP staining, is by-passed to some extent by the inhibition with 0.1 per cent phenylhydrazine which does not affect cell antigenicity. Nevertheless, endogenous peroxidase cannot be inhibited on eosinophils but their characteristic strong positivity is easily distinguishable from specific immunostaining (Figs 2 and 6).

Studies are in progress in our laboratory employing alternative immunohistochemical reagents such as antibodies conjugated with alkaline phosphatase [4] in order to avoid the need of inhibition of endogenous peroxidase. Although alkaline phosphatase is also present as an endogenous enzyme in some BM cells (fibroblastic reticular cells, capillary endothelium, osteoblasts), it can be completely and easily inhibited by levamisole [17].

The use of BM cryostat sections offers a new valuable tool to detect immunologically different normal and malignant cell populations together with morphological and histochemical details of the haemopoietic microenvironment.

*

We wish to thank Dr. Peter Beverley, ICRF London, for the gift of 2D1 and UCHT1 monoclonal antibodies. We are indebted to Carlo Vincenzi and Licia Montagna for excellent technical assistance.

References

1. Janossy, G., Thomas, J. A., Pizzolo, G., Granger, S. A., McLaughlin, J., Habeshaw, J. A., Stansfeld, A. G., Sloane, J.: Immuno-histological diagnosis of lymphoproliferative diseases by selected combinations of antisera and monoclonal antibodies. *Brit. J. Cancer 42*, 224 (1980).
2. Pizzolo, G., Sloane, J., Beverley, P., Thomas, J. A., Bradstock, K. F., Mattingly, S., Janossy, G.: Differential diagnosis of malignant lymphoma and nonlymphoid tumors using monoclonal anti-leukocyte antibody. *Cancer 46*, 2640 (1980).
3. Stein, H., Bonk, A., Tolksdorf, G., Lennert, K., Rodt, H., Gerdes, J.: Immunohistologic analysis of the organization of normal lymphoid tissue and non-Hodgkin's lymphomas. *J. Histochem. Cytochem. 28*, 746 (1980).
4. Mason, D. Y., Stein, H., Naiem, M., Abdulaziz, Z.: Immunohistological analysis of human lymphoid tissue by double immunoenzymatic labelling. *J. Cancer Res. Clin. Oncol. 101*, 13 (1981).
5. Chilosi, M., Pizzolo, G., Menestrina, F., Iannucci, A. M., Bonetti, F., Fiore-Donati, L.: Enzyme histochemistry on normal and pathologic paraffin embedded lymphoid tissues. *Amer. J. Clin. Path. 76*, 729 (1981).

6. Pizzolo, G., Chilosi, M., Cetto, G. L., Fiore-Donati, L., Janossy, G.: Immuno-histological analysis of bone marrow involvement in lymphoproliferative disorders. *Brit. J. Haemat. 50*, 95 (1982).
7. Chilosi, M., Pizzolo, G., Janossy, G., Bofill, M., Fiore-Donati, L.: Enzyme histochemical analysis on cryostat sections of human bone marrow. *J. Clin. Path. 35*, 1220 (1982).
8. Wood, G. S., Warnke, R. A.: The immunologic phenotyping of bone marrow biopsies and aspirates: frozen section techniques. *Blood 59*, 913 (1982).
9. Johnson, D. G., Nogueira Aranjo, G. M.: A simple method of reducing fading of immunofluorescence during microscopy. *J. Immunol. (Methods) 43*, 349 (1981).
10. Straus, W.; Peroxidase procedures. Technical problems encountered during their application. *J. Histochem. Cytochem. 27*, 1349 (1979).
11. Hanker, J. S., Ambrose, W. W., James, C. J., Mandelkorn, J., Yates, P. E., Gall, S. A., Bossen, E. H., Fay, J. W., Laszlo, J., Moore, J. O.: Facilitated light microscopic cytochemical diagnosis of acute myelogenous leukemia. *Cancer Res. 39*, 1635 (1979).
12. Wood, G. W., Travers, H.: Non-Hodgkin's lymphoma: identification of the monoclonal B lymphocyte component in the presence of policlonal immunoglobulin. *J. Histochem. Cytochem. 30*, 1015 (1982).
13. Beverley, P. C. L., Callard, R. F.: Distinctive functional characteristic of human T-lymphocytes defined by E rosetting or a monoclonal anti T-cell antibody. *Eur. J. Immunol.* 11, 329 (1981).
14. Reinherz, E. L., Schlossman, S. F.: The differentiation and function of human T lymphocytes. *Cell 19*, 821 (1980).
15. Poppema, S., Bhan, A. K., Reinherz, E. L., Posner, M. R., Schlossman, S. F.: In situ immunologic characterization of cellular constituents in lymph nodes and spleens involved by Hodgkin's disease. *Blood 59*, 226 (1982).
16. Beckstead, J. H., Bainton, D. F.: Enzyme histochemistry on bone marrow biopsies: reactions useful in the differential diagnosis of leukemia and lymphoma applied to 2-micron plastic sections. *Blood 55*, 386 (1980).
17. Ponder, B. A., Wilkinson, M. M.: Inhibition of endogenous tissue alkaline phosphatase with the use of alkaline phosphatase conjugates in immunohistochemistry. *J. Histochem. Cytochem. 29*, 981 (1981).

Haematologia 17 (2), pp. 259–266 (1984)

Molecular and Cellular Regulation of Pyruvate Kinase in Red Blood Cells

G. JACOBASCH, H. HOLZHÜTTER

Institute of Physiological and Biological Chemistry, Humboldt University, Hessischestr. 3–4, 104 Berlin, GDR

(Received 5 November 1982, accepted 19 January 1983)

The main sources of the heterogeneity of pyruvate kinase (PK) isoenzymes are discussed. The regulative role of the L-type PK for the energy metabolism of a tissue without glyconeogenesis has been examined in erythrocytes. For that purpose a kinetic model of the PK has been established taking into account the substrates and the effectors ATP and fructose-1,6.biphosphate. This PK model was incorporated into a complex mathematical model of glycolysis and the concentrations of metabolites and the glycolytic flux were calculated for normal PK activity and for a PK activity ten times lower than normal. From calculations it is concluded that under the conditions of PK deficiency the energy metabolism cannot be stabilized. This corresponds to the experimental finding that the filtration constant of PK deficient red blood cells increases with declining survival time.

Keywords: pyruvate kinase, red blood cells

*

Pyruvate kinase catalyses the last quasi irreversible reaction of glycolysis, in which pyruvate and ATP are formed from PEP and ADP. Pyruvate kinase belongs to the enzymes which have various molecular forms. Their heterogeneity is produced in three different ways. First, the heterogeneity is caused genetically by different genes. Three tetrameric isoproteins of PK may be distinguished in human tissues, which have been designated K_4, L_4 and M_4 according to the organs: kidney, liver and muscle from which they were first isolated. At a very early step of ontogenesis, all fetal tissues contain the isoprotein K, whereas the isoproteins L and M appear later. During activation of the M or L gene of PK the synthesis of the K isoenzyme is often repressed. Examples for that phenomenon are muscle and red blood cells [1, 2]. In other tissues such as the brain and kidney, the synthesis of the polypeptide chains of PK-K is only partly repressed resulting in heteromers of PK composed of K with L, and K with M, subunits, respectively [1, 3, 4]. The existence of L-M-hybrids in vivo can be excluded, since M- and L-type polypeptide chains of PK are never synthesized together in one and the same cell.

The second way of producing heterogeneity is the posttranslational modification by limited proteolysis. By electrophoresis three distinct forms of the isoenzyme L could be separated. These forms of PK have been termed L, R_1 and R_2 (Fig. 1). They cannot be distinguished immunologically and their kinetic properties are

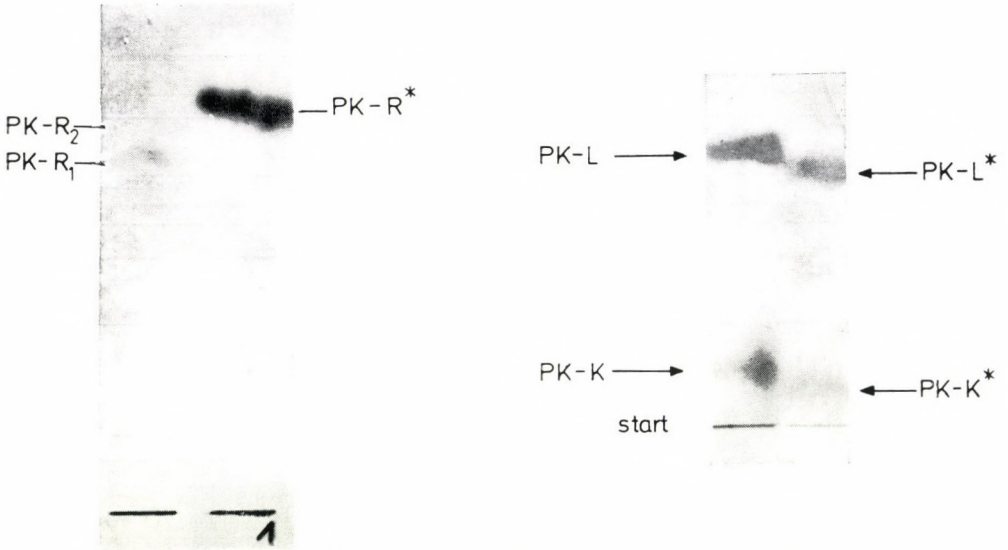


Fig. 1. Electrophoretic mobility of PK-L, PK-K, PK-R_1 and PK-R_2. L, K, R_1 and R_2 are native forms of PK; L*, K*, R_1^* and R_2^* are trypsinized forms of PK. Electrophoresis was carried out on cellulose polyacetate strips. PK activity was determined by means of NADH defluorescence produced enzymatically by coupling with the LDH-reaction

quite similar [5], but they differ in the molecular weight of their subunits indicating that the proteolytic cleavage takes place at different sites of the proisoenzyme in liver, kidney and red blood cells. Kahn et al. [5] and Daheki et al. [7] concluded from experiments by SDS polyacrylamide gel electrophoresis that PK-R_2 is composed of 4 identical subunits of about 63 000 daltons [6, 7]. PK-R_2 will be transformed into PK-R_1 during maturation of red blood cells, PK-R_1 is composed of two partially proteolysed L-type subunits with a molecular weight of about 58 000 daltons and two unchanged large subunits.

The third cause of producing heterogeneity of the L-type of PK is a modification by reversible phosphorylation. The 3'5'AMP dependent phosphorylation takes place at a specific seryl residue near the C-terminal of the polypeptide chain [8]. The main effect of phosphorylation is an increase of the $S_{0.5}$ constant for PEP without changing of V_{max} at saturating concentrations of PEP and an enhanced Hill coefficient. ADP and alanine inhibit the phosphorylated PK more intensely than they do the unphosphorylated enzyme and higher concentrations of Fru 1,6 P_2 are necessary to activate the phosphorylated than the unphosphorylated PK. Since in vivo PK is not saturated with PEP, the glucagon induced inhibition of PK by phosphorylation is of great importance for the hormonal regulation of gluconeogenesis. The inhibition of L-type PK in the liver prevents the switch to the futile cycle pyruvate → oxalacetate → phosphoenolpyruvate → pyruvate.

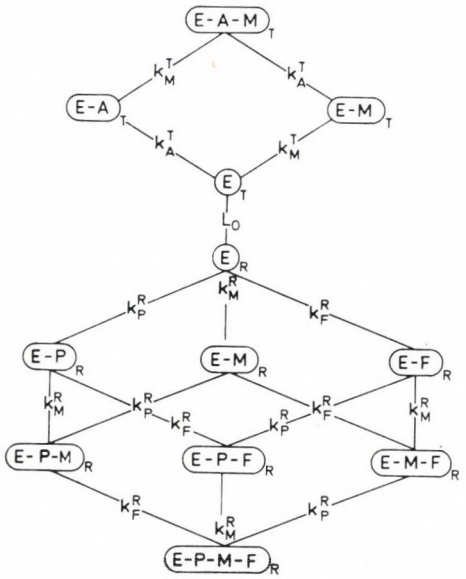


Fig. 2. Scheme of the pyruvate kinase model.
PEP = P; MgADP = M; Fru 1,6 P_2 = F; ATP = A

The PK-L of renal tubular cells is also regulated by reversible phosphorylation. In contrast to the hepatocytes, the gluconeogenesis of the renal cortex is independent from the food and the activation by glucagon [9]. A close relationship has, however, been observed between gluconeogenesis and the regulation of renal acid-base homeostasis. A proof of this is the stimulation of gluconeogenesis by parathyroid hormone under conditions of metabolic acidosis.

Recently it has been discovered that the pyruvate kinase of human red blood cells is regulated also by reversible phosphorylation [10, 11]. Both a c-AMP stimulated protein kinase and a phosphoprotein phosphatase, which can reverse the phosphorylation of PK, could be identified in erythrocytes and has been partially purified [11, 12]. Still the biological significance of the reversible phosphoryla-

$$V_{PK} = V_{max} \frac{\frac{PEP}{k^R_{PEP}} \cdot \frac{Mg \cdot ADP}{k_{Mg.\,ADP}}}{\left(1 + \frac{PEP}{k^R_{PEP}}\right)\left(1 + \frac{Mg \cdot ADP}{k_{Mg.\,ADP}}\right)(1 + L)}$$

$$L = L_0 \left[\left(1 + \frac{ATP}{k^T_{ATP}}\right) / \left(1 + \frac{PEP}{k^R_{PEP}}\right) / \left(1 + \frac{Fru1,6P_2}{k^R_{Fru1,6P_2}}\right) \right]^4$$

Fig. 3. Velocity function of the pyruvate kinase reaction

tion of the pyruvate kinase forms of red blood cells is not clear. The red blood cells do not exhibit gluconeogeneis, only glycolysis. Therefore, erythrocytes are a suitable object to study the significance of the pyruvate kinase reaction in glycolytic metabolism. From experimental data and calculations based on mathematical models of erythrocyte glycolysis it is known that the glycolytic flux normally is controlled by hexokinase and phosphofructokinase [13, 14]. Pyruvate kinase activity contributes, however, to the maintenance of a high cellular ATP level and is of importance for the control of 2,3-biphosphoglycerate concentration. Whenever there is a relative preponderance of the PK over the HK-PFK-system, the $2,3P_2G$ level is lowered. Such conditions exist in the red blood cells of newborn mammals that are not characterized by fetal hemoglobin. The result is a shift to the left of the O_2 saturation curve [15]. On the other hand, a relative preponderance of the HK-PFK over the PK-system, for instance in PK enzymopathies, increases the steady state level of $2,3P_2G$ [16]. In order to investigate the regulative role of PK in erythrocyte glycolysis and the effect of the pyruvate kinase enzymopathies on energy metabolism, it was necessary to work out an appropriate kinetic model which sufficiently reflects the main properties of the enzyme. Previous models were mainly used to elucidate the importance of metal ion complexes and neglected the allosteric properties of the PK, whereas our model takes into account the sigmoidal behaviour as well as the influence of the essential effectors ATP, and Fru $1,6P_2$. Following the allosteric concept of Monod et al. the four subunits of the tetramer are assumed to exist in two different conformational states R and T, forming an equilibrium characterized by the constant L_0 [17]. Owing to the heterogeneity of the PK forms of erythrocytes, the assumption of four identical subunits represents a certain simplification which should, however, be acceptable for the nonphosphorylated enzyme. In the binding scheme (Fig. 2) the substrates PEP and Mg.ADP, the activation by Fru $1,6P_2$ and ATP inhibition have been considered. Because the velocity-Mg.ADP-curves are hyperbolic in shape it was concluded that both conformations bind Mg.ADP with the same affinity ($K^R{}_M = K^T{}_M = K_{Mg.ADP}$). In that case the corresponding velocity function is given by Figure 3. The saturation function V_{PK}/V_{max} was measured by considering the four metabolites mentioned above within a physiological concentration range.

All experimental data were fitted simultaneously to the theoretical expression by means of nonlinear regression analysis using desk computer SILEX. The values obtained for the kinetic parameters are listed in Table 1. A good agreement of the theoretical and experimental curves has been achieved indicating the validity of

Table 1

Estimated values for the kinetic parameters $K^R{}_{PEP}$, $K_{Mg.ADP}$, $K^T{}_{ATP}$, $K^R{}_{Fru\ 1.6P_2}$ and L_0 obtained from nonlinear regression analysis based on the theoretical expression

$K^R{}_{PEP}$, mmol	$K_{Mg.ADP}$, mmol	$K^T{}_{ATP}$, mmol	$K^R{}_{Fru\ 1.6P_2}$, µmol	L_0
0.23 ± 0.02	0.47 ± 0.03	3.39 ± 0.71	5.0 ± 1.2	19 ± 1.3

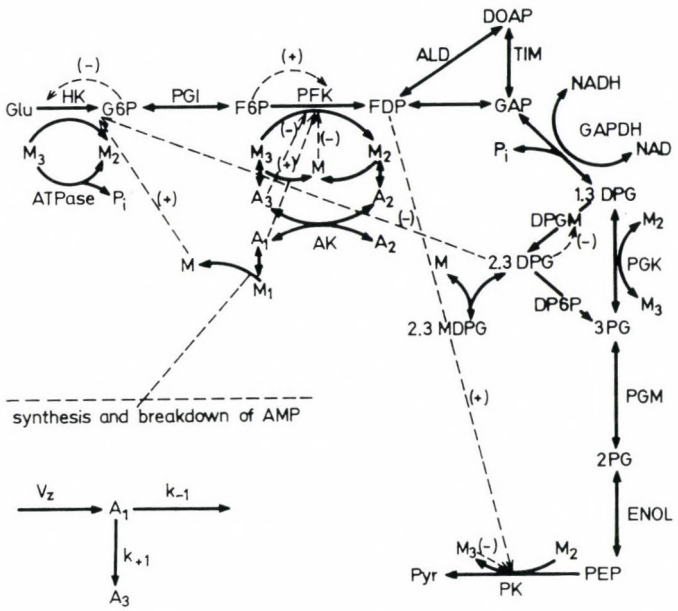


Fig. 4. Scheme of the mathematical model of glycolysis

the model. It should be emphasized that the kinetic model was constructed to be incorporated into the complex mathematical model of glycolysis but nevertheless it is suitable to describe the dependence of the velocity on the four relevant glycolytic metabolites PEP, Mg.ADP, Fru 1,6P_2 and ATP.

The aim of our investigation was to present a comprehensive mathematical model of glycolysis closely based on experimental findings. Figure 4 shows the scheme of the glycolytic model; it includes the reaction sequence with all the irreversible steps in the pathway and the important regulatory functions of enzymes, the adenylate kinase reaction, the ATP consuming reactions (ATPase) and a term which summarizes the synthesis and degradation of AMP. In addition to previous mathematical models [13, 18] the Mg-dependence of glycolytic reactions and a more realistic description of the reactions of the key enzymes hexokinase, phosphofructokinase and pyruvate kinase has been taken into account. The data in Table 2 illustrate the good agreement of the calculated concentrations of glycolytic metabolites and the rate of glycolysis with experimental findings in the normal case.

The mathematical model of glycolysis was used also to analyse the conditions of PK enzymopathies. In the given example the rate of the PK-reaction was lowered by one order of magnitude. The consequences of this drastic PK deficiency was a decrease of the glycolytic flux and the ADP level to 50 per cent, and the well known increase of the concentration of PEP, 2,3 PG, 3 PG and of the hexose phos-

Table 2

In vivo concentrations of glycolytic metabolites (μmol · l^{-1})

Parameter	Control		Pyruvate kinase deficiency (V_{max} 0.1 of control)	
	experimental	calculated	experimental	calculated
Gluc-6-P	38	46	↑	50
Fru 1,6P_2	7	6	22	23
3-PG	69	100	340	260
2,3P_2G_{total}	4800	4800	9500	9600
ATP_{total}	500	1500	820	1000
AMP_{total}	80	90	76	84
Mg free	200–500	455	—	340
PEP	26	26	120	75
V_{HK}	1300–1500	1300	↓	600

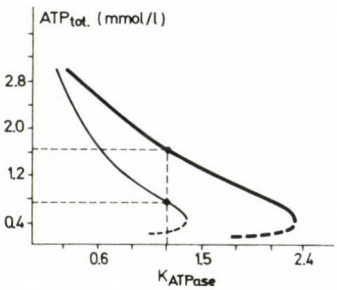


Fig. 5. Dependence of ATP concentration upon energy consumption upper curve: control

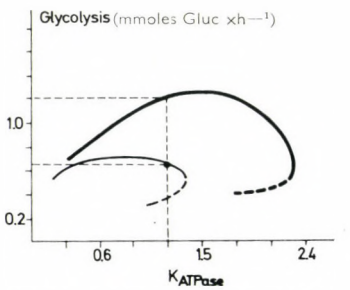


Fig. 6. Dependence of glycolytic flux upon energy consumption

phate (Table 2, Figs 5, 6). It is difficult to establish experimentally the effect of PK deficiency on the rate of glycolysis and the ADP level because both parameters are maturation dependent. Therefore, experimental data for blood samples with a high number of reticulocytes are often interpreted incorrectly.

Table 3

Control activity of glycolytic enzymes

Enzyme	Control	PK-deficiency (V_{max} PK 0.1 of control)
Hexokinase	0.78	0.42
Phosphofructokinase	0.07	0.16
Pyruvate kinase	0.10	0.71
Diphosphoglycerate-mutase	−0.40	−0.67
P_2G-phosphatase	0.40	0.62
ATPase	0.05	−0.24

For the calculated case of PK deficiency the control strength of PK is greater than the control strength of hexokinase or phosphofructokinase, indicating a limitation of the glycolytic pathway by the abnormal enzyme (Table 3).

Under conditions of a PK deficiency the computed ATP concentration as a function of the energy consumption of the red cell shows a steep slope above the in vivo point, i.e. the ATP level can not be stabilized (Fig. 5).

The breakdown of the energy metabolism of red blood cells in PK deficiency is probably connected with the mechanism of hemolysis. But until now attempts to find abnormalities in the composition and/or in the structure of the membrane of PK deficient red blood cells proved to be unsuccessful. We therefore preferred to test the filtrability of PK deficient red blood cells. The clogging kinetics of blood suspensions (HK 2.0) was determined under a constant pressure of a 10 ml water column [19]. In order to obtain a high reproducibility, silver filters with a pore width of 5 μm were used, corresponding to the pore width in spleen or bone marrow. The filtration curves were compared on the basis of a mathematical model which gives the filtration constant k as the exact parameter for the clogging kinetics [20]. Results of these experiments are listed in Table 4. The filtration constant k was increased in all cases of PK deficiency and was found to show a good correlation between k and the life span of red blood cells.

Table 4

Filtrability of pyruvate kinase deficient red blood cell suspensions

Proband	$k \cdot 10^{-3} \cdot s^{-1}$	Derivation
Control	1.2	0.4
Case 1 PK deficiency	2.2	0.1
2	4.3	0.1
3	7.9	0.1
4	10.7	0.3
5	20.0	1.3

References

1. Osterman, J., Fritz, P. J., Wuntch, T.: *J. Biol. Chem. 248*, 1011 (1973).
2. Tzanev, D., Schulz, J., Jacobasch, G., Richter, A., Gerth, C.: *Acta Biol. Med. Germ. 39*, 745 (1980).
3. Van Veelen, C. W. M., Rijksen, G., Vlug, A. M. C., Staal, G. E. J.: *Clin. Chim. Acta 110*, 113 (1981).
4. Cardenas, J. M., Richards, T. C., Gabourel, L.: *J. Cell Physiol. 96*, 189 (1978).
5. Kahn, A., Marie, J., Garreau, H., Sprengers, E. D.: *Biochim. Biophys. Acta 523*, 59 (1978).
6. Marie, J., Garreau, H., Kahn, A.: *FEBS Lett. 78*, 91 (1977).
7. Saheki, S., Saheki, K., Tanaka, T.: *FEBS Lett. 93*, 25 (1978).
8. Engström, L.: *Molecular Aspects of Cellular Regulation.* P. Cohen (ed.). Elsevier/North Holland Biomedical Press, Amsterdam 1980, Vol. 1, p. 11.
9. Jungermann, K., Möhler, H.: *Biochemie.* Springer, Berlin–Heidelberg 1980.
10. Marie, J., Tichonicky, L., Dreyfus, J. C., Kahn, A.: *Biochem. Biophys. Res. Commun. 87*, 862 (1979).
11. Kiener, P. A., Massaras, C. V., Westhead, E. W.: *Biochem. Biophys. Res. Commun. 91*, 50 (1979).
12. Kiener, P. A., Westhead, E. W.: *Biochem. Biophys. Res. Commun. 96*, 551 (1980).
13. Rapoport, T. A., Heinrich, R., Jacobasch, G., Rapoport, S. M.: *Eur. J. 42*, 107 (1974).
14. Jacobasch, G., Minakami, S., Rapoport, S.: In: *Cellular and Molecular Biology of Erythrocytes.* H. Yoshikawa, S. M. Rapoport (eds). University Tokyo Press 1974, p. 55.
15. Jelkmann, W., Bauer, C.: *Pflügers Arch. 375*, 189 (1978).
16. Jacobasch, G., Helbig, W., Syllm-Rapoport, I., Pester, H., Heine, K. M., Blese, Ch., Otto, F. M. G., Balu, H. J.: *Dtsch. Gesundh. Wes. 24*, 1441 (1969).
17. Monod, J., Wyman, J., Changeux, J. P.: *J. Molec. Biol. 12*, 88 (1965).
18. Rapoport, S. M., Heinrich, R., Rapoport, T. A.: *Progr. Biophys. Molec. Biol. 32*, 1 (1977).
19. Jacobasch, G., Schulz, J., Zanev, D., Richter, A., Gerth, C., Plonka, A.: *Acta Biol. Med. Germ. 40*, 547 (1981).
20. Jacobasch, G., Megow, D., Holzhütter, H. G.: *Stud. Biophys.* (In press.)

Haematologia 17 (2), pp. 267–279 (1984)

Release of Multivesicular Bodies from Platelets as Observed in SEM

J. POLASEK

Etobicoke General Hospital, Toronto, Ont., Canada

(Received 9 September 1982; accepted 17 February 1983)

Scanning EM was used to study platelet shape changes induced by collagen in citrated PRP. While using a modified fixation technique known to protect phospholipids from extraction during alcohol dehydration a release of multivesicular bodies from platelets was observed. Maximum frequency of their appearance correlated with the onset of a release reaction from platelets as seen in an aggregometer curve. Depicted structures may thus be related to the release of different platelet constituents.

Keywords: SEM, platelets, collagen, phospholipid vesicles.

Introduction

Morphological studies dealing with a platelet release reaction so far did not show convincingly the nature of a degranulation process of platelets [1, 2]. In contrast, only several authors noticed the release of presumably lipid vesicles from platelets using TEM [3, 4, 5]. Claims were made that multivesicular structures seen in TEM represent the morphological basis of a platelet release reaction [3] or the release of a platelet factor 3 [4], but no correlations were made between appearance of these structures and biochemical parameters of a release reaction.

Since the mentioned TEM observations did not find widespread acceptance, the present study was undertaken to reexamine platelet morphology with special attention to the onset of a platelet release reaction as seen in an aggregometer curve [6] and to preservation of platelet phospholipids. It has been shown that considerable amounts of phospholipids may be extracted from tissues during alcohol dehydration, a procedure necessary for preparing samples for SEM or TEM [7].

Material and Methods

Citrated platelet rich plasma (PRP) was prepared by differential centrifugation of whole blood obtained from healthy human volunteers. Nine volumes of whole blood were collected into one volume of sodium citrate (38 g/l). Aliquots (0.45 ml) of PRP containing 300×10^9 platelets/l were transferred to aggregometer

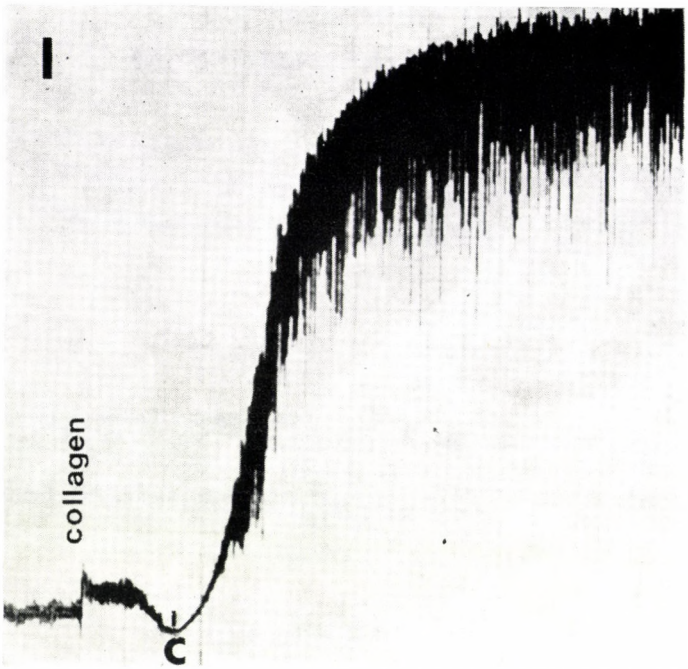


Fig. 1. A typical aggregation curve: after collagen addition to PRP under constant stirring at 37 °C, oscillations shift upward due to dilutional effect. During a lag phase oscillations remain unchanged, then they decrease in amplitude and the aggregation curve moves downward, indicating a decrease in light transmission as the platelets change shape. Letter "C" marks the point from which light transmission increases as platelets form larger and larger aggregates

cuvettes and activated by the addition of bovine acid soluble collagen from Sigma Chemical (St. Louis, Mo) at 37 °C. The final concentration was adjusted to give an aggregation lag phase approximately of 40–45 seconds. At different times the contents of the cuvettes were quickly transferred into cold (4 °C) glutaraldehyde (25 g/l) in 0.1 M cacodylate buffer with 3 mM $CaCl_2$ (pH 7.2). One volume of PRP was added to 20 volumes of the glutaraldehyde solution and kept at 4 °C for 30 minutes. This platelet suspension was then gently filtered through a Durapore 0.22 μm filter obtained from the Millipore Company or Nuclepore filters size 0.60 μm from the Nuclepore Company (Toronto, Ont.). The platelets retained on the filter were thoroughly washed using the glutaraldehyde solution and then placed for 5–10 minutes into a second fixative solution consisting of osmium tetroxide (10 g/l) in 0.1 M cacodylate and 3 mM $CaCl_2$ (pH 7.2). The filter was then washed twice with saline (9 g/l) and placed for 30 minutes into a third fixative consisting of uranyl acetate in H_2O (40 g/l). Aliquots of PRP were taken as controls

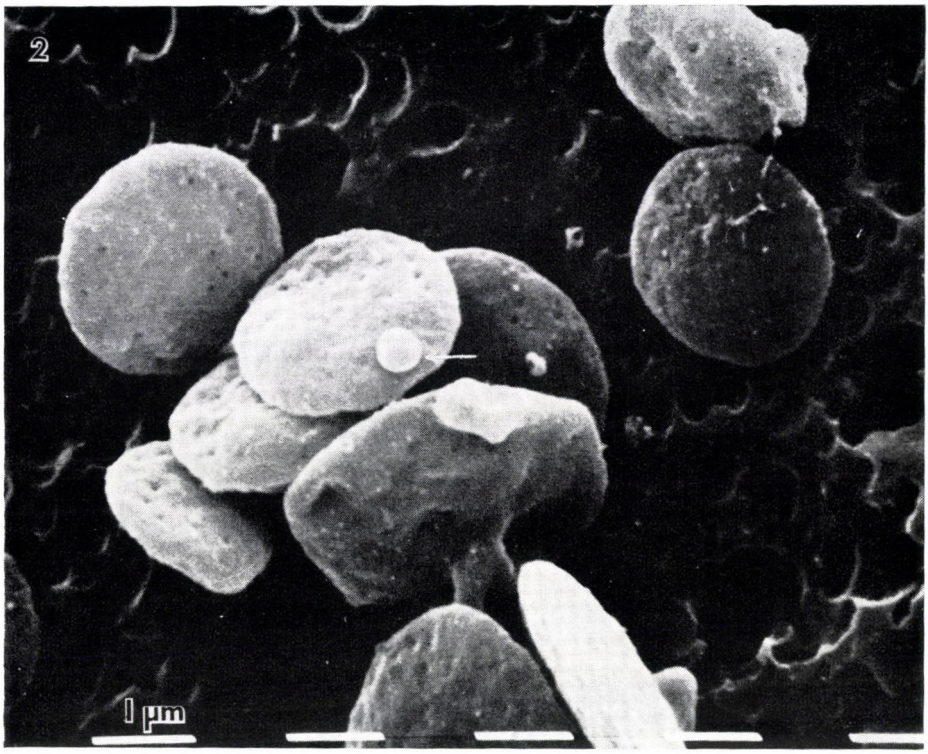


Fig. 2. Resting, disk shaped platelets. Arrow points to a balloon like structure occasionally seen in these preparations

at appropriate times where collagen was replaced by saline. Variations in fixation procedure are explained in Results. All samples were then dehydrated using gradually increasing concentrations of alcohol. Critical point drying using CO_2 and sputter coating with gold palladium followed. The samples were examined in a JEOL JSM-50A scanning electron microscope.

Results

Resting platelets sampled before addition of collagen showed typical disk forms and pores on the surface as described in the literature [1, 8]. Infrequently a new morphologic feature was noted, a balloon-like structure attached to the surface of a platelet (Fig. 2). During the lag phase after collagen addition the mor-

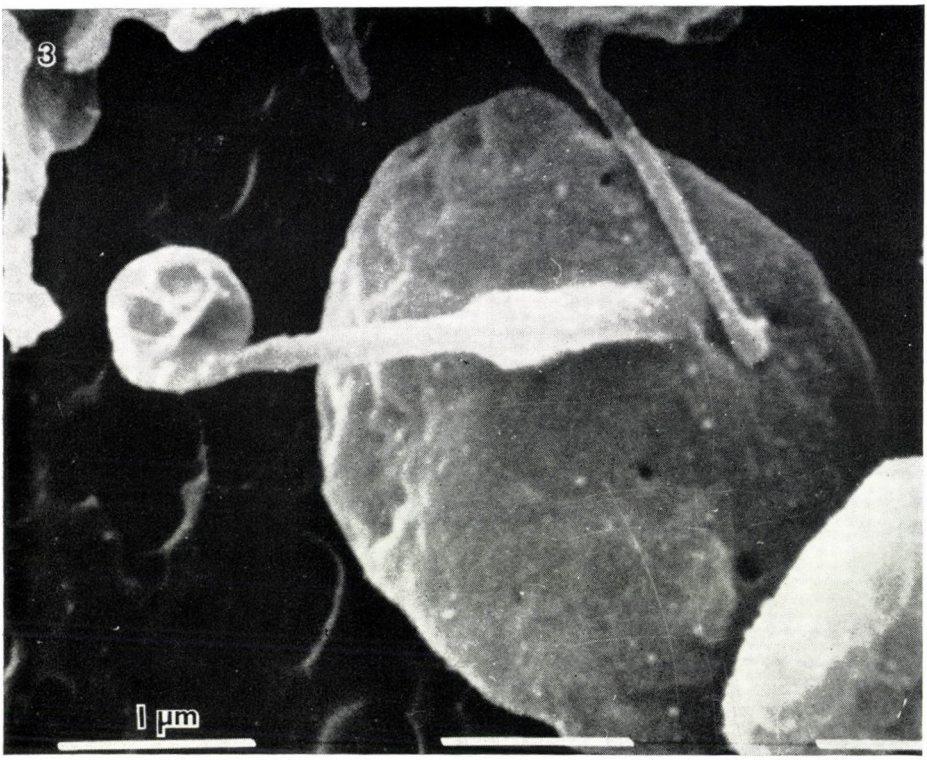


Fig. 3. Apparently a transitional form of a platelet changing from the resting stage into an activated one. Still disk shaped platelet has a long pseudopod with attached MVB

phology of platelets did not change much except for some increase of pseudopod formation and slight increase of multivesicular body (MVB) formation of which a representative picture is given in Fig. 3.

With the dip formation on the aggregation curve caused by platelet shape change into round irregular forms with many pseudopods nearly every platelet showed multivesicular structures hitherto not observed in SEM (Fig. 4). The optimal time for observation of MVB is marked in Fig. 1 by letter "C". Some seconds later the platelets start to form large clumps in which MVBs are difficult to see.

MVBs were usually but not always attached to the end of a pseudopod and were noticed basically in 2 forms. A balloon-like bleb occasionally seen with an opening as if discharging its contents into the surrounding milieu (Figs 5, 6), or as a typical MVB as seen enlarged in Fig. 7. Remnants of the structures could be seen separated from platelets on the filter as an empty shell (Fig. 5). Not infrequently a MVB has been seen in the middle of a pseudopod (Fig. 8). The MVBs ranged from 0.3–0.6 µm in diameter.

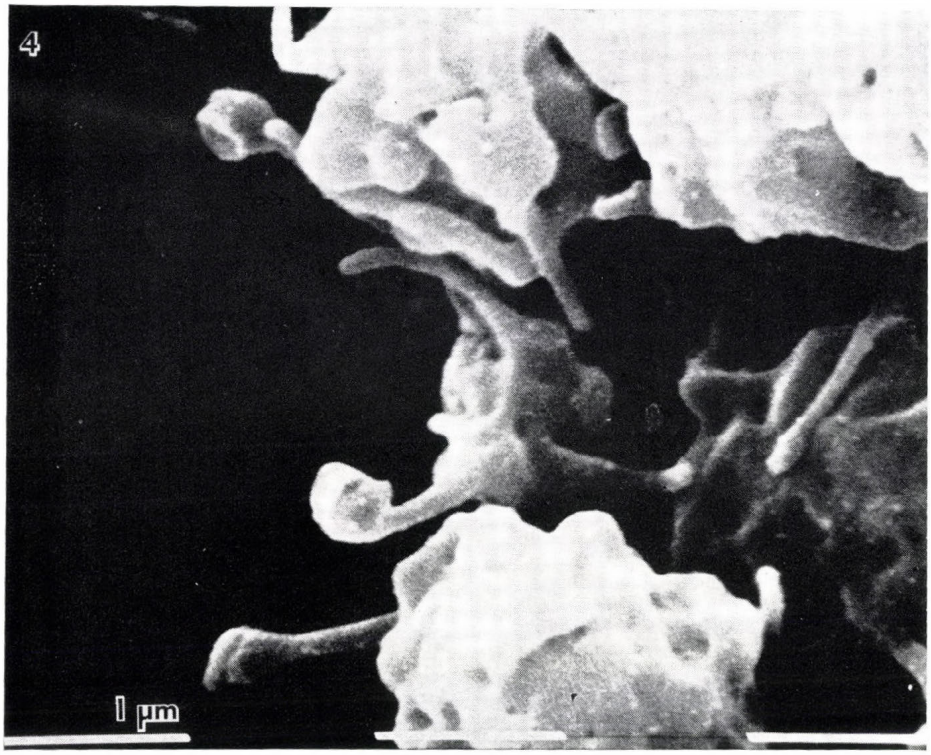


Fig. 4. Group of platelets with 3 MVBs apparently in different stages of development

Platelets fixed only in 2.5% glutaraldehyde in cacodylate at time "C" after collagen addition were almost completely devoid of MVBs and only a diligent search disclosed remnants of a material attached to pseudopods. As seen in Fig. 9 these remnants suggest a partial solubilization probably during a dehydration process.

Short use of OsO_4 as a second fixative preserved more MVBs as compared to glutaraldehyde only. Best details were, however, always seen with uranyl acetate as the 3rd fixative. For OsO_4 fixation a short time was chosen because preliminary experiments suggested a possible deleterious effect of longer times on MVB preservation.

continued on p. 277

Fig. 5. Group of platelets with 2 MVBs obviously draining its content into the surrounding milieu. Arrow points to what appears as an empty MVB separated from platelets, its walls collapsing

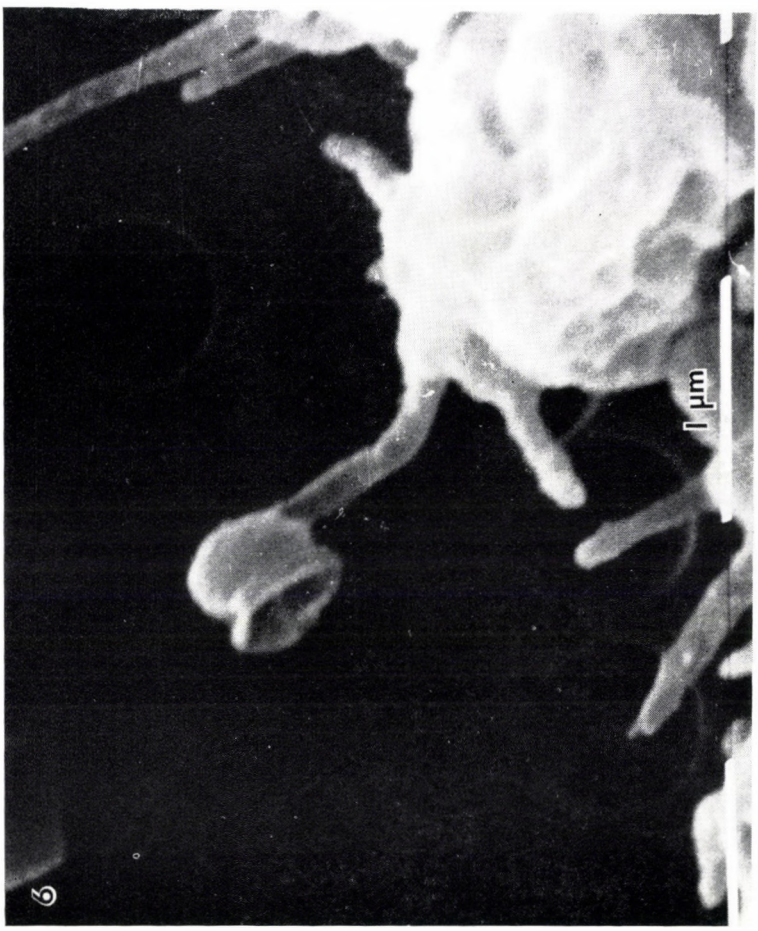


Fig. 6. Another MVB with what appears as an opening for discharge of its contents

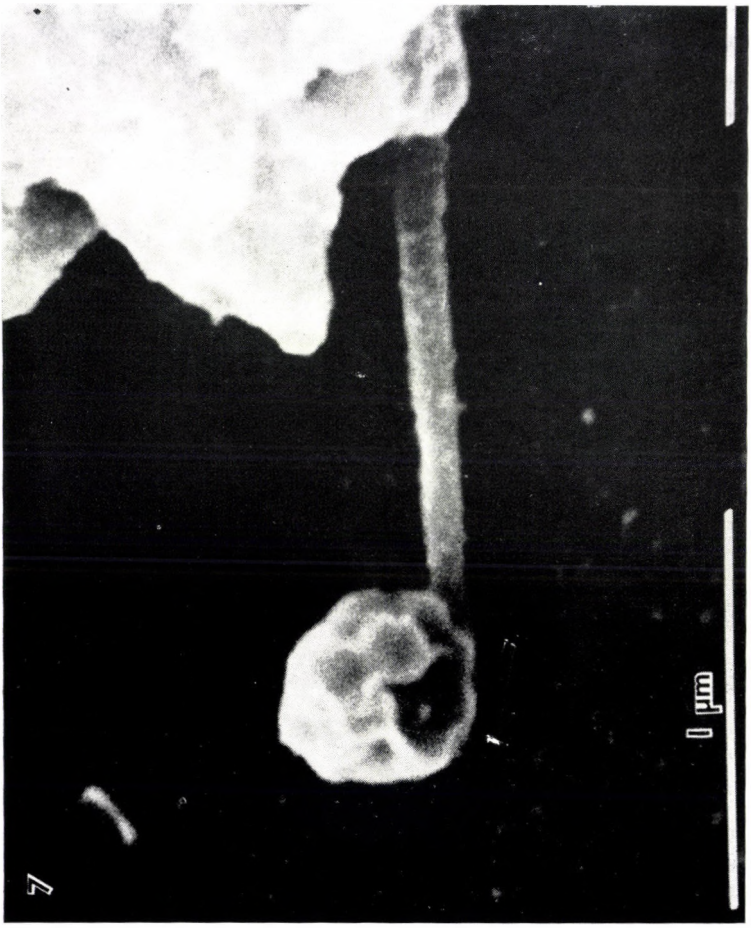


Fig. 7. A MVB in the form of morula, showing numerous thin walled vesicles

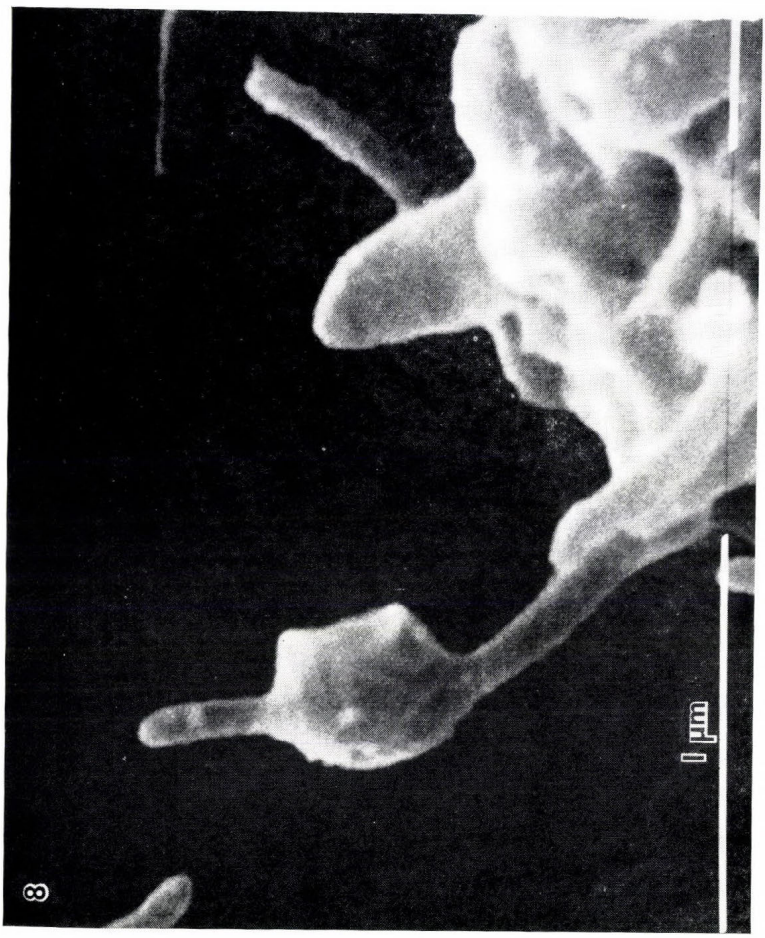


Fig. 8. A MVB in the middle of a platelet pseudopod

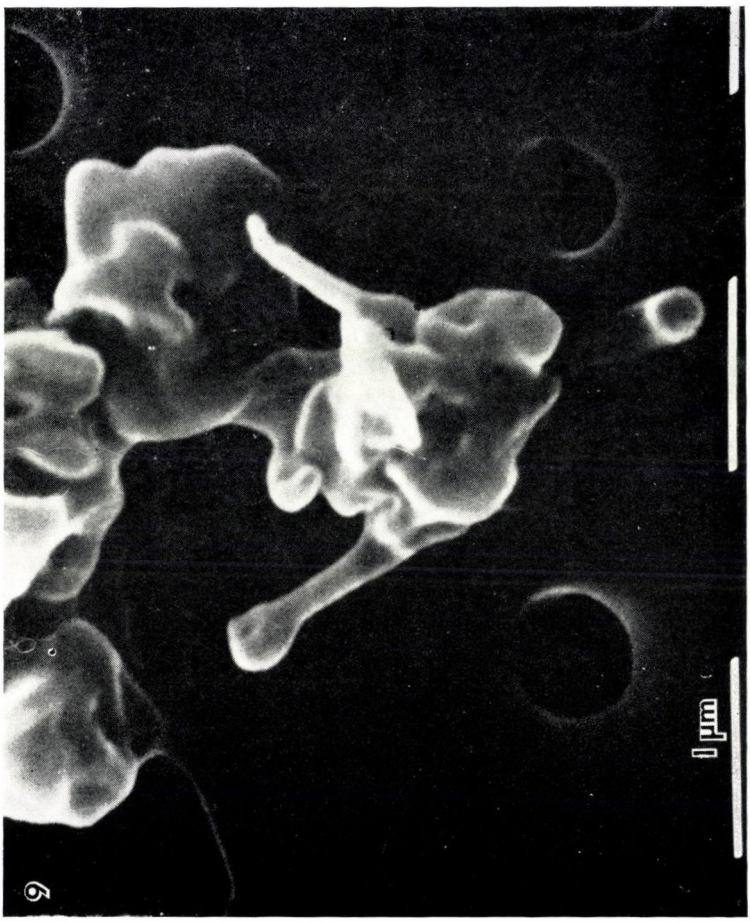


Fig. 9. Experimental conditions described in Results. Arrow points to what could be a partially washed off MVB

Discussion

Using uranyl acetate as a third fixative, new morphologic structures related to platelet activation have been described. Some of them appear as a balloon or bleb like structures, attached to a platelet surface, others, however, show ruffled surface or even presence of a multivesicular composition visible through thinned or lytic like lesions in a platelet plasma membrane.

These MVBs seem to be moving from the interior of a platelet through and toward the end of a platelet pseudopod, visibly bulging the platelet membrane in the process. Only the triple fixation as described, preserves MVBs best. Fixed only in glutaraldehyde, MVBs seem to dissolve promptly during alcohol dehydration. Preliminary experiments suggest that prolonged osmium fixation may further labilize them.

Possibility that described MVBs may be of phospholipoprotein nature is suggested by a protective action of uranyl ions on MVBs preservation.

Formation of phosphatidic acids early during platelet activation has been related to platelet shape changes, release reaction and aggregation [9]. Stabilizing effect of uranyl ions on synthetic lecithin and phosphatidic acid monolayer and thus inhibition of its solubilization was observed already in 1956 [10].

Uranyl acetate also significantly reduces the amount of phospholipids extracted during alcohol dehydration of a rat brain tissue [7]. Similarly a lung surfactant considered primarily to be dipalmitoyl lecithin and released from pneumocytes type II in the form of MVBs, to become a part of an alveolar lining, is only preserved for EM purposes by the use of uranyl acetate as a 3rd fixative [11, 12].

Furthermore, uranyl acetate has been reported to preserve multivesicular structures associated with the release of granules from rat peritoneal mast cells [13].

Although a claim has been made that blistering of the surface of avian embryonal fibroblasts may be an artefact caused by glutaraldehyde fixation [14], own unpublished results and work presently underway discount such a possibility for structures described in this paper and will be published soon. Also alcohol solubility or that of other organic solvents on avian fibroblast's blebs and blisters was not investigated in the mentioned study, making comparison difficult with platelet MVBs described here.

Similarly as in the case of secreting peritoneal mast cells [13], it was noted, that platelet MVBs seen even in the so called resting platelets were rather result of a partial platelet activation due to a mechanical manipulation of platelets. Therefore an extremely gentle technique was employed during PRP preparations. Platelet sensitivity to a mechanical manipulation was noted also while studying platelet secretion [15].

Extensive knowledge is available concerning release of platelet granules content extracellularly; for review see [16], but little is known that happens to platelet granule membranes during a release reaction [1, 2, 3, 4, 5], while using EM techniques. Biochemically, platelet granule membranes and platelet plasma

membranes have similar composition [17]. Labelling studies showed increase of glycoprotein I material derived from granules, on the surface of thrombin treated platelets [18]. Previously noted increase of procoagulant activities, phospholipid availability along with considerable changes of the phospholipids orientation in the plasma membrane of activated platelets [19, 20, 21], so far failed to find its counterpart in morphologic studies.

Platelet membranes and material derived from platelet granules have been detected in blood serum [22] and confirmed recently [23]. Presence of acid phosphatase activity in this material, called platelet dust, has been also noted [24, 25]. Rapidity by which platelet granules disappear from the platelet interior has been observed by Allen et al. [2].

Interaction of lysosomes with secretory granules and/or material taken up by pinocytosis is a general biological phenomenon seen in many secretory cells and gives rise to the so-called auto- and hetero-lysosomes, where MVBs represent practically combination of both [12]. Some of the structures described in this paper could thus represent a link between platelet lysosomes and secretory granules of platelets and explain why it has been so difficult to prepare fractions of platelet granules free of acid phosphatase activity – a typical lysosomal marker [26].

In a working hypothesis one could then anticipate a rapid movement of platelet granules during a release reaction toward the surface of platelets, where they could play manifold roles associated with platelet activation. Different approaches and techniques should help to elucidate this fascinating aspect of platelet physiology.

*

The author wishes to thank Professors P. Hjort, H. Stormorken and T. Hovig for making available to him the research facilities of Oslo University in Rikshospitalet during the summer of 1981.

This paper represents a poster presentation shown at the 19th International Congress of ISH held in Budapest in 1982.

References

1. White, J. G.: Current concepts of platelet structure. *Am. J. Clin. Path. 71*, 363 (1979).
2. Allen, R. D., Zacharski, L. R., Widirtsky, S. T., Rosenstein, R., Zaitlin, L. M., Burgess, D. R.: Transformation and motility of human platelets. Details of the shape change and release reaction observed by optical and electron microscopy. *J. Cell. Biol. 83*, 126 (1979).
3. Warren, B. A., Vales, O.: The adhesive dendritic pseudopodium of the platelet and the release reaction. *Microvasc. Res. 4*, 159 (1972).
4. Weber, A. J., Johnson, S. A.: Platelet participation in blood coagulation aspects of hemostasis. *Am. J. Path. 60*, 19 (1970).
5. Hugues, J.: Plaquettes sanguines et structure du vaisseau. *Nouv. Rev. Franç. Hemat. 9*, 529 (1969).

6. Kinlough-Rathbone, R. L., Cazenave, J. P., Packham, M. A., Mustard, J. F.: Effect of inhibitors of the arachidonate pathway on the release of granule contents from rabbit platelets adherent to collagen. *Lab. Invest. 42*, 28 (1980).
7. Silva, M. T., Guerra, F. C., Magalhaes, M. M.: The fixative action of uranyl acetate in electron microscopy. *Experientia (Basel), 24*, 1074 (1968).
8. Hovig, T.: Blood platelet surface and shape. A scanning electron microscopic study. *Scand. J. Haemat. 7*, 420 (1970).
9. Lapetina, E. G., Cuatrecasas, P.: Stimulation of phosphatidic acid production in platelet precedes the formation of arachidonate and parallels the release of serotonin. *Biochim. Biophys. Acta 573*, 394 (1979).
10. Anderson, P. J., Pethica, B. A.: *Biochemical problems of lipids.* Interscience, New York 1956, p. 24.
11. Gil, J., Weibel, E. R.: Improvements in demonstration of lining layer of lung alveoli by electron microscopy. *Respir. Physiol. 70*, 13 (1969).
12. Holtzman, E.: *Lysosomes: A survey.* Springer Verlag, New York 1976, p. 207.
13. Lawson, D., Raff, M. C., Gomperts, B., Fewtrell, C., Gilula, N. B.: Molecular events during membrane fusion. *J. Cell. Biol. 72*, 242 (1977).
14. Hasty, D. L., Hay, E. D.: Freeze-fracture studies of the developing cell surface. II. Particle-free membrane blisters on glutaraldehyde fixed corneal fibroblasts are artefacts. *J. Cell. Biol. 78*. 756 (1978).
15. Levine, S. P., Suarez, A. J., Sorenson, P. R., Knieriem, L. K., Raymond, N. M.: The importance of blood collection methods for assessment of platelet activation. *Thromb. Res. 24*, 433 (1981).
16. Niewiarowski, S.: Platelet release reaction and secreted platelet proteins. In: Haemostasis and Thrombosis. A. L. Bloom, D. P.Thomas (eds.) Churchill Livingstone, Edinburgh, London, 1981, p. 73.
17. Marcus, A. J., Ullman, H. L., Safier, L. B.: Lipid composition of subcellular particles of human blood platelets. *J. Lipid. Res. 10*, 108 (1969).
18. Jenkins, C. S. P., Clemetson, K. J.: The role of platelet membrane glycoproteins. in: Platelets and Thrombosis. Eds: Mills, D. C. B., Paroti, F. I.; Academic Press, New York, 1977, p. 15.
19. Morin, R. J.: The role of phospholipids in platelat function. *Am. Clin. Lab. Sci. 10*, 463 (1980).
20 Joist, J. H., Dolezel, C., Lloyd, J. V., Kinlough-Rathbone, R. L., Mustard, J. F.: Platelet factor 3 availability and the platelet release reaction. *J. Lab. Clin. Med. 84*, 474 (1974).
21. Zwaal, R. F. A., Hemker, H. C.: Blood cell membranes and haemostasis. *Haemostasis 11*, 12 (1982).
22. Wolf, P.: Platelet products in human plasma. *Br. J. Haematol. 13*, 269 (1967).
23. George, J. N. Thoi, L. L., McManus, L. M., Reimann, T. A.: Isolation of human platelet membrane microparticles from plasma and serum. *Blood 60*, 834 (1982).
24. Polasek, J.: Particulate nature of acid phosphatase released from thrombocytes. *Thrombos. Diathes. haemorrh. (Stuttg.) 19*, 465 (1968).
25. Polasek, J., Kubisz, P.: Acid phosphatases and platelet factor 3. *Scand. J. Haemat. 5*, 390 (1968).
26. Gogstad, G. O.: A method for the isolation—granules from human platelets. *Thromb. Res. 20*, 669 (1980).

Haematologia 17 (2), pp. 281–287 (1984)

Two Cases of Alpha Heavy Chain Disease in Venezuelans

MARIA L. GALLANGO, M. RAMÍREZ, R. SUINAGA

Instituto Venezolano de Investigaciones Científicas,
Departamento de Medicina Experimental, Laboratorio de Hematología,
Apartado 1827, Caracas 1060 A, Venezuela

(Received 21 January 1983; accepted 13 July 1983)

Two cases of alpha chain disease are described. The clinicopathological features of the two patients were very similar, and similar to the numerous cases described in the literature, presenting the gastrointestinal form of the disease. In both patients the pathological protein was not noticeable in the electrophoretic pattern. The diagnosis was established by immunoselection with electrophoresis. The α-HCD proteins belonged to the α_1 subclass and in no case was it found in the urine. The patients were young Venezuelan mestizos and seem to be the first cases reported in Venezuela.

Keywords: abnormal immunoglobulins, alpha chain disease, immunoblastic lymphoma, immunoglobulinopathy, immunoproliferative small intestinal disease (IPSID).

Introduction

Production of immunoglobulin molecules with incomplete heavy chains devoid of light chains is a characteristic of the immunoproliferative disorders known as heavy chain diseases (HCD). Since the discovery and description of the first case of a structurally abnormal immunoglobulin, the γ heavy chain disease [1], many other alterations have been observed. The α-chain disease (α-CD) is the most remarkable and common of these conditions and more than 150 cases have been reported to date [2].

Demonstration of the immunoglobulin heavy chains devoid of light chains is essential for diagnosis, but the disease can be suspected on the basis of clinical findings. Most patients are affected by the digestive form of α-CD, localized to the small intestine and mesenteric nodes. The respiratory form has been observed in a few cases only, with manifestations almost confined to the respiratory tract [3–5]. The IgA secretory system is mainly involved in α-CD.

As the existence of a premalignant stage has been considered in the evolution of the disease, early detection of the abnormal protein in serum is important for successful treatment and prevention of the malignant transformation.

In this paper we shall present two patients with α-chain disease; to our knowledge these were the first cases reported in Venezuela.

Report of Cases

Case 1. A 25 year-old female presented with a history of recurrent diarrhea during the past six months, with abdominal pain, marked weight loss, malabsorption, edema of the ankles, finger clubbing and hepatosplenomegaly. Her growth was stunted and the secondary sexual characteristics underdeveloped. Stool examination revealed giardiasis and malabsorption of D-xylose of which she excreted only 2.5 g/5 h (normal 4–9). Excretion of vitamin B_{12} given orally with intrinsic factor was only 6 per cent in 24 h (normal > 10 per cent), the fecal fat was increased. Gastrointestinal X-rays revealed coarse mucosal folds of the small bowel. Jejunal biopsy showed infiltration of the lamina propria with predominantly plasma cells and immature lymphoid cells. Bone marrow examination was normal.

The immunoglobulin levels were low (Table 2). Treatment with oral tetracycline (500 mg 4 times daily) caused improvement and a marked increase of body weight. Six months later the patient is doing well on tetracycline therapy, twice daily. The clinical condition is notably improved, but the α-HCD protein is still present in serum in a lower level.

Case 2. This 22 year-old man was admitted because of repeated episodes of diarrhea, accompanied by abdominal pain and meteorism. He had lost much weight and noted weakness, fatigue, and severe deterioration of the general condition. On physical examination, finger clubbing and moderate hepatospleno-

Table 1

Hematological findings

Case	Hb g/dl	Blood film	White cell count	Bone marrow
1	11.2	Hypochromic	8000 75% neutrophils	Normal
2	10.9	Hypochromic	9000 Monocytosis	Iron deficiency anemia

Table 2

Protein studies

Case	Total protein g/dl	Serum albumin g/dl	Serum Ig (mg/dl)				Free α chain		
			IgG	IgA	IgM	IgD	Serum	Jejunum	Urine
1 FH	6.4	3.6	944	706	76	9	+	+	−
1 FA	5.8	3.3	786	326	33	8	+	+	−
Normal		3.5–5.5	*1.406 ±413	261 ±90	86 ±31	0.3–40			

* Normal values for Venezuelan blood donors

megaly were noted. The peripheral lymph nodes were normal. X-rays showed accelerated transit with thickened mucosal folds of duodenum and jejunum.

Intestinal biopsy showed diffuse lymphocytic and plasmocytic infiltration in the small bowel with infiltration of the mesenteric lymph nodes and the liver. Stool examination revealed giardiasis and a fecal fat excretion of 12 g/24 h (normal value 5/24 h). The serum alkaline phosphatase value was high. Haematological values and protein studies are presented in Tables 1 and 2. This patient died from intercurrent infection during therapy.

Material and Methods

Protein studies. Electrophoresis of serum and urine was carried out on cellulose acetate in barbital buffer ionic strength 0.05, pH 8.6. For Ouchterlony agar gel diffusion studies, 1.5 per cent agar in barbital buffer pH 7.2 was used. Quantitation of the immunoglobulins was performed according to Mancini'] technique. The immunoselection plate method of Ràdl [6] as modified by Doe [7s

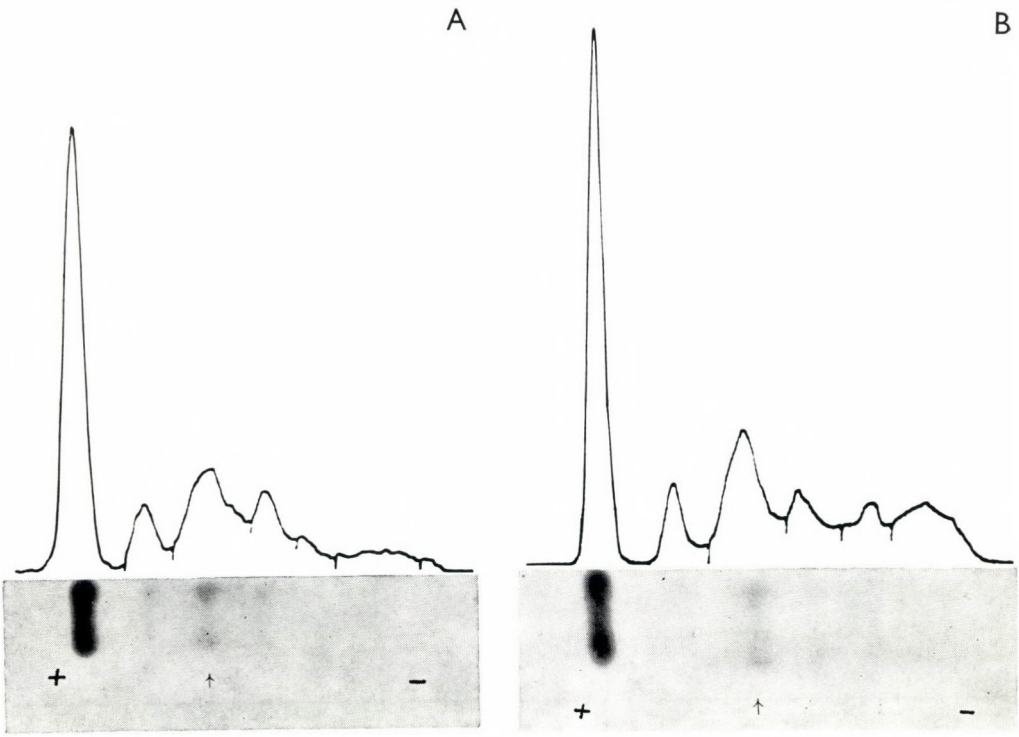
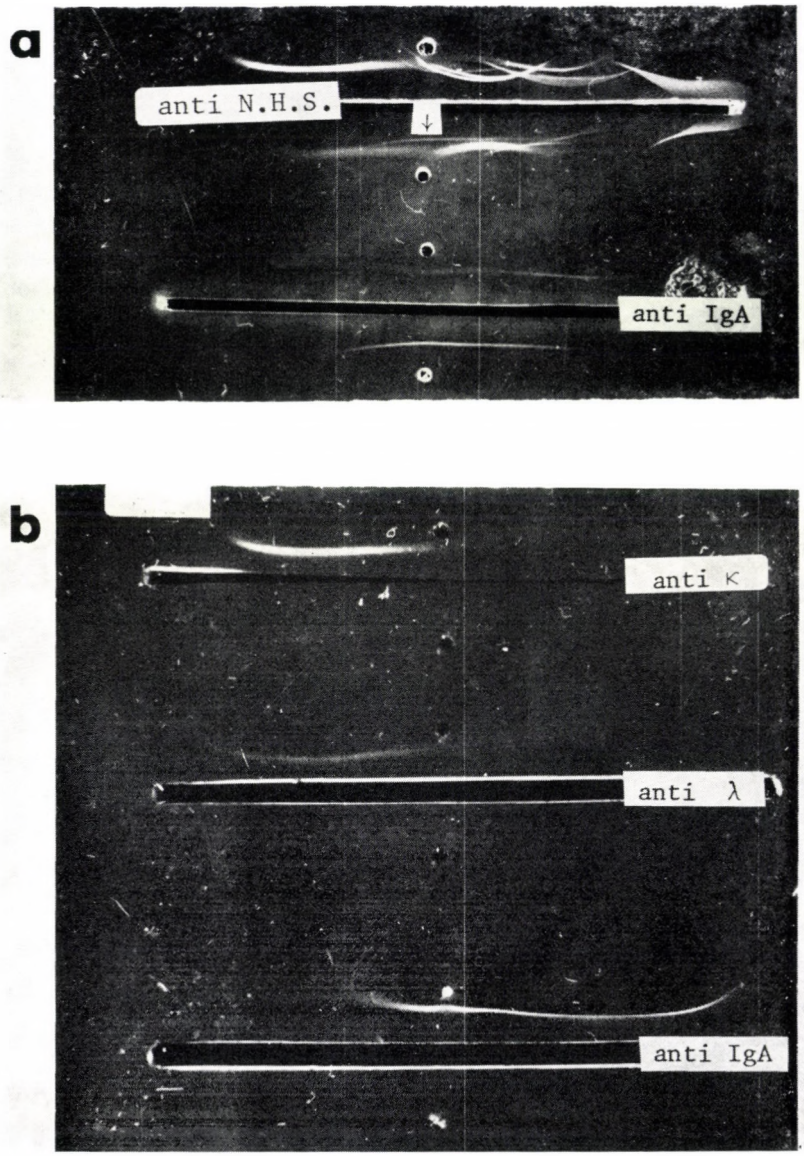


Fig. 1. Cellulose acetate electrophoretic pattern of sera from the two patients with α-HCD

was used to detect free alpha chains in the serum. For this purpose, equal amounts of anti-lambda and anti-kappa antisera were added to the agar at 2 per cent concentration and 1 to 5 μl of each sample was tested. Once the serum proteins containing light chains had precipitated during electrophoresis, the free alpha chains were also precipitated on using in the channels monospecific antisera to IgA or anti α-chains.



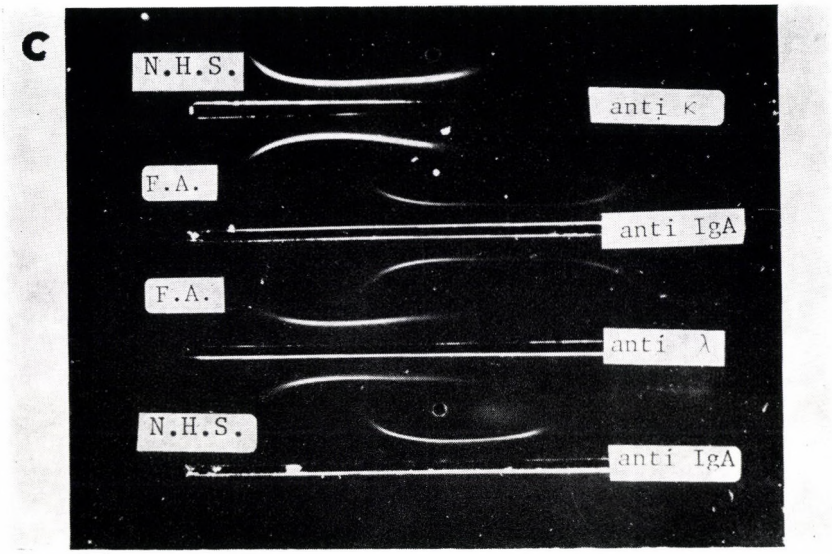


Fig. 2. Immunoelectrophoretic pattern of sera, urine and jejunal fluid of patients with α-HCD. (a) Upper wells: serum of Case 1; lower wells: jejunal juice. (b) Upper wells: serum of Case 1. Lower wells: concentrated urine serum of Case 1. (c) Serum of Case 2 with normal human serum as control

Antisera were prepared in rabbits by immunization with IgA myeloma protein and absorption was carefully performed to obtain antisera reacting only with IgA. Special care was given to select high titer antisera, some of which were obtained commercially.

Thin layer chromatography of sera, followed by immunodiffusion was performed according to Morris [8], using Sephadex G-200 and 0.5 M NaCl as solvent, and blue dextran 19S as marker. The precipitation arcs of the α-chains usually corresponded to the heavier molecular weight of the polymerized proteins.

Results

Haematological findings and results of the protein studies are seen in Tables 1 and 2.

Electrophoresis failed to detect abnormal proteins, it only revealed a moderate increase in alpha$_2$ globulins with a decrease of all other proteins and moderate hypogammaglobulinemia in both cases (Fig. 1).

Immunoelectrophoresis revealed an abnormally extended precipitin arc from α_1 to β regions but no anomalous components with anti-kappa and anti-lambda light chain antisera, in any case (Fig. 2).

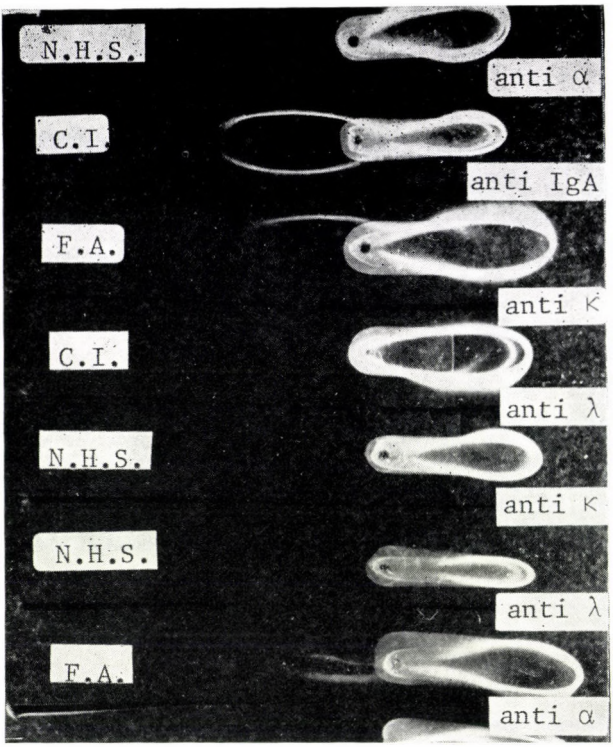


Fig. 3. Immunoselection by immunoelectrophoresis. Sera of the patients and of normal controls had immunoglobulins containing light chains, precipitated in the agar prepared with anti-kappa and anti-lambda as observed on the cathodal side by the anti-α serum contained in the wells

The immunoselection technique combined with immunoelectrophoresis allowed to detect free alpha chains in the serum and jejunal fluid of the patients but not in their concentrated urine (Fig. 3). The thermostability test for Bence–Jones proteins was always negative and the amount of excreted protein was low.

In both cases the alpha chains were present in polymerized form, as demonstrated by thin layer chromatography and immunodiffusion using monospecific antisera. The α-CD proteins were typed as A_1 subclass, with monoclonal antibodies.

Discussion

No exceptional characteristics have been found in these two patients. The hepatosplenomegaly was the only sign which is considered rare in patients with α-CD, but it might have been due to chance. Both patients were young adults of low socioeconomic status. This is a consistent feature of the disease which is common in all the underdeveloped South American countries. These countries

have a high proportion of the population living in underprivileged conditions, having parasites and malnutrition, but only a limited number of cases of α-CD have been reported to date [2].

Differences in geographical areas, environment, racial groups, and genetic factors could be the reason of this observation; and also the lack of systematic studies of immunoglobulins in the jejunal juice as well as of intestinal biopsy in cases of IPSID, where α-CD protein is not detected in the serum. These investigations are essential for the diagnosis of the disease as recommended by WHO [12]. In this respect immunoselection by immunoelectrophoresis is helpful in the search for the abnormal protein, even when instead of anti Fab_{α} antisera light chain antisera are incorporated into the gel.

References

1. Franklin, E. C., Lowenstein, J., Bigelow, B., Meltzer, M.: Heavy chain disease, a new disorder of γ globulins: report of the first case. *Amer. J. Med. 37*, 332 (1964).
2. Seligmann, M., Mihaesco, E., Preud'Homme, J. L., Danon, F., Branet, I. C.: Heavy chain diseases: current findings and concepts. *Immunol. Rev. 48*, 145 (1979).
3. Stoop, J. W., Ballieux, R. E., Hijmans, W., Zegers, J. M.: Alphachain disease with involvement of the respiratory tract in a Dutch child. *Clin. Exp. Immunol. 9*, 625 (1971).
4. Franx, J. A., Crain, J. D., Rosen, F. S., Merler, E.: An alpha heavy chain abnormality in a child with hypogammaglobulinemia. *Clin. Immunol. Immunopath. 1*, 282 (1973).
5. Florin-Christensen, A., Domiach, D., Newcomb, P. B.: Alpha-chain disease with pulmonary manifestations. *Brit. Med. J. 2*, 413 (1974).
6. Ràdl, J.: Light chain typing of immunoglobulins in small samples of biological material. *Immunology 19*, 137 (1970).
7. Doe, W. F., Danon, F. Seligmann, M.: Immunodiagnosis of alpha chain disease. *Clin. Exp. Immunol. 36*, 189 (1979).
8. Morris, C. J. O. R.: Thin-layer chromatography of proteins on Sephadex G-100 and G-200. *J. Chromat. 16*, 167 (1964).
9. Seligmann, M., Mihaesco, E. Hurezm, D., Mihaesco, C., Preud'Homme, J. L., Rambaud, J. C.: Immunochemical studies in four cases of alpha chain disease. *J. Clin. Invest. 48*, 2374 (1969).
10. Skinner, J. M., Manousos, O. N., Economidou, J., Nicoloau, A., Merikas, G.: Alpha chain disease with localized plasmacytoma of the intestine. Immunoperoxidase study. *Clin. Exp. Immunol. 25*, 112 (1976).
11. Doe, W. F., Henry, K., Hobbs, J. R., Avery Jones, F., Dent, C. E., Booth, C. C.: Five cases of alpha chain disease. *Gut 13*, 947 (1972).
12. Alpha-chain disease and related small-intestinal lymphoma: a Memorandum. *Bull. Wld. Hlth. Org. 54*, 615 (1976).

Haematologia 17 (2), pp. 289–295 (1984)

Immunofixation Electrophoresis: An Immunochemical Method for Testing Protein Anomalies

Sz. Ottó

National Institute of Oncology, P. O. Box 114/21, 1525 Budapest, Hungary

(Received 12 May 1982; revised 28 October 1982; accepted 8 January 1983)

The practical problems of immunofixation electrophoresis are discussed and the advantages of the method are stressed on the basis of data in the literature and own experience mainly in tests of monoclonal gammopathies. Technical modifications are recommended by which the tests can be performed easily and quickly with small amounts of immune sera.

Keywords: immunofixation electrophoresis, protein anomalies.

*

By enhancing the resolving power of electrophoresis by immunochemical separation, immunoelectrophoresis [12] has increased the effectiveness of the examination of proteins and it has become a primary means of laboratory testing of protein anomalies. One of its limitations is, however, that the formation and strengthening of the precipitation bands takes several hours during which the position of the separated fractions undergoes changes; owing to diffusion the individual components occupy more and more space and cover each other partly or completely. This is why the precise recognition of small extra components or proteins of atypical mobility is often difficult or impossible.

Immunofixation electrophoresis [3] eliminates the disturbing diffusion and the coalescence of the bands and is currently one of the most valuable immunochemical methods applied in research and laboratory diagnostics. By placing a cellulose-acetate sheet saturated with specific immune serum or else a thin strip of filter paper on the gel area in question, prompt fixation in situ of the separating protein fractions can be achieved. Depending upon the specificity of the antibody, the protein complex formed remains where it is; it does not diffuse, nor can it be washed out of the gel.

The present paper besides surveying the practical problems of immunofixation electrophoresis, recommends some technical modifications in order to decrease the cost of the assay.

Materials and Methods

Glass plates 7.6 × 7.6 cm or 7.6 × 26 cm in size were used onto which 6 or 21 ml agarose–acrylamide gel was layered by means of veronal buffer pH 8.2. The agarose–acrylamide ratio was 2 : 1 (1 g agarose and 0.5 g acrylamide to 100 ml buffer). The samples (serum, urine) diluted 1 : 1 with the above gel mixture were placed into the 6.0 × 0.2 mm starting holes at 2 cm distance from the cathode and with the small plates the intensity was 20 mA and with the big ones, 70 mA. The duration of the

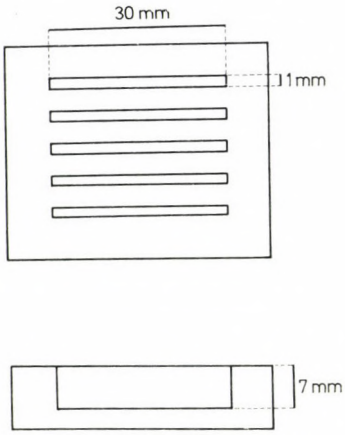


Fig. 1. Immune serum container recommended for immunofixation, viewed from above and from the side

electrophoresis was one h. Immediately after electrophoresis 7.0 × 20 mm cellulose-acetate strips soaked in specific immune serum were placed on the previously localized gel surface (Fig. 1) in such a manner that they touch the immune sera in the wells of a container and absorb the sera evenly, ensuring that there is no air bubble between the membrane and the gel. The duration of the precipitation at room temperature was one h. Then the strips were removed from the gel surface, the plates soaked overnight in physiological saline for a minimum of 6 h, then dehydrated at room temperature, covered with a wet filter paper and dried. They were stained with 0.5 per cent Coomassie Brilliant Blue R-250 for 2–3 min, then differentiated in methanol–acetic acid–distilled water 150 : 100 : 450 until a clean background was attained.

Results

Immunofixation electrophoresis is a very effective method for the investigation of monoclonal gammopathies. Acrylamide mixed to agarose moderately decreases electro-osmosis and changes the net structure of the agarose gel, it favourably influences the conditions of electrophoresis, promotes the separation of certain

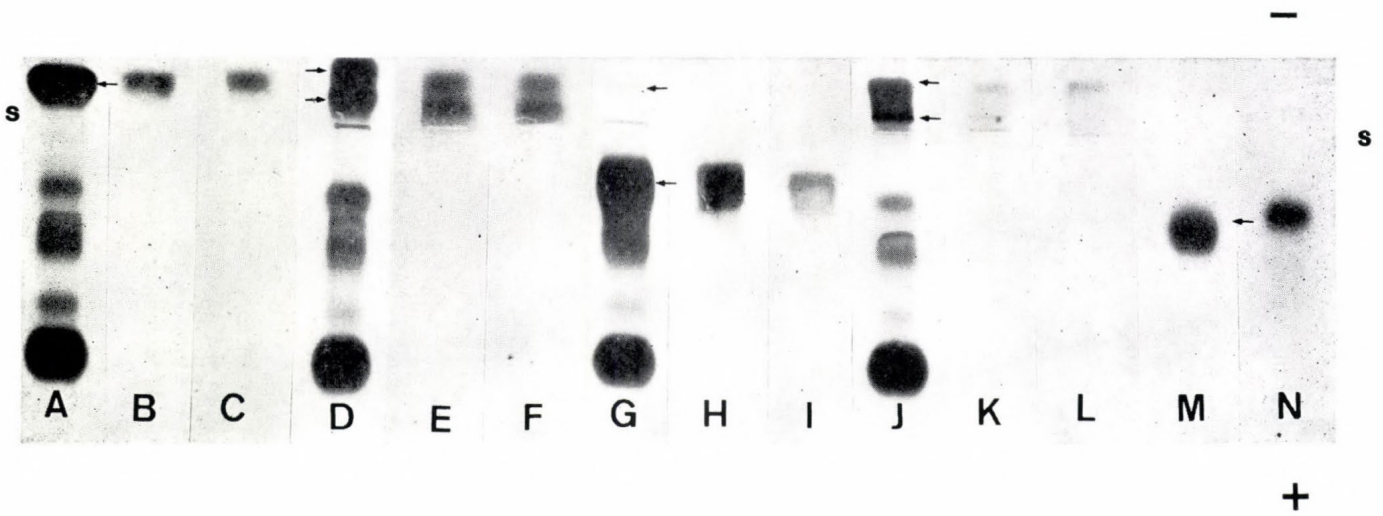


Fig. 2. *A:* M-component of γ-mobility; *B:* pattern of immunofixation using anti-γ (IgG) immune serum; *C:* immunofixation by anti-λ immune serum. Dilution of patient's sera in *B* and *C* samples is 1 : 256. *D:* M-component of double γ-mobility; *E:* immunofixation by anti-γ (IgG) and *F:* anti-λ immune sera. The two M-components of identical class and type specificities are well isolated. *G:* high concentration M-component of wide base under the starting trough while above it the M-component is narrow, hardly noticeable. *H:* the large M-component with anti-α (IgA) and *I:* with anti-k immune sera. *J:* directly above the starting trough a narrow M-component, while nearer to the negative electrode an M-component with wide base contacting the former. *K:* immunofixation by anti-μ (IgM) immune serum. Both M-components are recognizable; *L:* with anti-k immune serum only the larger one reacted; the *E*, *F*, *H*, *I*, *K*, *L* were diluted 1 : 64. *M:* M-component from concentrated urine; *N:* immunofixation by anti-λ immune serum. The protein concentration is 200 mg/l; the *S* on the *A* and *N* samples indicates the position of the starting trough. The arrows point to M-components

components, increases the decomposition ability and shortens the duration of electrophoresis; in this low concentration it does not disturb development of the precipitate.

According to literary data the immune serum requirement is generally 25 $\mu l/cm^2$, 60–100 μl are required for each strip. Provided the field in question is correctly localized 30 μl are sufficient [19, 25]. The 7×20 mm membrane fragments used by us absorbed only 16–18 μl of material from the troughs, so the 6 mm wide trough still ensures adequate separation of the components. A wider trough is not practical because it requires more immune serum.

The above band size was found adequate as proved also by Figure 2 where *A*, *B*, *C* samples show monoclonal IgGL protein, while the *D*, *E*, *F* samples show a double IgGL gammopathy. The migration speed of the two IgGs is so close to each other that no double precipitation band can be traced by immunoelectrophoresis. Namely, the components coalesce in the course of diffusion, they cover each other and only one precipitation band will be seen. The *G*, *H*, *I* samples show a wide base IgAK-protein under the starting trough while above it a narrow, pale extra-component can be seen. This does not react with anti-immunoglobulin sera and so it may not be of immunoglobulin character. On the *J*, *K*, *L* samples a narrow component of the IgMK-protein reacted only with the IgM immune serum produced against heavy chain Ig, while no precipitation band is formed with immune serum produced against the light chain. The *M*, *N* samples show the λ-type protein of an isolated light chain disease.

Discussion

An increase of immunoglobulin may be caused by the increased activity of normal immune response or likewise by the clone formation from single immunocyte that has turned malignant. The former produces a heterogeneous molecule population, while the latter produces a homogeneous immunoglobulin (M-component. (The difference between the two is not always distinct; the M-component may be microheterogeneous [1, 2, 10] or it is present in such a low concentration that normal protein components may prevent its recognition, thus causing eventually difficulties in differential diagnostics.

Attempts have been made to increase the resolving power of immunoelectrophoresis by methods of direct immunoelectrophoresis [1, 33] and after the usual gel electrophoresis, the immune sera were not loaded into the trough next to the starting trough but were layered directly on the gel surface. In this way, of course, a stronger precipitation was achieved but the resolving power decreased in most cases. Although the two-dimensional immunoelectrophoresis [18] increased the resolving power, nevertheless the method is technically difficult and the evaluation of samples is often a problem.

Immunofixation electrophoresis or simply immunofixation recently has become useful in immunochemical studies. The method [2] described originally for studying polymorphism of protein was soon applied in several fields for investigat-

ing the phenotypes [4, 20, 26] of certain plasma proteins; the nature of protein components of abnormal mobility [30]; the monoclonal immunoglobulins [8, 17, 27, 30]; the immunocomplexes [5, 28, 29]; the heterogeneity of specific antibodies [16, 21] and the cerebrospinal fluid [7, 14].

The new fields of application resulted in technical changes [4, 7, 8, 11, 15, 19, 22, 25]. The original carrier was agarose gel [2] and the immune serum was placed onto the gel surface directly. Today a number of carriers are used, thus cellulose-acetate membrane and its varieties [9, 13, 15, 24] and also polyacrylamide [14]. The antigen is usually added by filter paper or cellulose-acetate saturated with immune serum [19, 25]. The combination of isoelectrofocusing with immunofixation [4, 6, 16, 21] has further refined the method and ensures a good resolution.

The immunofixation method is most useful for the detection of monoclonal gammopathies [8, 23, 27, 30, 31]. It also facilitates recognition of bi- or oligoclonal components, and in case of the presence of smal 'occult' or of M-components of atypical mobility it is valuable in differential diagnostics. It is also suitable for demonstrating Bence–Jones proteinuria [32] and for investigating the nature, e.g. the homogeneity of free light chains. With its help a free light chain derived from the monoclonal cell process may be differentiated from the normal immunoglobulin components.

As regards the sensitivity of immunofixation and immunoelectrophoresis [17, 19, 23, 30] in the case of testing M-components of low concentration, immunofixation had a higher resolving power and sensitivity. The sensitivity was further increased when the precipitation was performed at 40 °C. In this case, however, filter paper bands have to be used [19] because no such effect was observed with cellulose-acetate. On the other hand, filter paper absorbs more immune sera and is therefore less economic. At the proper protein concentration, however, cellulose-acetate will also be effective.

Although immunofixation electrophoresis can be applied more favourably than immunoelectrophoresis in several fields, it should not be regarded as a more sophisticated procedure. When applying it a number of factors have to be considered that may cause difficulties in evaluation of the results, viz.

(i) only pure, high titre immune sera comply with the requirements of the method;

(ii) the immune serum should not be cloudy or containing precipitate;

(iii) the use of immune serum produced in horse is not favourable as the evolving antigen–antibody complex dissolves more easily either in the antigen or antibody excess;

(iv) in the case of a high antigen concentration, in the precipitation field a central, light area appears that covers the details;

(v) it is more difficult to find the optimal antigen-antibody ratio than with immunoelectrophoresis. The territory is smaller in which a properly evaluable precipitate is formed.

Still, the suitable dilution or protein concentration can always be found. In our investigations a concentration of 400–2000 mg/l serum-immunoglobulin was

the optimum, and in the case of urine samples it was 200–400 mg/l light chain concentration. This ratio may, however, be modified according to the properties of the immune serum [24].

In clinical investigations, immunoelectrophoresis and immunofixation are of equal value. Experience and the desired type of information decide which of the two, or perhaps both, should be applied. The two methods will usefully supplement each other.

An earlier described methodological variant, the reversed immunofixation agar-gel electrophoresis [22] may be used not only for detecting monoclonal gammopathies, but also for a quick control of the protein content of biological fluids or of their immunochemical purity.

The recommended methodological modifications may be well utilized in the clinical laboratory. As the immunoglobulin producing lymphoid plasma cell tumours raise several problems in diagnostics, classification and treatment, every method deserves attention that may help to characterize such tumours. Their logical selection will help in attaining the correct diagnosis and also to decrease the cost of materials.

References

1. Afonso, E., Proenca, J. S.: Electrophoretic heterogeneity of IgG. *Clin. Chim. Acta 21*, 143 (1968).
2. Afonso, E.: Brief study of a myeloma protein. *Clin. Chim. Acta 21*, 283 (1968).
3. Alper, C. A., Johnson, A. M.: Immunofixation electrophoresis, a technique for the study of protein polymorphism. *Vox Sang. 17*, 445 (1969).
4. Arnaud, P., Wilson, G. B., Koistinen, J., Fudenberg, H. H.: Immunofixation after electrofocusing: improved method for specific detection of serum proteins with determination of isoelectric points. 1. Immunofixation print technique for detection alpha-1-protease inhibitor. *J. Immunol. Meth. 16*, 211 (1977).
5. Baumstark, J. S.: Quantitative immunofixation of proteins following zone electrophoresis in agarose gel: application of the determination of the stoichiometry of the alpha-1-antitrypsin-elastase interaction. *J. Immunol. Meth. 23*, 79 (1978).
6. Burnett, D., Bradwell, A. R., Ramsden, D. B.: Demonstration of thyroxine-binding globulin (TBG) microheterogeneity by isoelectric focusing of whole human serum in agarose followed by immunofixation. *Science Tools 27*, 19 (1980).
7. Cawley, L. P., Minard, B. J., Tourtelotte, W. W., Ma, B. I., Chelle, C.: Immunofixation electrophoresis techniques applied to identification of protein in serum and cerebrospinal fluid. *Clin. Chem. 22*, 1262 (1979).
8. Cejka, J., Kithier, K.: A simple method for the classification and typing of monoclonal immunoglobulins. *Immunochemistry 13*, 629 (1979).
9. Chang, C. H., Inglis, N. R.: Convenient immunofixation electrophoresis on cellulose acetate membrane. *Clin. Chim. Acta 65*, 91 (1975).
10. Fahey, J. L.: Heterogeneity of myeloma proteins. *J. Clin. Invest. 42*, 111 (1963).
11. Gerson, B., Labrie, J., Copeland, B. E.: Immunofixation on thin-layer agarose. *Clin. Chem. 25*, 197 (1973).
12. Graber, P., Williams, C. A.: Méthode immunoelectrophorétique d'analyse de mélanges de substances antigéniques. *Biochim. Biophys. Acta 17*, 67 (1955).

13. Janik, B.: Immunofixation electrophoresis. In: *Electrophoresis Today*. Gelman Sciences Inc. 1980, Vol. 1, No. 1.
14. Kahn, S. N., Thompson, E. J.: Rapid quantitative surface immunofixation of proteins in polyacrylamide gels. *Clin. Chem. 21*, 253 (1975).
15. Karinkanta, H. H., Nieminen, E. J.: An improved technique for immunofixation of electrophoretograms. *Clin. Chem. 24*, 1639 (1978).
16. Keck, K., Grossberg, A. L., Pressman, D.: Specific characterisation of isoelectrofocused immunoglobulins in polyacrylamide gel by reaction with ^{125}I-labelled protein antigens or antibodies. *Europ. J. Immunol. 3*, 99 (1973).
17. Keshgegian, A. A., Pfeiffer, P.: Immunofixation as an adjunct to immunoelectrophoresis in characterisation of serum monoclonal immunoglobulins. *Clin. Chim. Acta 110*, 337 (1981).
18. Laurell, C. B.: Antigen-antibody crossed electrophoresis. *Anal. Biochem. 10*, 358 (1965).
19. Marshall, M. O.: Comparison of immunofixation and immunoelectrophoresis methods in the identification of monoclonal immunoglobulins in serum. *Clin. Chim. Acta 104*, 1 (1980).
20. Martin, W., Ziegler, Ch.: Bf-Immunofixation auf Cellogel-Folien. *Blut 42*, 23 (1981).
21. Nye, L., Roitt, J. M.: Isoelectric focusing of human antibodies directed against a high molecular weight antigen. *J. Immunol. Meth. 35*, 97 (1980).
22. Ottó, Sz.: Reversed immunofixation agar gel electrophoresis. *Immunol. Letters 4*, 85 (1982).
23. Pedersen, N. S., Axelsen, N. H.: Detection of M-components by an easy immunofixation procedure: comparison with agarose gel electrophoresis and classical immunoelectrophoresis. *J. Immunol. Meth. 30*, 257 (1979).
24. Pizzolato, A. M., Goni, F. R., Salvarezza, R. C.: Immunofixation on cellulose acetate: an improved screening method for monoclonal immunoglobulins. *J. Immunol. Meth. 26*, 365 (1979).
25. Ritchie, R. F., Smith, R.: Immunofixation. I. General principles and application to agarose gel electrophoresis. *Clin. Chem. 22*, 497 (1976).
26. Ritchie, R. F., Smith, R.: Immunofixation. II. Application to typing of alpha-1-antitrypsin at acid pH. *Clin. Chem. 22*, 1735 (1976).
27. Ritchie, R. F., Smith, R.: Immunofixation. III. Application to the study of monoclonal proteins. *Clin. Chem. 22*, 1982 (1976).
28. Strong, R., Watkins, J.: A simple electrophoretic technique for the estimation of complement C3 conversion: specific application to the investigation of anaphylactoid response to I.V. agents. *J. Immunol. Meth. 29*, 293 (1979).
29. Sudo, K., Kanno, T.: Membrane associated enzyme complexes containing lambda type immunoglobulin A. *Clin. Chim. Acta 107, 49* (1980).
30. Sun, T., Lien, Y. Y., Degnan, T.: Study of gammopathies with immunofixation electrophoresis. *Amer. J. Clin. Path. 72*, 5 (1979).
31. Vilpo, J. A., Irjala, K.: IgD-lambda myeloma with separate heavy- and light-chain M-components. *Clin. Chem. 26*, 1760 (1980).
32. Whicher, J. T., Hawkins, L., Higginson, J.: Clinical applications of immunofixation: a more sensitive technique for the detection of Bence–Jones protein. *J. Clin. Path. 33*, 779 (1980).
33. Wilson, A. T.: Direct immunoelectrophoresis. *J. Immunol. 92*, 431 (1964).

Open Forum on Identification of Blood Group HLA Antigens from Blood Spots and Other Body Fluids

Haematologia 17 (2), pp. 299–305 (1984)

Experience in the Detection of Red Cell Antigens and HLA Antigens in Bloodstains

P. J. LINCOLN, BARBARA E. DODD

Department of Haematology, The London Hospital Medical College, Turner Street, London E1 2AD, Great Britain

(Accepted 27 January 1983)

The red cell antigens A, B, H, C, C^w, c, D, E, e, S, s, K, Fy^a, Fy^b, Jk^a and Jk^b can all be detected on bloodstained threads using a modified Landsteiner elution technique. Bloodstain material is often very limited in quantity and far from fresh so every effort is made to produce optimum conditions throughout the test including extension both of the absorption period and the elution time, using a low concentration of indicator cells and the use of LISS. Detection of the HLA antigens in stains is less advanced but inhibition of the lymphocytotoxicity of HLA sera by bloodstained threads has produced some promising results in experiments involving the HLA-A1, A2, B8 and CW3 antigens.

Keywords: bloodstains, elution, inhibition

*

For many years tests for inherited characters in bloodstains were confined to the detection of the A and B antigens and somewhat later, H, by an inhibition technique. Today, tests for these antigens take their place among a wide range of polymorphisms which include almost all the systems currently used in paternity testing.

Since the topic of this paper is blood group antigens the many enzyme polymorphisms and serum groups which play an important role as useful tools in the

Table 1

Antigens detectable in bloodstains

Antigen	Usual method of detection
A, B, H M Ss K Fy^a, Fy^b Jk^a, Jk^b D, C, c, E, e, C^w	Absorption-elution
Gm, Km	Inhibition
HLA-A1, A2, A3, B7, B8, CW3	Inhibition

identification of stains are not within our scope. The range of antigens detectable in stains is illustrated in Table 1. The antigens listed have all been successfully detected in bloodstains and a good many of them are routinely used in casework.

It can be seen from this table that all the red cell antigens can be detected by an elution test which is a modified version of the well known Landsteiner technique. The detection of the white cell antigens is at a less advanced stage but for these an inhibition test appears to be the most promising technique investigated up to now.

Detection of Red Cell Antigens

An elution technique has been developed which will detect in stains antigens of the ABO, Rh, MNS, Kell, Duffy and Kidd systems. The red cells which are to be tested for the presence of a particular antigen have usually been dried on threads of material and it is these bloodstained threads that are allowed to absorb the appropriate antibody which is subsequently eluted and its presence then demonstrated using fresh red cells. However, the amount of bloodstain material available is often very small and far from fresh, so it is essential to obtain a high degree of sensitivity in this test and in our effort to achieve this the technique has been developed to attempt to produce the optimum conditions throughout.

Concentration of antigen and antibody during absorption

The relative concentrations of antigen and antibody are important and at the absorption stage of the test there will be an optimum amount of antibody for the given bloodstained thread. To use more than this optimum amount is not only unnecessary but may lead to false positive results due to the difficulty of washing away uncombined antibody from the thread [1].

Failure of an elution test can lead the novice to use *more* bloodstain material, i.e. more antigen, but this can sometimes ensure failure of the test rather than success. This is particularly so in the ABO system where it has been shown that increasing the amount of antigen may not increase the yield of antibody in the eluate when more than the optimum amount of bloodstain is used, but in fact the yield has decreased [1].

In practice we find that a 2–3 mm thread is satisfactory for testing with each ABO serum, while 5–10 mm threads are usually ideal for other red cell antigens.

Absorption may take place by adding the antiserum to the threads in tubes but for large scale work attachment of the threads to polycarbonate sheets is a time saver [2]. The tip of each thread is attached to the polycarbonate sheet using cellulose acetate glue and sufficient antiserum is added to the sheet to cover the thread.

Absorption of the antibody by the bloodstain takes place overnight at 4 or 37 °C depending upon the antigens being investigated. A shorter incubation time

will not necessarily result in a failure of the test but experiments have shown that such extended incubation does increase the sensitivity.

At the end of the absorption period, when the uncombined antiserum has to be washed away, the polycarbonate sheets can simply be plunged into a tank of saline at 4 °C, whereas the threads in tubes must be washed four or six times with the cold saline. The washing is carried out at 4 °C to ensure minimum dissociation of antibody from antigen and loss of antibody at this stage. The washing process is spread over a period of about 2 h. When polycarbonate sheets are used the threads are cut off and transferred to tubes at the end of the washing procedure.

Elution

One generous drop of 0.3% bovine albumin is added to each tube and elution is performed in a shaking water bath. Ideally the optimum temperature should be selected for each individual antibody [1]. In practice, 15 min at 55 °C is selected for ABO antibodies which may be a mixture of both IgG and IgM. A temperature of 60 °C is found suitable for antibodies of most other specificities which are mostly IgG antibodies and, moreover, extension of the elution time to 30 min has been found to improve the antibody yield.

Detection of eluted antibody

At the end of the elution period, in contrast to the normal elution procedure, the eluates are not removed from the antigen but the indicator cells are added directly to the eluate without removal of the stain material. Experiments have shown that stronger agglutination is obtained when the eluate is tested in the pres-

Table 2

Effect of presence of stain material in eluates during testing using anti-D

D type of 1 cm thread of bloodstain	Age of stain in months	Tests on eluate for anti-D	
		Stain material removed from eluate	Stain material remaining in eluate
+	2	(+)	++++
+	6	+	++++
−	6	−	−
+	8	−	(+)
+	10	+	+++
−	12	−	−
+	12	+	++++
+	15	++	++++
+	15	++	++++

Data from Dodd, Lincoln and McDowall [3]

ence of the stain. It appears that the indicator cells continue to acquire antibody at this stage both by competing successfully for any antibody remaining combined with the antigen of the stain and by reacting with antibody which, continues to be eluted. The advantage of not separating the eluate before testing is well illustrated by the results shown in Table 2.

For the detection of the very small quantities of eluted antibody, the concentration of the indicator cells must be very low in order to approach the optimum situation of antibody excess. Experience has shown that the optimum concentrations of indicator cells are 0.5% for ABO, 0.1% of papain treated cells for Rh, and 1% cells for all antibodies requiring anti-globulin for their detection.

Benefits of using LISS

The finding of a low ionic strength solution (LISS) by Löw and Messeter [4], which would improve the antigen–antibody reaction without inducing detectable non-specific uptake of antibody has proved to be exceedingly useful in our work with the elution technique. The introduction of LISS, both at the absorption stage and from the detection of eluted antibody, dramatically increases the sensitivity of the test [5, 6]. The LISS is used in the technique without any reduction in the incubation time because our objective is to gain increased sensitivity rather than a reduction in the time required for testing.

Results given in Table 3 illustrate the added sensitivity gained by incorporation of LISS in tests for the detection of various Rh antigens, the Duffy, S and Kidd antigens.

Table 3

Typing of bloodstains for various antigens showing the enhancement obtained using LISS

Antiserum	Phenotype of stain	Age of stain in weeks	Activity of eluate	
			without LISS	with LISS
anti-D	CcDEe	8	++	++++
anti-D	CcDee	60	(+)	++++
anti-C	CcDEe	3	(+)	++++
anti-E	CcDEe	20	+	++++
anti-S	Ss	36	++	++++
anti-Fy^a	Fy(a+b+)	33	++	++
anti-Fy^b	Fy(a+b+)	21	(+)	++
anti-Jk^a	Jk(a+b+)	21	+	++
anti-Jk^a	Jka+b+	33	−	+
anti-Jk^b	Jka−b+	33	−	++

++++ macroscopic agglutination
++ good microscopic agglutination
+ definite microscopic agglutination
(+) weak agglutination

Often the advantage of LISS may not be apparent on the fresher stains whereas on some of the older stains equivocal results obtained in the normal test became clear cut positive reactions when the LISS technique was used.

The Fy^a antigen was found to be detected just as well without LISS in the stains selected which were up to eight months old, but $Duffy^b$ showed a marked improvement on stains which were five months old.

Results obtained from Jk^a and Jk^b typing demonstrated that added sensitivity was achieved on the introduction of LISS into the test. Also the use of papain-treated cells in the LISS ahg test was found to produce further advantage for Jk^a and Jk^b antigen detection.

Detection of HLA Antigens

In addition to the impressive range of red cell antigens which can be detected in bloodstains, there is of course a wealth of white cell antigens of the HLA system. Ability to detect but a few of the antigens of this highly polymorphic system is a tantalising challenge for forensic serologists and there have been a few reports of attempts to do this over the past years.

Work carried out in our own Laboratory in conjunction with Professor Festenstein and also independently by Newall [7] working in Toronto has investigated the use of an inhibition technique. The lymphocytotoxic activity of HLA antisera is inhibited by HLA bearing bloodstains and this inhibition is then demonstrated using target lymphocytes in a standard lymphocytotoxicity test as used in normal HLA typing.

In our own work we first of all selected the optimum dilution of each potential serum by determining the highest dilution that would produce 100% killing of the target lymphocytes. For the inhibition test the appropriately diluted antiserum is added to a 4 cm length of bloodstained thread and after overnight incubation the absorbed serum is used in a microlymphocytotoxicity test using target lymphocytes which had been frozen until required. All tests were performed in duplicate and results averaged, and each batch of tests included a control test of the antiserum incubated without addition of a bloodstained thread. This control always produced 100% killing of the lymphocytes.

After assessing the suitability of individual antisera, three were usually selected for use in parallel for each HLA specificity. The results obtained were assessed by taking the mean inhibition of the three antisera.

Inhibitions of 55–100% were taken as indicative of the presence of the HLA antigen whereas inhibition of 0–45% indicated absence of the antigen. Inhibitions in the range 46–54% were regarded as indeterminate.

Our first investigations which we reported in 1979 [8] and 1980 [9] produced some promising results for detection of the HLA-A1 antigen. Only one out of 41 HLA-A1 positive stains was incorrectly typed and three out of 40 HLA-A1 negative stains were incorrect. Moreover further investigation showed that two

of the incorrect results on the HLA-A1 negative stains could be explained by known HLA cross-reactions. Age seemed to have little effect on the results obtained from stains up to several months old.

Results obtained from typing for the HLA-A2 antigen on stains of various ages are shown in Table 4. Generally these results are less promising than those obtained for HLA-A1. However, when categorised according to the age of the stains, 39 out of 42 HLA-A2 positive and 35 out of 37 HLA-A2 negative stains which were not more than thirty days old were correctly typed. The older stains showed a greater tendency to be mistyped.

Table 4

HLA-A2 typing of stains

Age of stain in days	HLA-A2 positive		HLA-A2 negative	
	Correct	Incorrect	Correct	Incorrect
0—9	17	1	15	1
10—19	6	0	6	1
20—39	16	2	14	0
40—79	4	5	12	0
>80	4	8	9	1

The HLA-B8 antigen was selected from the B locus for investigation but the results were disappointing. Over 50% of the HLA-B8 positive stains which were no more than ten days old, failed to show significant inhibition of the antisera.

Ability to detect the antigen was again the problem when testing for the HLA-CW3 antigen. From the results presented in Table 5 it can be seen that 31 out of 39 HLA-CW3 positive stains less than forty days old were correctly typed but the success rate appeared to drop dramatically in older stains.

Table 5

Results of HLA-CW3 typing of bloodstains

Age of stain in days	HLA-CW3 positive stains			HLA-CW3 negative stains		
	Correct	Incorrect	Intermediate	Correct	Incorrect	Intermediate
0—9	15	6	1	17	0	0
10—19	9	1	0	7	0	0
20—39	7	1	1	8	0	0
40—79	9	3	0	6	2	0
>80	4	4	0	2	0	0

It appears from these results that the inhibition test has produced some very promising results but certainly too many failures in detecting the antigen to be acceptable as a reliable technique for use in casework at the moment.

With the arrival of monoclonal antibodies we were able to investigate the alternative technique of direct detection of the HLA antibodies taken up by the bloodstain using ^{125}I-labelled antiglobulin. However, using a range of dilutions of a monoclonal anti-HLA-A2 antibody we did not appear to see a specific uptake of the HLA antibody in our initial experiments and we therefore proceeded to investigate the sensitivity of our technique. We found that we needed far more lymphocytes than would be available in 4 cm of bloodstained thread in order to obtain a satisfactory differentiation between the uptake of labelled globulin by the HLA-A2 positive and negative lymphocytes. In fact, part of the problem appeared to be the non-specific uptake of ^{125}I-labelled globulin. Further investigation must now be made using other examples of monoclonal antibodies and other techniques.

Thus, although a very wide range of red cell antigens are routinely used in our casework, the white cell antigens must still remain as a tantalising possibility because even the results gained by the inhibition test need more consolidation before they can be used confidently in casework and presented to the Courts.

References

1. Lincoln, P. J., Dodd, B. E.: An evaluation of factors affecting the elution of antibodies from bloodstains. *J. Forens. Sci. Soc. 13*, 37 (1973).
2. Howard, H. D., Martin, P. D.: An improved method for ABO and MN grouping of dried bloodstains using cellulose acetate sheets. *J. Forens. Sci. Soc. 9*, 28 (1969).
3. Dodd, B. E., Lincoln, P. J., McDowall, M. J.: Observations on the use of an autoanalyser (LISP) and a manual technique for the detection of red cell antigens in bloodstains. In: *Proc. 8th Technicion Int. Congr. London 1978.*
4. Low, B., Messeter, L.: Antiglobulin test in low-ionic strength salt solution for rapid antibody screening and crossmatching. *Vox Sang. 26*, 53 (1974).
5. Lincoln, P. J., Dodd, B. E.: The use of low ionic strength solution (LISS) in elution experiments and in combination with papain treated cells for the titration of various antibodies including eluted antibody. *Vox Sang. 34*, 221 (1978).
6. McDowall, M. J., Lincoln, P. J., Dodd, B. E.: Increased sensitivity of tests for the detection of blood group antigens in stains using a low ionic strength medium. *Med. Sci. Law 18*, 16 (1978).
7. Newall, P. J.: The identification of HLA-A2 and HLA-B5 antigens in dried bloodstains. *Canad. Soc. Forens. Sci. J. 12*, 1 (1979).
8. Hodge, D., Festenstein, H., Wolf, E., Lincoln, P., Dodd, B.: Results from a preliminary study of the detection of HLA antigens in bloodstains. In: *Proc. 8th Int. Congr. Soc. Forensic Haemogenetics, 1979*, p. 175.
9. Hodge, D. G., Wolf, E., Lincoln, P. J., Festenstein, H., Dodd, B. E.: The detection of the HLA-A1 antigen in bloodstains. *Med. Sci. Law 20*, 213 (1980).

Haematologia 17 (2), pp. 307–310 (1984)

The Change in Criminalistic Methods for Bloodstain Analysis in California

B. A. MYHRE

Department of Pathology, Harbour/UCLA Medical Center, 1000 W. Carson Street, Torrance, CA 90509, USA

(Accepted 30 January 1983)

The field of bloodstain analysis is undergoing considerable change. Newer technics are being used, and new legal decisions are providing specimens which are of better diagnostic use. However, the cost of the equipment, and the necessary training needed, has caused a considerable decrease in the number of individuals who are performing studies for the defense. At the same time the technics have made it possible for the courts to have better scientific evidence available to help them in their decisions. It is hoped that defense experts will keep pace with those of the prosecution, and ensure the right to fair trial for any individual.

Keywords: bloodstains, criminalistics, forensic hematology, medico-legal issues

*

In most of the United States, until the last 5 years, stain analysis in criminal cases was usually confined to chemical tests for the presence of blood or semen, simple serological tests for species, and the determination of the ABO group factors. These studies were usually not of too much help to the courts in the determination of guilt because of the small power of exclusion. However, in a few cases it was a help, since it was possible to show a blood group similarity or lack of similarity between a blood or seminal fluid stain and the accused. Sometimes these studies were actually a hindrance, since they were occasionally done with no or minimal controls, and were inaccurate.

About 5 years ago, a new type of criminalist appeared, working for the prosecution. These criminalists were well trained individuals, mostly chemists, who started using some very sophisticated tools in their trade. These were the same tools which had been used in Europe for a number of years, but in this area we were very slow. Most of the newer tests involve electrophoresis, and by the use of these, the results can show great discrimination. Therefore the studies now coming from the prosecution are rather inclusive and can be very helpful. Several problems have arisen with the use of these new technics. Due to the expense of setting up the newer style laboratories, and gaining skill in performing and interpreting the tests many laboratory scientists have now decided that they do not wish to be bothered with the time, the expense, and the trouble involved in performing analyses for the defense and giving court testimony. Therefore there has been a decrease in the number of people who do this type work, even though it is certainly necessary

[1]. Further, in California several decisions have been reversed in appellate court by the failure of the defense to use adequate expert testimony. The question then is who does the work for the defense to challenge the prosecution, or at least to serve as a quality control for their findings and testimony?

In order to answer this question, one must make several observations about the American court system and how it differs from those in Europe. The American system is a dedicated adversary one. In this system, both sides try to present their evidence as the only correct interpretation, and the jury makes the decision as to who is correct. It is most unusual for a laboratory scientist to be invited into the courtroom as a friend of the court, and give impartial testimony that is accepted by both sides. Usually each side has its experts, and the lawyers try to show that their expert is the only possessor of true knowledge, while the other expert is incompetent, if not liable for malpractice. The state or country criminalist and the defense expert are treated comparably. If it is possible to find an opposing expert, the defense lawyer will try to discredit the prosecution's testimony as much as possible. The blow to a scientists pride to hear his hard-won knowledge and findings deprecated can be devastating if one is not used to it. Legitimate findings are discredited or misinterpreted and the expert is rarely allowed to give an unbiased scientific opinion. The basic attitude is that if the opposing lawyer does not understand the problems, it is his problem. For this reason, many scientists do not like to testify in court.

Another problem which the defense analyst encountered, was the condition of the specimens which were to be tested. Usually, as soon as a crime has been committed, the specimens were transmitted to the local Scientific Investigation Unit (the Peoples Laboratory), where after a little while they are studied. Then the accused criminal is incarcerated, a defense lawyer is appointed, and after 9 months to 2 years, the defense analyst will be contacted. The specimens are therefore almost always 1 – 2 years old. Blood samples, by this time, are often contaminated, vaginal aspirates are growing mold, many of the blood tubes are dried out, and clothing may have been stored in such a manner that it is almost impossible to solubilize a clearly visible stain. This does not lead to precise analysis.

In 1980, the Supreme Court of California handed down the decision of the case of People vs Nation [2]. In this decision they found that it is the duty of the people (prosecution) to preserve specimens in such a manner that they can be analyzed at a later date by the defense experts thus confirming or contradicting the results of the people. Since this ruling there has been more interest in the preservation of the specimens, and also in performing work for the defense.

Until the Nation decision, many blood samples were not even refrigerated after being studied, but rather were stored in the evidence locker at room temperature. Currently, they are being refrigerated in most localities but many still do not freeze them. For this reason, it still is unusual for a defense analyst to be able to perform a large number of enzyme analyses which are valid, however some usually react. Further, it is possible by reexamining vaginal slides for sperm, by repeating all blood groups, and by testing chemically for blood and seminal

fluid, to perform enough analyses and interpret the results in such a way that the defendant is given a fair trial.

Most of the studies performed in the crime laboratories of either the city or county use a variation of the system which was developed by Beckman Laboratories under a contract from the Law Enforcement Assistance Administration and administered by the Aerospace Corporation. These methods are based on work done and published by Culliford [3]. They were given to the Aerospace Corp. as a final report, but the work has not been detailed in a reviewed scientific journal. Following completion of the work, the technics were disseminated throughout the country by the use of a series of workshops presented by the advisers of the project. The methods are based on a gel electrophoresis method in which an eluate of the stain is absorbed on a cloth fiber and then embedded into an appropriate gel. Usually three different gels are prepared. The first, for so-called group I enzymes, determines erythrocyte acid phosphatase (EAP), phospho-gluco mutase (PGM), and glyoxalase (GLO) simultaneously. The second gel is used to run esterase D (EsD), amino deaminase (ADA), and adenylate kinase (AK). The third gel is used to for group component (Gc), and haptoglobins, but this has not been as effective, and for that reason, these tests are not as often done.

The technics used in my laboratory are slightly different in methodology, but achieve comparable results. As do most laboratories, we begin by testing the blood stain chemically to see if it is blood or not. We have found as has everyone else, that all red stains are not necessarily blood. We have used the old and reliable benzidine procedure because it is sensitive and we like it, but we are in the process of change, since benzidine is now classified as a carcinogen, and cannot be obtained. If a positive reaction for blood is obtained, we then set up a series of Ouchterlony plates with appropriate antisera for species identification. This is necessary to assure that the blood stain is not from some animal, but is indeed human. Some defenses are based on the alleged fact that the defendants killed some animal on the night of the crime and the specimens must be studied to assure that the bloodstain is actually human. If the stain is suspected of being seminal fluid we localize it by using fluorescent illumination. A sample is dissolved and confirmed with an acid phosphatase reaction. We usually confirm the stain's identity by electrophoresis rather than using a spot test. Although, it is very difficult to demonstrate diagnostic isoenzyme bands in seminal fluid with this technic, if the material migrates in an electric field and reacts with the correct reagents, then one is certain that the material studied is acid phosphatase, and not a whitener from soap or some other foreign material which will fluoresce.

After it has been confirmed that the stain is blood or seminal fluid, and that it is of human origin, the ABO blood group testing is done using neutralization if the stain is large, and absorption-elution if it is small. The methods for these studies are outlined in current texts [5]. To save time and increase accuracy in the study, we use the Howard and Martin modification of the absorption-elution test where threads soaked in the blood stain are attached to a cellulose acetate plate and processed together [6]. We do not use the mixed agglutination procedure, since we have had

difficulty in standardizing it. If possible, we confirm all ABO studies with the Lattes technic in which one attempts to find blood group antibodies in the stain.

Once we have determined the blood group, we then attempt to extend the identification by determining as many isoenzyme groups as possible. The electrophoresis method that we use is that popularized by Grunbaum [7] and employs a cellulose acetate membrane as the supporting medium. When this method is used, each enzyme is studied on a separate membrane. After the run, the membrane is placed on agar gel containing the desired substrate, and the colors develop. The method has several advantages. It is very fast, does not require a cooling bath, and usually gives clean, easy to read lines. Further, it is possible to dry the membranes and store them as a permanent record.

Using this method, we normally test EAP, EsD, Gc (performed with immunofixation), PGM and GLO. We do not do ADA or AK because of the poor discrimination value due to the skewed gene frequencies. We feel that it is not cost effective to perform these studies.

By the use of these methods, it is possible to achieve a discrimination probability of 1 in 200. This index may even be higher if the suspect or the blood stain is of a rare type. Probability indices are always calculated for the lawyer as an aid in planning the defense. The gene frequencies used for these calculations are found in the paper by Grunbaum et al. [8]. The subject of further calculations on the similarity of the accused and the blood or semen stains has been the subject of a recent communication [9] and will not be presented here in more detail. Prior to the use of the more sophisticated technics the probability index was rather low; so no calculations were necessary. Now, however, it is possible to show a rather high discrimination probability and the calculation of probabilities is going to be a fact of great interest in the future, and will undoubtedly generate considerable controversy.

References

1. Lincoln, P. J.: Blood group evidence for the defense. *Med. Sci. Law. 20*, 239 (1980).
2. People vs. Nation — Supreme Court Decision 26 Cal. 3d 169.
3. Culliford, B.: *The Examination and Typing of Bloodstains in the Crime Laboratory*. U. S. Govt. Printing Office, Wash., D. C., (1971).
4. Wraxall, B., Bordeaux, J., Harmor, G., and Walsh, J.: *Final Report—Bloodstain Analysis System*. Contract No. J-LEAA-025-73 of the Law Enforcement Assistance Administration, U. S. Dept. of Justice, Beckman Advanced Technology Operations (1978).
5. Boorman, K. E., Dodd, B. E., and Lincoln, P. J.: *Blood Group Serology*. 5th ed. Churchill Livingstone, London 1977 p. 415.
6. Howard, H. D., and Martin, P. D.: An improved method for ABO and MN grouping of dried bloodstains using cellulose acetate sheets. *J. Forens. Sci. Soc. 9*, 28–30, (1969).
7. Grunbaum, B. W.: *Handbook for Forensic Individualization of Human Blood and Bloodstains*. Sartorius GmbH, Göttingen, 1982.
8. Grunbaum, B. W., Selvin, S., Myhre, B. A., and Pace, N.: Distribution of gene frequencies and discrimination probabilities for 22 human blood genetic systems in four radical groups. *J. Forens. Sci. 25*, 428 (1980).
9. Selvin, S.: The probability of non-discrimination or likelihood of guilt of an accused: criminal identification. *J. Forens. Sci.* in press, (1982).

Importance of the Choice of the Means in the Study of Bloodstains

A. André, B. Hoste, L. Kamenev

University of Liège and Red Cross Blood Group Laboratory and Transfusion Centre
39, rue Dos Fanchon, 4020 Liège, Belgium

(Accepted 20 January 1983)

The determination of blood groups has taken on an ever increasing importance as judicial proof, due to the latest immunological advances and to technical progress. The fact that one can determine in a small bloodstain a great number of genetic markers, now enables us to elucidate a large number of criminal affairs. This, of course, requires a thorough knowledge in the field of blood groups. Their study in biological stains concerns blood, saliva, sweat and semen. It sometimes enables one to exclude a suspect, or it can yield reliable information if gene frequencies are used.

Cases where bloodstains studies are applied:

- homicide
- suspect injured on the scene of a crime
- injuries and wounds
- reconstruction of a criminal act
- rechecking of blood samples in cases of suspicion of inverted tubes

It is mainly the search for blood group antigens in stains which is most frequently carried out.

A systemic study of our investigations has been done. It concerns the various biological mediums. The distribution of which is given in Table 1. The "alcoholaemia" column represents the identification of blood samples when there is a suspicion of inverted tubes.

Table 1

Classification of 229 investigations according to the nature of the biological stain 1977–1982

sweat	saliva	blood	semen	alcoholaemia
1	3	169	28	6
		19		
	3			

Table 2

200 investigations of blood, semen and saliva stains
1977–1982

Nature of crime	per cent
homicide	40.5
assault and battery	12
rape, indecent assaults	17
robbery with violence	5.5
simple robbery	4.5
traffic	5
blood sample identification (for alcoholaemia)	3
various: unexplained death	6
corpse identification	1
vandalism	1
suspect stains	1
escape	1
others	2.5

Table 3

200 investigations of blood, semen and saliva stains
1977–1982
Judicial value of the results

Type		negative	incomplete	positive
homicide		2	15	64
assault and battery		2	5	17
rape, indecent assault		0	15	19
robbery with violence		0	1	10
simple robbery		2	3	4
traffic		0	1	9
blood sample identification (for alcoholaemia)		0	0	6
various		1	5	19
Total	200	7	45	148
per cent	100	3.5	22.5	74.0

The investigations can first be studied in function of the nature of the facts (Table 2), homicides being the most frequent.

Analysis of the results can be negative, or incomplete when some information is lacking from the beginning or when, through lack of biological material, the investigation could not be completed. Finally, it can be positive when reliable results can be given to the magistrate, that is to say whether or not there is human

blood: whether or not the blood groups can be determined; and whether, through this determination, a suspect can be excluded or proof of his presence be given.

These positive results are added to other elements of the investigation. They sometimes even carry definitive weight of the judge's decision.

This analysis, given in Table 3, shows that in 74% of the cases, positive results were obtained.

We have been particularly concerned with the study of 159 investigations on blood groups (Table 4). Using the same criteria with regard to the judicial value, i.e. negative, incomplete or positive results, it can be seen that the study of blood stains gave positive results in 77.3% of the cases.

Table 4

159 investigations of bloodstains
1977–1982
Judicial value of the results

Results	Judicial value			Number of cases
	negative	incomplete	positive	
no blood	1	4	16	21
blood	0	3	0	3
animal blood	0	0	4	4
human blood				
without groups	4	7	1	12
with groups	1	16	102	119
Total	6	30	123	159
per cent	3.8	18.9	77.3	100

In Table 5, the study of these investigations was done in function of the number of individuals concerned, the number of exhibits, the number of stains analysed, the number of searches for evidence of human origin, and the number of blood groups that were determined.

Table 5

159 investigations of bloodstains (1977–1982)

number of cases	159
number of persons involved	241
number of exhibits	747
number of analysed stains	774
number of searches for human origin	705
number of determined blood groups	1 654

We may draw the conclusion that if the study is without doubt an important one, it should not be forgotten that it yields useful results in 77.3% of the cases (Table 4).

Table 6 shows the blood group systems that have been particularly investigated and the frequency of these searches. In some cases the choice of the latter was made in function of blood samples of the victim or victims, of the suspect or suspects that were referred to us, which allowed to choose the blood groups that could differentiate the individuals and, as a consequence, to identify the bloodstains in relation to the individuals concerned.

Table 6

159 investigations of bloodstains (1977-1982)

Number of determined blood groups	:	1654
ABO	:	361
MN	:	266
Ss	:	62
Rhesus	:	75
Gm, Km	:	424
Hp	:	202
PGM_1	:	146
AK	:	92
ADA	:	26

Table 7

Relative efficiency of the blood group systems in dried blood

Systems	positive results for 10 searches
ABO	9
Gm, Km	8
MN	8
Rhesus	7
AK	7
Hp	7
PGM_1	6
Ss	6
ADA	3

Unfortunately, this is not always the case, and we often have to proceed blindly if we do not have, from the start, blood samples of the individuals involved. The results for each blood group system vary in function of their persistence in the exhibits or of the technique that can be used for evidencing them. For instance (Table 7), if we take 10 searches for each system listed in Table 6, we may classify the blood group systems which will most frequently give useful results.

Table 8

An average bloodstain investigation (1977–1982)

1 to 2	persons involved
5	exhibits
5	stains examined → 4 blood
4	searches for origin → 3 to 4 human blood
10 to 11	blood groups determined

As a conclusion (Table 8), we may say that in each investigation one or two individuals are concerned and about five exhibits are examined. On the average five bloodstains are examined, 4 are blood and 3 to 4 are human blood, in which 10 to 11 blood group systems are determined. This shows the utility of such researches, despite the amount of work required for each investigation.

As it can be seen in Table 4, a result is obtained in 77.3% of the cases.

Haematologia 17 (2), pp. 317–322 (1984)

ABO and Lewis Typing of Semen, Saliva and Other Body Fluids

MARGARET PEREIRA

Home Office Forensic Science Laboratory, Chepstow, Great Britain

(Accepted January 10, 1983)

It is well known that ABH group specific substances are usually present in high concentrations in body fluids of secretors. In normal circumstances these substances can withstand drying and retain their antigenic activity over a prolonged period. This enables the forensic serologist to assist in the investigation of various crimes by grouping stains of body fluids such as semen and saliva. It is possible, for instance, to group saliva and lip mucosa stains on cigarette ends, gags, masks, postage stamps and envelope flaps etc. but it is the grouping of seminal stains in the investigation of sexual crimes which predominates. The results of such tests can be extremely valuable in either including or excluding suspects..

Keywords: ABH group-specific substances, body fluids, forensic serology

Grouping methods in the ABO system

The grouping of dried bloodstains in the ABO system can be undertaken both by absorption-inhibition and absorption-elution. Because of the sensitivity of the latter, this has tended to be the method of choice of many workers during the last twenty years. Both methods can also be applied to the grouping of body fluid stains. Because of the normally high concentrations of blood group substance in stains from secretors, the less sensitive absorption-inhibition method is usually very satisfactory. For testing by the absorption-elution method, it is essential to work on a series of dilutions of stain extract prepared in distilled water. The dilution series can then be dried onto either cotton thread or polycarbonate sheet for testing. The very high concentration of blood group substance present in body fluids of secretors causes a 'prozone' type effect. This is due to an excess of water soluble antigen which combines with antibody so that there is insufficient antibody available to combine with that portion of the antigenic material bonded to the substrate. However, a series of dilutions starting with negative or weak reactions and showing an initial increase in activity in a dilution series is very convincing of the presence of a high concentration of antigen.

Absorption-inhibition tests are also carried out using a dilution series (usually 10-fold dilutions) of stain extract in saliva. With both techniques, 3 aliquots of each dilution are tested with anti-A, anti-B and anti-H (Ulex europaeus) respectively [4].

Possible pitfalls of interpretations

On reading basic textbooks on serology, one might be tempted to think that the presence of the secretor gene produces clearly defined segregation between secretors and non-secretors in all body fluids. In practice, it is not always possible to distinguish one from the other; sometimes this may be attributed to poor samples but there is also a problem due to the variability of the level of blood group substances between individuals. There is also fluctuation in the levels produced by individuals at different times. This is scarcely surprising since most of the fluids concerned are not homogeneous; in saliva there is a variability in the contributions from the different salivary glands and in vaginal secretion one would expect variations due to the influence of the menstrual cycle. Semen is, of course, a very complex mixture. Occasionally one also encounters individuals where the substance is not expressed at a particular site; for example, one may obtain typical secretor reactions in the semen but fail to detect substance in the saliva. Stains from group AB secretors can sometimes present problems in that one or other of the antigens, particularly the A antigen, may be very weakly expressed and therefore difficult to detect except by the more sensitive absorption-elution technique. It is frequently possible to detect blood group substances in the body fluids of non-secretors especially when using the absorption-elution technique.

Occasional false A and B reactions are also encountered although these are generally weak compared with true groups specific substances in secretors. Jenkins et al. [3] reported the production of pseudo B reactions in seminal stains after inoculation with E. coli 086. Others studied saliva samples which gave false ABH reactions and isolated certain strains of micro-organisms which gave A, B or H activity corresponding to the false reactions obtained from the original saliva samples. False reactions likely to be bacterial in origin are also encountered with vaginal fluid.

Dorrill and Whitehead [1] investigated the levels of A, B and H group specific substances in semen and saliva. They found that on average semen contained 2–3 times more A and B substances than saliva and 20 times more H substance. They found a much wider range of levels of substances in semen compared with saliva.

It is not uncommon to obtain positive reactions from the unstained substrate. This is frequently experienced when dealing with stains on underwear. This is a serious limitation and can render group determination impossible.

Having regard to all of these problems, it is obvious that great care must be taken in interpretation if reliable results are to be obtained in the grouping of body fluid stains in the ABO system. In my opinion absorption-inhibition and absorption-elution tests should be conducted in parallel; opinion as to the group should be given only when the two sets of tests are compatible. A typical example of discrepancy between the two sets of results is as follows: both tests show strong B reactions but A activity is detected only by absorption-elution. In these circumstances, although one can be confident about the presence of the B antigen, the weak A reaction may be the result of a group AB stain with suppressed A activity

Table 1

Example of group A secretor saliva stain reactions

		Inhibition			Elution		
		Anti A	Anti B	Anti H	Anti A	Anti B	Anti H
Control	Neat	3	4	3	0	0	0
	1/10	4	4	3	0	0	0
	1/100	4	4	4	0	0	0
	1/1000	4	4	4	0	0	0
Stain	Neat	0	3	0	1	0	3
	1/10	0	4	0	3	0	2
	1/100	1	4	3	4	0	2
	1/1000	2	4	4	3	0	1

Key to score

4. Almost all cells agglutinated in one large clump
3. Several large clumps
2. Small clumps
1. Most cells free, slight agglutination

Table 2

Lewis group stain reactions

Stain extract dilutions	Le (a−b+)		Le (a+b−)		Le (a−b−)	
	Anti Le^a	Anti Le^b	Anti Le^a	Anti Le^b	Anti Le^a	Anti Le^b
Neat	0	0	0	4	3	3
1/5	0	0	0	4	3	4
1/10	0	0	0	4	4	4
1/20	3	0	0	4	4	4
1/30	4	0	0	4	4	4
1/40	4	0	2	4	4	4

Unabsorbed sera (goat)

Anti Le^a	Anti Le^b	Both antisera diluted
4	4	1/4

or it may be a false reaction probably due to the presence of micro-organisms. It has been argued that one should not refer to secretor status when grouping dried stains because of the variation in the levels of blood group substance produced both by secretors and non-secretors. I am of the opinion that when very strong

reactions are obtained by both inhibition and elution techniques, one can be confident that the stain originates from a secretor. It would also be most unlikely to obtain negative A, B and H reactions from a stain which was apparently a neat seminal stain.

Lewis grouping

One can have greater confidence in the interpretation of secretor status if the ABO results are supported by compatible Lewis group results. Now that good quality Lewis sera are more readily available their use has become a matter of routine in cases involving body fluid grouping. It is usual practice in British Forensic Science Laboratories to request samples of both blood and saliva from individuals involved in an offence for the purpose of obtaining blood groups and secretor status. In the Chepstow Forensic Science Laboratory, secretor status of individuals is initially made by Lewis typing of the blood samples providing they are in good condition and are Lewis positive, not Le (a−b−). The saliva is available for Lewis negative samples and can also be kept frozen and tested later in parallel with any relevant stains. Although Lewis sera are costly this practice results in considerable saving of labour.

Lewis grouping of body fluid stains using absorption-inhibition is reasonably successful. Saliva stains from Le (a−b+) individuals usually inhibit both anti Le^a and anti Le^b although the anti Le^b is consistently inhibited to a greater degree. Stains from Le (a+b−) individuals show strong inhibition of anti Le^a and little or no inhibition of anti Le^b. Lewis negative Le (a−b−) stains show little or no inhibition. There have been differing reports regarding the Le^a activity found in seminal stains from Le (a−b+) individuals. Grubb [2], Pereira and Martin [5] and Shutler and Dawson [7] reported the absence of Le^a in seminal stains of this type but Piner and Sänger [6] found that it was present. The experience in the Chepstow Forensic Science Laboratory using Lewis typing sera prepared in goats is that Le^a can be detected in semen samples from Le (a−b+) individuals but at a consistently lower concentration than Le^b. Like the inhibition technique for ABO typing of body fluid stains, the Lewis antisera are exposed to a series of dilutions of stain extract and are tested after an overnight absorption period at 4°C. Because of the fragility of agglutination, Lewis typing requires a high standard of serological expertise. Absorption-elution has not proved satisfactory for Lewis grouping.

Seminal stains – sexual offences

Much of the work involving body fluid stain grouping is related to the investigation of sexual offences. Although in some cases one may be dealing with uncontaminated seminal stains, they are frequently contaminated with vaginal secretion (which can contain high levels of blood group substance) or even saliva. Seminal stains are occasionally found on skin surfaces; this is an obvious source

of background interference since it is easy to detect blood group substances in sweat. An unstained portion of skin must be swabbed for control purposes. In the average rape case, examinations are made of vaginal swabs and clothing of the victim. For semen grouping, drainage stains tend to give better results than vaginal swabs. No assessment can be made of the group of the semen without knowledge of the group of the victim. Blood and saliva samples from the victim are therefore a prerequisite for grouping seminal stains in these circumstances. It is also necessary to consider whether or not the victim has had intercourse with anybody else which might contribute to the reactions obtained from the material examined. If enquiries reveal that this is so, then it is also necessary to obtain the appropriate samples from the man concerned. Swabs and clothing are screened for the presence of semen using tests for acid phosphatase. This is followed by a microscopic examination of extracts from swabs or clothing for the detection of spermatozoa and an assessment of their concentration and also for an assessment of the possible presence of vaginal epithelial cells. Absence of such cells cannot be taken as definite elimination of the presence of vaginal secretion. No accurate assessment of the relative concentrations of seminal and vaginal material is possible although strong acid phosphatase reactions and numerous spermatozoa indicate a high concentration of semen from which one would expect to obtain group specific reactions if the semen originates from a secretor.

Stains of mixed origin

When dealing with stains of mixed origin the interpretation of the results obviously depends very heavily on the group and secretor status of the victim. There would be little point in conducting the tests if she were a group AB secretor. If the victim were of group O, then reactions for A (and H) would clearly indicate the presence of group A semen. Given a reasonably high concentration of semen and reactions for A and H from a group A secretor victim, one could only eliminate men who were secretors of groups B or AB as possible sources of the semen.

Discussion

In this account I have laid great emphasis on the problems which exist in the grouping of body fluid stains in the ABO system and the care which must be taken in the interpretation of results. Despite all these difficulties and despite the many occasions when one is unable to report grouping results, nevertheless much useful information can be obtained. Body fluid grouping is chiefly concerned with serious crime such as sexual assaults and murder. In many sexual offences it is possible to either include or eliminate suspects on the basis of seminal stain grouping. The elimination of suspects is of great assistance to the police in their investigation in cases where there are many suspects. Sometimes grouping tests have provided

an important linking factor in a series of crimes especially when associated with PGM typing. Therefore, although there is a relatively high percentage of stains which give results which are inconclusive, the work is of sufficient value to justify the effort.

References

1. Dorril, M., Whitehead, P. H.: The species identification of very old human blood-stains. *Forensic Sci. Int. 13* (2), 111 (1979).
2. Grubb, H.: *Acta Pathol. Microbiol. Scand.* 28, 61 (1951).
3. Jenkins, G. C., Brown, J., Lincoln, P. J., Dodd, Barbara E.: *J. Forensic Sci. Soc. 12*, 597 (1972).
4. Pereira, M., Martin, P. D.: Problems involved in the grouping of saliva, semen and other body fluids. *J. Forensic Sci. Soc. 16* (2), 151 (1976).
5. Pereira, M.: Determinations of Lewis secretions in body fluids. *J. Forensic Sci. Soc. 17* (2–3), 135 (1978).
6. Piner, S. C., Sänger, M. S.: Lewis grouping of human secretion stains. *Forensic Sci. Int. 15* (2), 87 (1980).
7. Shutler, G. C., Dawson, D. E.: *Can. Soc. Forensic Sci. 13*, 11 (1980).

Haematologia 17 (2), pp. 323–325 (1984)

Identification of Human Lymphocyte Antigens (HLA) in a Semen Stain

S. LONTAI-SANTORA, E. GYÓDI, S. R. HOLLÁN

Institute of Forensic Science, Budapest 1903, P. O. Box 314/4 and National Institute of Haematology and Blood Transfusion, Budapest, Hungary

(Accepted 30 April 1983)

A method developed for the determination of HLA-A and HLA-B antigens in blood stains has been applied for the identification of HLA antigens in a semen stain, on the basis of the inhibition of the lymphocytotoxic activity of HLA-A and HLA-B antigens of semen stains.

Keywords: bloodstain, HLA-A2 antigen, HLA-Bw38 antigen, lymphocytotoxic activity or inhibition of lymphocytotoxic activity, semen stain

Introduction

Although the existence of the HLA system has been known for more than twenty years [1], and this extremely polymorphous system would be of utmost importance in criminalistic investigations, up to now few papers reported on HLA antigen determination from blood stains [2, 3, 4, 5] and even less have dealt with the identification of HLA antigens from secretion's and stains [5].

In the Serological Laboratory of the Institute of Forensic Science, identification from stains of some antigens of the HLA system beside the the antigens of the ABO, Rh and Gm systems has been done successfully.

In this paper we report on the identification of HLA-A2 and HLA-Bw38 antigens from a semen stain in a criminal case.

Report of a case

On the face and hair of the victim of a sexual crime, stains of biological origin were found. Part of the biological material proved to be semen on the basis of specific enzyme tests and of the morphological picture. Semen samples were collected on gauze soaked in physiological saline solution.

Blood and serum group determinations from the semen stain for ABO and for Gm_1 and Gm_4 were carried out using the absorption inhibition method [6]. Determination of the same groups from the blood of a suspect person gave A and Gm_1^-, Gm_4^-. As to the HLA antigens of the semen stain, we used the same procedure as for blood stain HLA antigen determination. Since in the blood sample of

the suspect, HLA-A2, HLA-Aw30 and HLA-Bw38 were found, these antigens were sought directly in the semen stain sample.

The principle of HLA antigen determination from stains was that the lymphocytotoxic activity of HLA-A and HLA-B antisera is inhibited by the appropriate HLA-A and HLA-B antigens of the stain. The morphological changes in target lymphocytes were studied under the phase contrast microscope after eosin staining.

The control in every experiment was such a system in which there was a direct reaction between the antiserum and the appropriate HLA-antigen-containing target cells. This antigen–antibody reaction resulted in a total killing of target cells.

Another control was sterile gauze without any biological material. We had antisera of sufficiently high titre for the determination of the A2 and Bw38 antigens, but not for Aw30. For the determination of the A2 antigen the antiserum with 1 : 8 titre signed Ste was used, while for the determination of Bw38 two antisera signed OTCsP and Bp168 were used. Both of them had a titre of 1 : 8.

The results with antiserum Ste for determination of the A2 antigen were as follows. The semen stain sample from the face of the victim caused a decrease of the original 1 : 8 titre to 1 : 2, while the other sample from the hair of the victim caused a decrease of the titre to 1 : 4. This proved the presence of the A2 antigen in the semen stain.

The result with the antisera OTCsP and Bp168 for the determination of Bw38 antigen was as follows.

The semen stain from the face of the victim caused the titre to decrease from the original 1 : 8 to 0. The semen stain from the hair of the victim caused the decrease of the titre of OTCsP antiserum from 1 : 8 to 1 : 4, while the Bp168 antiserum showed a decrease from 1 : 8 to 1 : 2. These results pointed to the presence in the semen stain of Bw38 antigen.

Our results can be best interpreted as evidence that 1) the suspect carried the A blood group antigen, the Gm_1^-, Gm_4^- serum group antigens, and the A2, Aw30 and Bw38 HLA antigens, and 2) the semen stain from the hair and face of the victim contained A blood group antigen, Gm_1^-, Gm_4^- serum group antigens, and the A2 and Bw38 antigens.

Thus the semen sample may have originated from the suspect. As to the calculated frequency of the above mentioned properties in Hungary, the frequency of A blood group is 40% [7]; the frequency of A2 and Bw38 haplotypes is 0.97% (8); of Gm_1 negativity, 60%, while the frequency of Gm_4 negativity is 89% [7].

The frequency of the joint presence of A-Gm_1 negative, Gm_4 negative, HLA-A2 and HLA-Bw38 antigens in the Hungarian population on basis of the above data is 0.002%.

References

1. Dausset, J.: Isoleuco-anticorps. *Acta Haematol.* (Basel) *20*, 156 (1958).
2. Rittner, Ch., Waiyawuth, J.: HLA typing in dried bloodstains. I. Specificity and reliability of the inhibition test. *J. Immunogenet. 1*, 99 (1974).
3. Rittner, Ch., Waiyawuth, J.: HL-A typing in dried bloodstains. II. Comparative studies on microcytotoxicity and microcomplement fixation tests. *J. Immunogenet. 2*, 211 (1975).
4. Newall, P. J.: The identification of HLA-A2 and HLA-B5 antigens in dried bloodstains. *Can. Soc. Forens. Sci. J. 12*, 1 (1979).
5. Newall, P. J.: The identification of HLA-A9 in dried stains of blood and body secretions. *Can. Soc. Forens. Sci. J. 14*, 104 (1981).
6. Lontai-Santora, Zs.: ABO és Gm tulajdonságok meghatározása vérfoltokból. C. Sc. Thesis, Budapest (1982).
7. Gyódi, E., Tauszik, T., Petrányi, Gy., Kotvász, Á., Takács, I., Nemák, P., Hollán, S. R.: The HLA antigen distribution in the Gipsy population in Hungary. *Tissue Antigens 18*, 1 (1981).

OBITUARY

Dr L. P. HOLLÄNDER, who served as a Member of our Editorial Board from 1967, our Journal's start to the present, passed away in Zurich on 5th November 1983.

Dr Holländer was born in Kassa, Hungary. He completed his medical studies at the School of Medicine of German University, Prague in 1935 and was Chief Physician of the Department of Child Health Care at Prague State Hospital.

He left for Switzerland and joined the staff of Prof. Fanconi at the Kinderspital Zurich in 1948. While there, he was appointed head of the Blood Group Serology Laboratory. Recognizing his outstanding services, they had sent him to the Central Laboratory of the Netherlands Red Cross, Amsterdam, and to the Lister Institute in London for postgraduate training. He became head of the Blood Transfusion Centre of Bürgerspital Basel in 1951. Within a short period of time he had built up a fine Centre with high standards. With Dr Karger and Peter Holländer Basel became the cradle of Vox Sanguinis, the first really international periodical in the field of blood transfusion. (One of the founders of the popular "Vox", Dr Holländer was European Editor-in-Chief for 17 years, from 1960–1977.)

The Blood Transfusion Centre in Basel became a meeting point for experts from all over the world. And in this milieu Peter Holländer was not only an internationally acknowledged expert and pioneer in blood transfusion but had remained a lovable person and a dear friend. The Editorial Board of our Journal feels bereaved by his unexpected death and will keep the memory of his achievements and eminent services. HAEMATOLOGIA also extends condolences to the family.

Susan R. Hollán

Abstracts

Regulation of calcium accumulation and efflux from platelet vesicles. Possible role for cyclic-AMP-dependent phosphorylation and calmodulin. C. J. Le Peuch, D. A. M. Le Peuch, S. Katz, J. G. Demaille, M. T. Hincke, R. Bredoux, J. Enouf, S. Levy-Toledano, J. Caen (U-249 de l'Institut National de la Santé et de la Recherche Médicale, Centre de Recherches de Biochimie Macromoléculaire du Centre National de la Recherche Scientifique, and Faculté de Médecine, 34033 Montpellier, U-150 de l'Institut National de la Santé et de la Recherche Médicale, 75010 Paris, France). *Biochim. Biophys. Acta 731*, 456 (1983).

Calcium-accumulating vesicles were isolated by differential centrifugation of sonicated platelets. Such vesicles exhibit a (Ca^{2+}+ + Mg^{2+})-ATPase activity of about 10 nmol (min · mg)$^{-1}$ and an ATP-dependent Ca^{2+} uptake of about 10 nmol (min · mg)$^{-1}$. When incubated in the presence of Mg[γ-^{32}P]ATP, the pump is phosphorylated and the acyl phosphate band is sensitive to hydroxylamine. The [^{32}P]phosphate-labeled Ca^{2+} pump exhibits a subunit molecular weight of 120 000 when analyzed by lithium dodecyl sulfate-polyacrylamide gel electrophoresis. Platelet calcium-accumulating vesicles contain a 23 kDa membrane protein that is phosphorylatable by the catalytic subunit of cAMP-dependent protein kinase but not by protein kinase C. This phosphate acceptor is not phosphorylated when the vesicles are incubated in the presence of either Ca^{2+} or Ca^{2+} plus calmodulin. The latter protein is bound to the vesicles and represents 0.5% of the proteins present in the membrane fraction. Binding of ^{125}I-labeled calmodulin to this membrane fraction was of high affinity (16 nM), and the use of an overlay technique revealed four major calmodulin-binding proteins in the platelet cytosol (M_r = 94 000, 87 000, 60 000 and 43 000). Some minor calmodulin-binding proteins were enriched in the membrane fractions (M_r = 69 000, 57 000, 39 000 and 37 000). When the vesicles are phosphorylated in the presence of MgATP and of the catalytic subunit of cAMP-dependent protein kinase, the rate of Ca^{2+} uptake is essentially unaltered, while the Ca^{2+} capacity is diminished as a consequence of a doubling in the rate of Ca^{2+} efflux. Therefore, the inhibitory effect of cAMP on platelet function cannot be explained in such simple terms as an increased rate of Ca^{2+} removal from the cytosol. Calmodulin, on the other hand, was observed to have no effect on the initial rate of calcium efflux when added either in the absence or in the presence of the catalytic subunit of the cyclic AMP-dependent protein kinase, nor did the addition of 0.5 μM calmodulin result in increased levels of vesicle phosphorylation.

G. Gárdos

Formation of aqueous pores in the human erythrocyte membrane after oxidative cross-linking of spectrin by diamide. B. Deuticke, B. Poser, P. Lütkemeier, C. W. M. Haest (Abteilung Physiologie, Medizinische Fakultät der Rheinisch-Westfälischen Technischen Hochschule, D-5100 Aachen, FRG). *Biochim. Biophys. Acta 731*, 196 (1983).

Oxidation of erythrocyte membrane SH-groups by diamide and tetrathionate induces cross-linking of spectrin. This cross-linking was now shown to go along with a concentration- and time-dependent enhancement of membrane permeability for hydrophilic nonelectrolytes and ions. The enhancement is specific for oxidative SH-group modifications, is reversible by reduction of the induced disulfides, can be suppressed by a very brief pre-treatment of the cells with low concentrations of N-ethylmaleimide and is strongly temperature-dependent. The pathway of the induced permeability discriminates nonelectrolytes on the basis of molecular size and exhibits a very low activation energy (E_a 3–8 kcal/mol). These findings are reconcilable with the formation of a somewhat inhomogeneous population of aqueous pores with radii probably $\leq$0.65 nm. Estimated pore numbers vary with the size of the probe molecule. Assuming a diffusion coefficient as in bulk water within the pore, at least 20 pores per cell have to be postulated; more realistic lower diffusion coefficients increase that number. Alterations of the lipid domain by changes of cholesterol contents and insertion of hexanol or nonionic detergents alter the number or size of the pores. Since aggregation of skeletal and intrinsic membrane proteins also occurs after the SH-oxidation, in parallel to the formation of membrane leaks, one may consider (a) defects in the disturbed bilayer interface, (b) a mismatch between lipid and intrinsic proteins or (c) channels inbetween aggregated intrinsic proteins as structures forming the pores induced by diamide treatment.

G. Gárdos

The stoichiometry of the Ca^{2+}-pumping ATPase of erythrocytes. A. Clark, E. Carafoli (Laboratory of Biochemistry, Swiss Federal Institute of Technology, Zürich, Switzerland). *Cell Calcium 4*, 83 (1983).

The stoichiometry of the erythrocyte Mg^{2+} dependent Ca^{2+}-stimulated ATPase has been determined in a reconstituted system. Purified Ca^{2+}-ATPase was incorporated into calcium impermeable liposomes and the ATP dependent calcium uptake was determined simultaneously with the hydrolysis of ATP. The results indicate that 1 gram atom of calcium is transported før each gram molecule of ATP hydrolysed, i.e., an ATP/Ca^{2+}-stoichiometry of 1.

G. Gárdos

Spontaneous inactivation of the Ca^{2+}-sensitive K^+ channels of human red cells at high intracellular Ca^{2+} activity. B. Vestergaard-Bogind (Zoophysiological Laboratory B, August Krogh Institute, DK-2100 Copenhagen Ø, Denmark). *Biochim. Biophys. Acta 730*, 285 (1983).

Ionophore A23187-mediated Ca^{2+}-induced oscillations in the conductance of the Ca^{2+}-sensitive K^+ channels of human red cells were monitored with ion specific electrodes. The membrane potential was continuously reflected in CCCP-mediated pH changes in the buffer-free medium, changes in extracellular K^+ activity were followed with a K^+-selective electrode, and changes in the intracellular concentration of ionized calcium were calculated on the basis of cellular ^{45}Ca content. An increased cellular ^{45}Ca content at the successive minima of the oscillations where the K^+ channels are closed indicates that the activation of the channels might be a (dCa^{2+}/dt)-sensitive process and that accommodation to enhanced levels of intracellular free calcium may occur. An incipient inactivation of the K^+ channels at intracellular ionized calcium levels of about 10 μM and a concurrent membrane potential of about -65 mV was observed. At a membrane potential of about -70 mV and an intracellular concentration of about $2 \cdot 10^{-4}$ M no inactivation of K^+ channels took place. Inactivation of the K^+ channels is suggested to be a compound function of the intracellular level of free calcium and the membrane potential. The observed sharp peak values in cellular ^{45}Ca content support the notion that a necessary component of the oscillatory systems is a Ca^{2+} pump operating with a significant delay in the activation/inactivation process in response to changes in cellular concentration of ionized calcium.

Ilma Szász

LADY TATA MEMORIAL TRUST INTERNATIONAL AWARDS FOR RESEARCH ON LEUKAEMIA AND ALLIED CONDITIONS

ACADEMIC YEAR 1984—1985

The Trustees of the Lady Tata Memorial Trust invite applications for Awards for personal support for research on leukaemia, in the Academic Year beginning 1st October, 1984. In view of the affinity between leukaemia and other forms of neoplastic disease, candidates with programmes of research on any aspect of malignant diseases which may throw light on problems of leukaemia will be eligible for consideration. The Trustees specially wish to encourage studies of the leukaemogenic viruses in animals, the epidemiology, pathogenesis, and immunology of leukaemia.

Awards are open to suitably qualified investigators of any nationality, working either in their own institutions or in other centres abroad.

Stipends will be adjusted according to the place and Institution at which the work will be done, with provision for annual increments and superannuation if required (Awards in recent years have usually been between 5,000 and 10,000).

Awards are tenable for 1 year in the first instance, renewable up to a maximum of 3 years.

Further particulars and forms of application may be obtained by writing to the Secretary for Europe to the Scientific Advisory Committee, Lady Tata Memorial Trust, Mrs. MIGLIERINA, Unité 108, Bâtiment INSERM, Hôpital Saint-Louis, 2, place du Dr. Fournier, 75475 PARIS CEDEX 10, France.

Application must be submitted before 1st March, 1984, and awards will be announced by the Trustees in June.

INSTRUCTIONS TO CONTRIBUTORS

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

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24
H-1113 Budapest, Hungary

Three copies of the manuscript should be submitted. They should be typed double-spaced on one side of a good quality paper with proper margins. The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which the work has been carried out; (4) name and full postal address of the author to whom communications regarding the manuscript should be directed; (5) a short title not to exceed 40 characters (including space) to be used as a running head. The second page should contain an **abstract** of 50—100 words, summarizing the reasons for the study, the methods used, the results, and the major conclusions. This page should also contain 4—8 **keywords** placed in alphabetical order. Original papers should not exceed 15 printed pages including tables, figures, and references. Case reports should not be more than four, technical notes and Letters to the Editor not more than two printed pages in length. In the manuscripts the approximate location of tables and figures should be indicated in the margin. The manuscript of original papers should be divided into summary, introduction, materials and methods, results, discussion, acknowledgements and references. Review articles should also be appropriately divided. SI units should be used in the manuscript, except that, for the time being, litre (l) may be used as a unit of volume.

References: Only papers closely related to the author's work should be referred to. In the text citations should include the name of the authors and/or the reference number in parentheses. A list of references, numbered according to the order of their occurrence in the text, should be compiled at the end of the paper. It should be typewritten, double-spaced, on a separate sheet.

References to periodicals should be typed double-spaced and mention: (1) name(s) and initials of all the authors; (2) title of the paper; (3) international abbreviation of the periodical; (4) volume; (5) first page number; (6) year of publication in parentheses. Thus: 4. Liebermann, J. E., Gordon-Smith, E. C.: Red cell pyrimidine 5'-nucleotidase and glutathione in myeloproliferative and lymphoproliferative disorders. *Brit. J. Haemat. 44*, 425 (1980).

References to books should include: (1) author(s)' name(s); (2) title; (3) editor(s); (4) publisher; (5) place and year of publication. Thus: 5. Guyton, A. C., Taylor, A. E., Granger, H. J.: *Circulatory Physiology. II. Dynamics and Control of Body Fluids.* Saunders, Philadelphia 1975. Or: 6. Rappaport, J. M.: Granulocyte transfusion. In: *Haematology of Infancy and Childhood.* D. G. Nathan, F. A. Oski (eds). Saunders, Philadelphia 1974, p. 875.

Illustrations must be cited and numbered in the order they appear in the text. All line drawings should be submitted as clear, glossy, black and white originals or good quality photographs. The author's name and the title of the paper together with the serial number of the figure should be written on the back of each print. Legends for illustrations should be typewritten, double-spaced, on a separate sheet, and included at the end of the manuscript. Tables, each bearing a title, should be self-explanatory and numbered consecutively.

Authors will receive one set of proofs which must be corrected and returned to the Editor-in-Chief *within three days of receipt*. Major alterations of the text cannot be accepted.

Authors are entitled to 50 reprints free of charge.

HAEMATOLOGIA
VOLUME 17 · NUMBER 2 19

Contents

Index: 26861

VOLUME 17 • NUMBER 3 • 1984

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

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.

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

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24.
H-1113 Budapest
Hungary

Technical Editor:
R. J. Valenti

Distributor for

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

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

For all other countries subscriptions should be sent to

VNU SCIENCE PRESS
P.O.B. 2073
3500 GB Utrecht, The Netherlands

Subscription price for volume 17

DM 279 incl. surface mail
DM 305 incl. air mail

VOLUME 17 · NUMBER 3

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

VNU SCIENCE PRESS, UTRECHT, THE NETHERLANDS

AKADÉMIAI KIADÓ, BUDAPEST

1984

Printed in Hungary
Akadémiai Kiadó és Nyomda, Budapest

Haematologia 17 (3), pp. 333–340 (1984)

Spleen Alterations in Hairy Cell Leukemia: A Scanning Electron Microscopic Study*

G. LAMBERTENGHI-DELILIERS, D. SOLIGO, E. COLAJORI, E. POLLI

Istituto di Clinica Medica I (Padiglione Granelli), Università di Milano, Via Francesco Sforza 35, 20122 Milan, Italy

(Received 12 April 1983; accepted 22 July 1983)

In Hairy Cell Leukemia (HCL) peripheral blood and bone marrow cells show under the scanning electron microscope (SEM) a characteristic surface with numerous ruffles and microvilli. The spleen of a patient affected by HCL was studied by SEM after fresh sectioning and routine preparation. Cells with the typical "hairy" surface were observed infiltrating the red pulp, altering the normal reticular meshwork and causing red blood cell distortion. In the sinuses, hairy cells adhered to the endothelial cells causing sinus dilatation and destruction. Aggregates of hairy cells delimiting pooled erythrocytes were also observed and may represent the "pseudosinuses" described in previous light and transmission electron microscopic studies.

These preliminary findings may explain the condition of hypersplenism which characterizes HCL. In addition, SEM is proposed as a rapid and simple method to identify HCL spleen involvement.

Keywords: hairy cell leukemia, scanning electron microscopy, spleen ultrastructure

Introduction

In Hairy Cell Leukemia (HCL) circulating and bone marrow cells show under the transmission electron microscope (TEM) a very irregular cell profile with characteristic cytoplasmic projections and a variety of intracytoplasmic organelles, such as mitochondria, small vesicles, Golgi bodies, ergastoplasmic reticulum strands and sometimes ribosome-lamellar complexes [1, 2, 3]. Under the scanning electron microscope (SEM), these cells are characterized by a typical surface with numerous large-based ruffles and well developed microvilli [4, 5, 6].

More recently, attention has been devoted to the ultrastructural changes in HCL involved organs and particularly the spleen. Different authors [7, 8, 9] have observed by TEM a conspicuous infiltration of the red pulp by hairy cells, emphasizing their capacity to adhere to endothelial cells and to cause sinus dilatation and destruction. This process is thought to lead to the formation of pseudosinuses and consequently to blood pooling [8, 9]. These ultrastructural data have therefore contributed to our understanding of the pathogenesis of hypersplenism, which characterizes HCL.

* This work was supported by the "Alberto e Antonietta Matarelli Foundation".

This paper presents a SEM study of the spleen from a patient affected by HCL in order to show how this technique can improve our knowledge of the pathological changes which occur in this organ. In addition, SEM is recommended as a rapid and simple method, not reported in the literature until now, for the diagnosis of spleen involvement in HCL.

Materials and Methods

A 50-year old male patient affected by HCL was admitted to the hospital to undergo splenectomy for worsening of his clinical condition and hematological parameters. The condition was diagnosed a few months before on the basis of clinical findings, morphological and cytochemical data obtained in bone marrow and peripheral blood samples [10].

Tissue from the spleen was obtained at the time of surgery and processed for routine histology. In addition, 1×1 mm slices were freshly cut with a new stainless surgical blade, mostly from the capsular surface of the organ, quickly rinsed in saline solution with added 1% sodium citrate and subsequently fixed in 1% glutaraldehyde in 0.05 M cacodylate buffer for 12–24 hours. After fixation specimens were rinsed in distilled water, dehydrated in graded alcohol, dried in a Balzers critical point drier, mounted on aluminium stubs, sputter-coated with gold and examined at 15 kV under a Philips SEM 505 scanning electron microscope.

Reference spleens from three subjects who underwent explorative laparotomy for surgical staging of lymphoma were also studied in the same way. Before splenectomy, the peripheral blood and bone marrow leukocytes of the patient were separated by Ficoll-Hypaque centrifugation, attached to glass coverslips pretreated with poly-L-lysine, fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and subsequently processed for SEM examination as described above.

Results

More than 80% of peripheral blood and bone marrow mononuclear cells of the subject under study were characterized under the SEM by the presence of a mixture of broad based surface ruffles and well developed microvilli (Fig. 1).

Light microscopic examination of HCL spleen specimens revealed a diffuse involvement of the red pulp of the organ, as classically described in HCL [7, 11, 12], while in reference spleens routine histology gave normal results. Cells with the same surface features as those described in peripheral blood and bone marrow were also observed in the spleen samples studied under the SEM; these cells generally adhered to one another and to the surrounding spleen structures with numerous cytoplasmic bridges and interdigitations (Fig. 2).

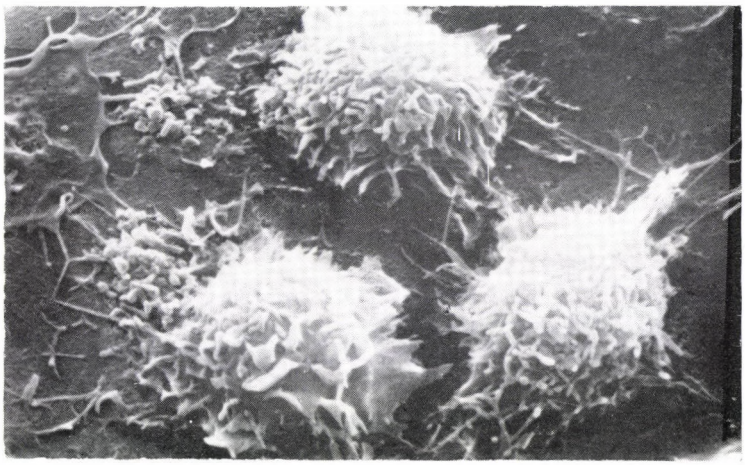


Fig. 1. HCL bone marrow. Cells show a typical surface with ruffles of large base and well developed microvilli. × 5700

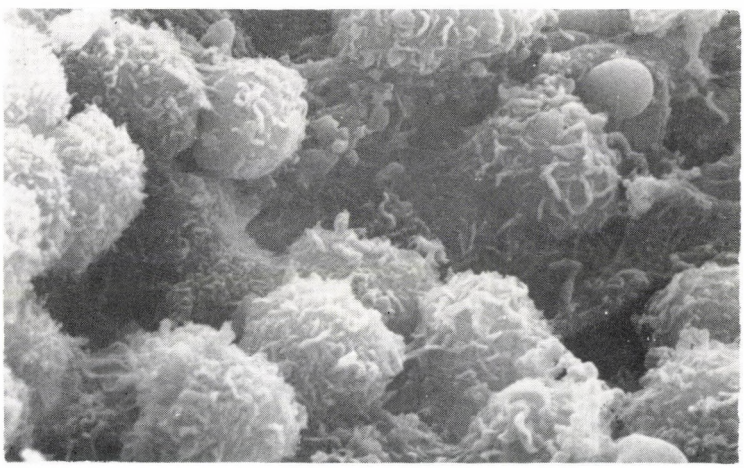


Fig. 2. HCL spleen (red pulp). Numerous hairy cells are seen adhering to one another and to the surrounding structures. × 4800

White pulp

The central arteries were observed throughout the cut section and appeared completely preserved with connective tissue, muscle cells and endothelial cells regularly arranged and never infiltrated. The periarterial lymphatic sheath was generally reduced in comparison to the control spleens and consisted of few round cells, classifiable on the basis of their surface appearance as lymphocytes, which surrounded the arterial adventitial surface (Fig. 3). Lymphatic follicles were rarely observed.

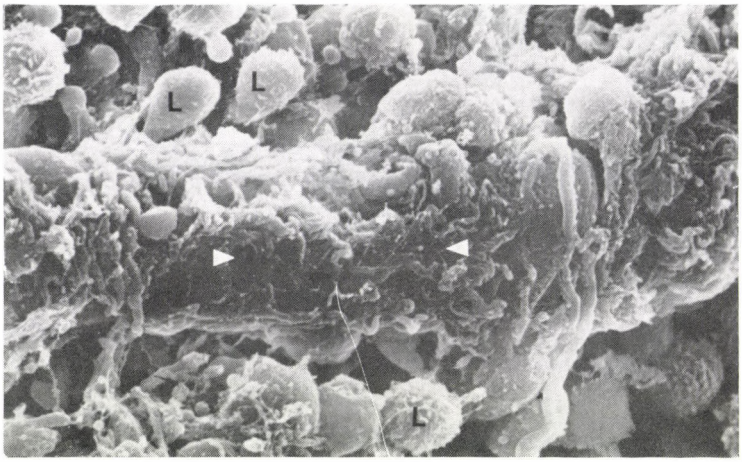


Fig. 3. HCL spleen (white pulp). The external surface of a central artery (arrows) is surrounded by few small round cells (L) with smooth surface (probably lymphocytes). × 2500

Red pulp

a) Cords

While in the control samples a regular cord pattern was seen (Fig. 4), in the HCL spleen (Fig. 5) cells with the typical ruffled surface were observed infiltrating the cord spaces, admixed to cells described in previous SEM studies as reticular cells [13]. The reticular cord pattern was always altered with fragmentation of the

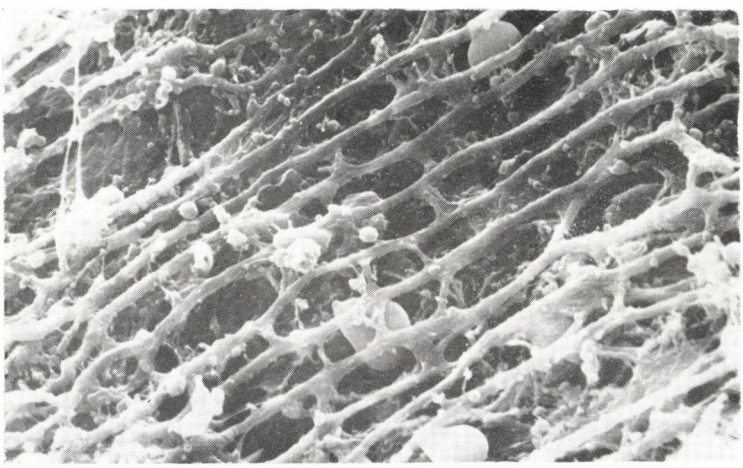


Fig. 4. Normal spleen. Red pulp showing a regular meshwork of reticular fibers. × 2600

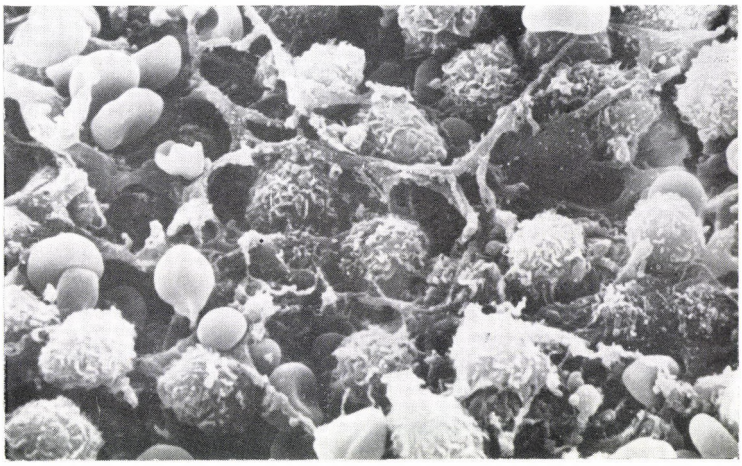


Fig. 5. HCL spleen (red pulp). The reticular cord space is distorted and fragmented by a hairy cell infiltrate. Numerous erythrocytes altered in shape. × 2700

fibers and shape distortion of the erythrocytes, which were seen in close contact with the hairy cell infiltrate. Sometimes hairy cells appeared clumped together in larger aggregates, which filled and substituted the cord spaces.

b) Sinuses

Normal sinuses with a regular alignment of intact endothelial cells were sometimes observed (Fig. 6). More frequently, however, hairy cells adhered to the endothelial cells (Fig. 7) and sometimes infiltrated the intercellular spaces. Endo-

Fig. 6. HCL spleen. The inner surface of a sinus shows regular alignment of intact endothelial cells. × 1600

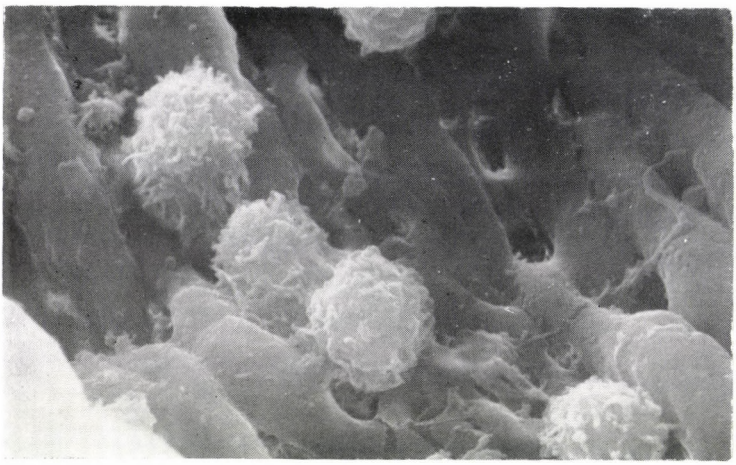


Fig. 7. HCL spleen. Hairy cells adhere to the sinus endothelial cells, which appear swollen, with smooth surfaces. Dilated intercellular spaces. × 4800

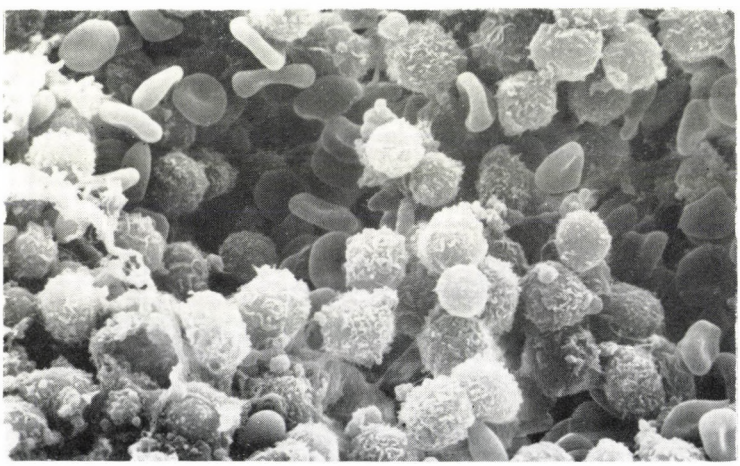


Fig. 8. HCL spleen (red pulp). Hairy cells circumferentially arranged seem to delimit spaces filled with erythrocytes. × 2200

thelial cells showing degenerative changes, such as swollen aspects with smooth surfaces and dilated intercellular spaces, were also frequent (Fig. 7).

In addition, hairy cells circumferentially arranged and mixed to fragmented ring fibers and endothelial cells were observed and seemed to delimit spaces filled with erythrocytes (Fig. 8).

Discussion

The peculiarity of hairy cell surface morphology under SEM, already reported by numerous authors in peripheral blood and bone marrow cells [4, 5, 6], was clearly evident also in spleen samples. In fact, from our study it appears that it is quite easy to recognize hairy cells in the spleen and to distinguish them from other structures. Therefore, HCL is one of the few examples in which SEM allows a three-dimensional study of the different structures and their relationship in a solid tissue, since we are able to identify pathological cells by their surface architecture.

Our SEM findings on HCL-involved spleen are substantially comparable with those obtained by TEM [7, 8, 9]. The capacity of hairy cells to adhere to the different spleen elements, i.e. endothelial cells, reticular elements, ring fibers and cord fibers was immediately and strikingly evident in our micrographs. In the red pulp HCL infiltrates were clearly revealed by SEM, both filling the cord spaces, deforming and destroying the stromal spleen structure. The regular cord meshwork seen in normal spleens is finally altered completely.

These findings could explain how the cord blood flow may be obstructed and slowed down in HCL. In addition, such alterations of the erythrocyte pathway may evidently lead to red cell membrane alterations and consequently enhance splenic sequestration and phagocytosis by macrophages. Although some authors described an increased number of cord macrophages in HCL spleens [8, 11], this was not the case in our samples, where a predominance of hairy cells was seen. It cannot, however, be ruled out that our case was in a later stage of the disease, where hairy cells could finally have overlapped the macrophage population. In addition, based on surface morphology alone, macrophages could be underestimated, when surrounded by many adhering cells. In an earlier phase there might be an increase of macrophage cells and may contribute to cell destruction.

The sinuses presented variable aspects since the sequence hairy cell adhesion to endothelial cells, infiltration of intercellular spaces, endothelial cell damage and death could be seen in the different stages. The process may eventually lead to complete destruction of the sinus, which is then replaced by pseudo-cavities surrounded by hairy cells mixed to sinusal residual structures and containing pooled erythrocytes. These structures may probably be considered the pseudosinuses observed by light microscopy [12] and TEM [9].

The morphological findings described in this preliminary report seem to confirm that, in the HCL spleen, architectural alterations exist which may explain the marked blood cell sequestration and pooling in the altered sinuses with exposure of blood cells, in particular erythrocytes, to the lienal phagocytes. Further SEM investigations of the HCL spleen are, however, needed to substantiate our interpretation.

Nevertheless, SEM is recommended as a simple and rapid technique to diagnose HCL not only in peripheral blood and bone marrow, but also in spleen specimens.

References

1. Katayama, I., Li, C. Y., Yam, L. T.: Ultrastructural cytochemical demonstration of tartrate-resistant acid phosphatase isoenzyme activity in "Hairy Cells" of Leukemic Reticuloendotheliosis. *Am. J. Pathol. 69*, 471 (1972).
2. Quan, S. G., Poolsawat, S. S., Golde, D. W.: Ultrastructure and tartrate-resistant acid phosphatase localization in a T-cell Hairy-Cell Leukemia cell line. *J. Histochem. Cytochem. 28*, 434 (1980).
3. Schnitzer, B., Kass, L.: Hairy-cell Leukemia. A clinicopathologic and ultrastructural study. *Am. J. Clin. Pathol. 61*, 176 (1974).
4. Catovsky, D., Frisch, B., Okos, A., Van Noorden, S.: Scanning electron microscopy and the nature of the hairy cell. *Lancet 1*, 462 (1975).
5. Golomb, H. M., Braylan, R., Polliack, A.: "Hairy" Cell Leukaemia (Leukaemic Reticuloendotheliosis): a scanning electron microscopic study of eight cases. *Br. J. Haemat. 29*, 455 (1975).
6. Polliack, A., Braylan, R., Golomb, H.: Scanning electron microscopy of hairy cells in Hairy-cell Leukemia. *Lancet 2*, 1013 (1974).
7. Bouroncle, B. A.: Leukemic Reticuloendotheliosis (Hairy Cell Leukemia). *Blood 53*, 412 (1979).
8. Burke, J. S., Mackay, B., Rappaport, H.: Hairy Cell Leukemia (Leukemic Reticuloendotheliosis). II. Ultrastructure of the spleen. *Cancer 37*, 2267 (1976).
9. Pilon, V. A., Davey, F. R., Gordon, G. B., Jones, D. B.: Splenic alterations in Hairy-Cell Leukemia: II. An electron microscopic study. *Cancer 49*, 1617 (1982).
10. Katayama, I., Finkel, H. E.: Leukemic Reticuloendotheliosis. A clinicopathologic study with review of the literature. *Am. J. Med. 57*, 115 (1974).
11. Namba, K., Jaffe, E. S., Soban, E. J., Braylan, R. C., Berard, C. W.: Hairy Cell Leukemia: enzyme histochemical characterization, with special references to splenic stromal changes. *Cancer 39*, 2323 (1977).
12. Namba, K., Soban, E. J., Bowling, M. C., Berard, C. W.: Splenic pseudosinuses and hepatic angiomatous lesions. *Am. J. Clin. Pathol. 67*, 415 (1977).
13. Weiss, L : A scanning electron microscopic study of the spleen. *Blood 43*, 665 (1974).

Haematologia 17 (3), pp. 341–351 (1984)

Plasmapheresis and Cytotoxic Drugs for Mixed Cryoglobulinemia

M. VALBONESI, F. MONTANI, L. MOSCONI, G. FLORIO, C. VECCHI

Immunohematology Service, Hospital of Saronno, 21047 Saronno, Italy

(Received 5 April 1983; accepted 20 May 1983)

Fifteen patients with symptomatic cryoglobulinaemia were subjected to apheretic treatment when acute renal insufficiency, glomerulonephritis, severe generalized vasculitis and polyneuropathy unresponsive to conventional therapy or complications due to steroids, such as vertebral collapse, peptic ulcer and steroid diabetes, had appeared. Treatment was performed by discontinuous flow centrifugation or cascade filtration: when discontinuous flow centrifugation was employed, a mixture of saline, gelatin and fresh frozen plasma was used for replacement. Cytotoxic drugs were administered to patients with lymphoma (4 patients) or chronic active hepatitis (5 patients) and also to patients suffering from essential mixed cryoglobulinaemia. Exchanges were organized into courses of 3 to 5 sessions over 5 to 10 days and employed as a supportive measure. No patient underwent long-term treatment. A complete resolution of kidney damage, skin involvement and neurologic signs was observed when treatment was started early in the course of the disease, whereas unequivocal but moderate improvement was obtained in the case of long-lasting symptoms such as polyneuropathy. Relapses were seen in most patients when cytotoxic drugs had been discontinued abruptly. In 8 patients the solubility of cryoglobulins was studied by a recently developed turbidimetric assay. Following treatment the solubility increased; when solubility decreased, 2 patients of this group had a relapse. On the basis of these preliminary observations it appears that the possibility of predicting relapsing disease or the need of continuing therapy can eventually be achieved.

Keywords: plasmapheresis, cryoglobulinaemia, cytotoxic drugs, plasma treatment.

Introduction

Growing evidence indicated that cryoglobulinaemia is a human model of immune complex diseases [1–8]. Different therapeutic approaches have been attempted in this condition, such as steroid and non-steroid drugs, cytotoxic drugs and eventually plasma exchange (PEX) by itself [6, 21] or combined with steroids and cytotoxic drugs [8]. Because of the relative rarity of the disease, none of these therapies has undergone controlled evaluation. In particular, the limited number of cases treated by PEX makes difficult any evaluation of the effect of this treatment in severely affected patients. Recently, evidence has been provided that circulating immune complexes (IC) other than cryoglobulins are present in the sera of patients with cryoglobulinaemia [6]: it has also been shown that cryoapheresis [6, 22] is

relatively inefficient [9] whereas PEX has been confirmed as the best technique for the removal of these offending agents. In this paper we report on our experience with 15 patients treated by combined apheretic and cytotoxic therapy. A method for the study of cryoglobulin solubility is also proposed.

Apheretic treatment

PEX was carried out by discontinuous flow centrifugation as described previously [10]. Replacement fluid was a mixture of saline and gelatin for the removal of 1 to 1.5 liters of plasma; after that only fresh frozen plasma was employed for preventing immunoglobulin and coagulation factor deprivation. In some patients cascade filtration (CF) was employed: EVA 5A hollow fibre filters were used, as described previously [11]. The treatments were organized into courses of 3 to 5 sessions over 5–10 days. The volume of plasma removed or treated at each session ranged from 3.4 to 5.2% of the patient's body weight.

All patients were on steroids when referred to us: steroids were maintained at the usual dosage if there were no complications due to their use, such as vertebral collapse, diabetes or peptic ulcer. Cyclophosphamide (CFX) or azathioprine (Aza) were given following the first apheretic session at a dosage of 2–2.5 mg/kg/day, according to our general protocol [10]. A single patient received Vincristine instead of CFX.

Laboratory evaluation

Cryoglobulins were isolated at 4 °C for 72 hours. The precipitate was washed 3 times at 0 °C and analysed for immunoglobulins and complement fractions. Protein quantification and percent cryocrit were obtained immediately prior to and following each apheretic session. The solubility of cryoglobulins a' 37 °C was studied employing a turbidimetric assay recently developed by us. Briefly, samples of sera collected prior to and following each session were kept at 4 °C for 48 hours. After centrifugation, the concentration of cryoproteins was brought into the same range (5% cryocrit) by removing cryodepleted serum from post-treatment samples. Cryoglobulins were then resuspended and kept at 0 °C. The study of cryoglobulin solubility was started employing an aggregometer operating at 37 °C, at a wavelength of 640 nm; 200 μl of each sample was analysed simultaneously. The sensitivity of the aggregometer was adjusted so that the transmission of samples kept at 0 °C was 0%. On warming a progressive decrease in cold induced turbidity was recorded as a curve of increase in light transmission whose slope depended on the physical characteristics of cryoglobulins. Two examples of these curves are given in Figure 1.

IC were measured according to Manca et al. [12] and expressed as μg of heat-aggregated human IgC equivalent per ml. The upper limit of normality was

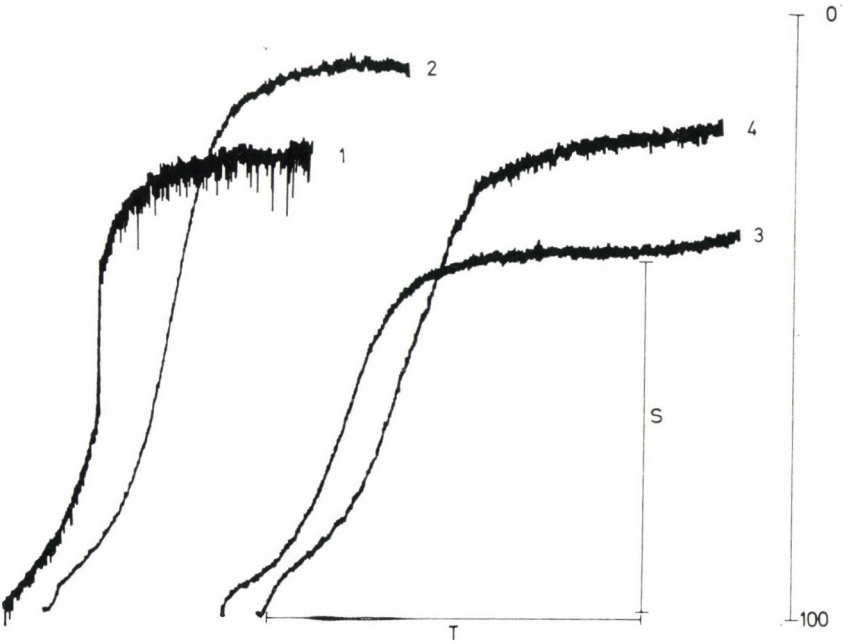


Fig. 1. Representative curves of cryoglobulin solubility prior to (curves 1 and 3) and following a single plasma exchange (curves 2 and 4) in 2 different patients. T: 2.5 minutes. S: solubility in percent of the 0–100 scale

considered to be 31 μg/ml (mean $\pm$ 2 SD) of the values in the control group. All tests needed for the confirmation of the clinical diagnosis were done by employing conventional methods.

Patients and effects of treatment

Fifteen patients whose diagnosis was clear and whose informed consent had been obtained, were subjected to PEX therapy combined with cytotoxic drugs. To be eligible for this treatment the patients had to have complications from prolonged use of steroids or to be unresponsive to high dosage steroids. A severe membranoproliferative glomerulonephritis or vasculitis or polyneuropathy were also regarded as indications of treatment. The clinical condition was graded from 0 (normal) to 4 (life threatening disease or loss of function). Eleven patients were treated by discontinuous flow centrifugation and 4 by cascade filtration: this was done for obtaining comparative results on the clinical efficacy of the technique which is still considered to be in the experimental stage. Table I and III describe patients admitted to the study, as grouped according to diagnosis; the significant clinical and laboratory data are given with some details of apheretic procedure.

Table I

Relevant clinical and laboratory data of patients with cryoglobulinaemia in the course of lymphomas

Pat.	Diagnosis	BW	Sessions	Volume liters	Cryocrit per cent pre/post	CIC pre/post	Clinical score pre/post	Follow-up
1	Lymphoma: ARI, skin ulcers	69	3 DFC	10.8	27/12	76/31	4/1	32 months: 3 relapses
2	Lymphoma: hyperviscosity syndrome	41	2 DFC	6.9	32/19	91/45	4/1	patient died 4 months later
3	Hodgkin lymphoma: ARI	79	1 DFC	3.1	8/1	58/21	4/0	37 months
4	Lymphoma: polyneuropathy, vertebral collapse	71	4 DFC	13.6	14/3	47/18	4/2	19 months

Table II

Relevant clinical and laboratory data of patients with cryoglobulinaemia complicating the course of chronic active hepatitis. All patients but No. 9 were HBsAg positive

Pat.	Diagnosis	BW	Sessions	Volume, liters	Cryocrit per cent pre/post	CIC pre/post	Clinical score pre/post	Follow-up
5	Nephritis, severe generalized vasculitis	58	3 DFC	9.1	21/7	67/32	4/1 (2)	11 months
6	Nephrotic syndrome	63	5 DFC	13.8	14/4	59/23	3/0	26 months
7	Vasculitis, steroid diabetes	72	3 CF	7.7	14/2	61/17	3/0	24 months, relapsing disease; 3 PE needed
8	Vasculitis, peptic ulcer	70	3 CF	7.9	7/1	42/11		11 months, relapsing disease; 2 PR needed
9	Polyneuropathy	68	3 DFC	10.1	11/3	51/32	4/2	13 months, relapsing disease when azathioprine was discontinued

Table III

Clinical and laboratory data of patients with essential mixed cryoglobulinaemia and cryoglobulinaemia in the course of mixed connective tissue disease (patient 15)

Pat.	Diagnosis	BW	Sessions	Volume, liters	Cryocrit per cent pre/post	CIC pre/post	Clinical score pre/post	Follow-up
10	Nephrotic syndrome	51	4 DFC	9.7	11/3	53/26	2/0	13 months
11	Polyneuropathy	67	3 DFC	9.3	14/5	48/23	4 (3)/2	7 months
12	Polyneuropathy	71	3 CF	8.8	17/6	51/16	4 (3)/2	11 months
13	Cerebral vasculitis	41	4 DFC	9.7	21/9	49/23	4/0 (1)	5 months
14	Polyneuropathy, vertebral collapse	63	3 CF	9.1	11/4	39/13	4 (3)/2	15 months. Exchanges on as needed basis.
15	Cerebral vasculitis, skin and kidney involvement	57	3 DFC	6.1	7/2	63/41	4/ (3)	This patient died 7 days after last PE

Rheumatoid activity was found in all patients; this activity was monoclonal in the 3 patients with non-Hodgkin's lymphoma (IgMk). Some improvement was achieved in all patients and was striking in some cases.

Patient 1, with non-Hodgkin's lymphoma, presented with relapsing acute renal insufficiency due to inadequate management with cytotoxic drugs: 3 PEX sessions were able to halt and to reverse the renal damage almost completely.

Patient 2 presented with hyperviscosity syndrome in the course of lymphoma; his cryocrit prior to PEX treatment was 32%. Following the first apheretic session an improvement of speech and movements was apparent and after the second session the hyperviscosity symptoms disappeared: at this time the patient's cryocrit was 19%.

Patient 3 had an acute renal insufficiency complicating the course of Hodgkin's lymphoma. Surprisingly, a single PEX session combined with Vincristine (1 mg) was able to reverse completely the renal failure. Within 11 days, blood urea nitrogen decreased from 250 to 31 mg/dl and proteinuria from 23 g/day to trace amounts. Diuresis prior to PEX was 200–650 ml/day; 3 days later it reached 2 870 ml and the patient lost 9 kg in a week. No relapse occurred in 37 months: during this period cryoglobulins were always detectable but in trace amounts.

Patient 4 presented with subacute polyneuropathy, poorly managed by steroids which determined vertabral collapse (D9) at the mainteinance dosage of 25 mg prednisone/day. A grade 2 improvement was obtained following 4 PEX sessions combined with cyclophosphamide, azathioprine and prednisone (5 mg/day) and no relapse occurred in the following 19 months.

Five patients with chronic active hepatitis underwent therapy when cryoglobulinaemia became symptomatic. Four patients were HBsAg positive and the last one was negative.

Patient 5 was treated for glomerulonephritis complicating a clinical picture very close to polyarteritis nodosa, even though the vascular involvement was prominent at the venous level. Both vascular and renal involvement benefited from treatment and this patient has been relatively well for 11 months.

Patients 6, 7 and 8 presented with nephrotic syndrome, steroidal diabetes and peptic ulcer, respectively, patient 9 suffered from multiple mononeuritis. Patient 6 recovered completely after therapy and did not relapse in the subsequent 26 months, whereas patients 7 and 8 who had optimal response to therapy with complete regression of their skin lesions, relapsed to pre-exchange condition due to poor control of their disease by low dose steroids (5 mg/day) and cytotoxic drugs. Relapses were treated by PEX therapy which once more produced good but transient benefits. Patient 9 had grade 2 regression of his paralytic symptoms lasting 8 months; when his home physician tried to suspend azathioprine, the patient had relapsing polyneuropathy which required a second course of exchanges producing grade 2 improvement.

Patients 10 to 14 underwent therapy because of essential mixed cryoglobulinaemia: 2 patients with glomerulonephritis (case 10) and renal and cerebral vasculitis (case 13) had complete remission of their symptoms. Patients 11 and 12, with

long-lasting polyneuropathy, had only grade 1 or 2 improvement following therapy. Patient 14, with polyneuropathy had been treated by CF 11 months before, with success. In this case steroids were suspended when vertebral collapse became symptomatic. Polyneuropathy relapsed slowly, requiring a second course of exchanges which produced grade 2 improvement. Now exchanges are performed on an "as needed" basis to maintain clinical benefits.

Kinetics of cryoglobulin solubilization following plasma exchange

Cryoglobulin solubility was studied in 8 patients employing the turbidimetric assay. In Table IV the changes in cryoglobulin solubility following plasma exchange therapy are reported. From these data it is apparent that with treatment cryoglobulin solubility increased significantly; $p < 0.01$. This could not be the consequence of drug induced phenomena since it is found following the first exchange. It appears, instead, that the removal of cryoglobulins (and antigens), giving back normal proteins as replacement, can alter the composition of circulating IC, eventually

Table IV

Solubility of cryoglobulins studied by turbidimetric assay in 8 patients. The extent of solubility prior to PEX treatment is compared with the values obtained immediately following PEX and in the follow-up period

Pat.	Cryoglobulin solubility, per cent			
	Prior to PEX	following PEX	1 month later	4 months later
1	61	87	75	81
5	63	77	74	72
10	64	89	81	75
11	65	88	73	73
12	57	72	75	77
13	63	81	76	74
14	59	78	73	65/77 (after PEX)
15	57	70	NT	NT

increasing their solubility, probably with the help of fresh complement. Several studies of these patients have been performed in the follow-up period; 2 patients of the group relapsed, and the relapse was heralded by a distinct decrease in cryoglobulin solubility. These are obviously very preliminary data which have to be confirmed by sequential studies in a larger series of patients, but they suggest the possibility of predicting relapse or the need of further treatment.

Discussion

The results give further support to the effectiveness of PEX therapy for the reduction of clinical severity in patients with symptomatic cryoglobulinaemia. Combined therapies and exchange modalities certainly play a role in determining the outcome of treatments: in our experience, which confirmed the efficacy of DFC for removing cryoglobulins and IC, it was also possible to show the effectiveness of CF for the same purpose. CF has several advantages over DFC such as a considerable saving in fluid replacement costs, a reduction in risks of transmitting viral hepatitis compared with fresh frozen plasma replacement and reduced risks of coagulation abnormalities compared with albumin replacement. Undoubtedly, new variations of filters for CF will be forthcoming and the costs will be reduced when it will be possible to reuse filters. Technical problems, however, are not the central point. The focus, instead, is the place occupied by plasma exchange in the management of patients with symptomatic cryoglobulinaemia. Two main strategies have been proposed: PEX was employed as a primary therapeutic measure aimed at reducing the use of steroids and cytotoxic drugs and, on the other hand, PEX was employed as a supportive measure and considered an additional treatment of the underlying disease. According to the first strategy, prolonged treatment is given and patients may reach even 70 runs in 12 months [6]. Even if we believe that PEX is a benign procedure, we have some concern about the risks connected with prolonged treatments: infectious complications [8], cardiovascular complications with fatal cardiac arrest [13] and fatal embolization [14] as well as augmented risks of atherosclerosis [15] have been observed. Several cases of fatal pulmonary oedema have occurred in France [16] when fresh frozen plasma was used as replacement fluid. On the other hand, when fresh frozen plasma is employed, viral hepatitis and allergic complications are to be considered inherent risks of each procedure. For these reasons we favour short-term treatments of 3 to 5 runs over 5 to 10 days, combined with low-dosage immunosuppressants for preventing rebound effects which can easily be recorded following the removal of cryoglobulins [6].

Cytotoxic drugs are an important part of the management of most patients with lymphoma and chronic active hepatitis, so that these patients have no further risk. For patients with essential mixed cryoglobulinaemia unresponsive to steroids or with complications due to their prolonged use, and only when severe and life-threatening complications occur, the additional risk of immunosuppressive treatment can be accepted only after possible benefits and risks have been weighed. From a clinical point of view it appears that the strategy we applied produced satisfactory results in most patients. Complete recovery was observed when treatment had started early and with adequate volume exchanges: even when patients were treated late when some lesions have become anatomical, even the signs and symptoms which developed the latest showed some improvement as we observed in patients with vascular and neurologic involvement. This means that PEX should not be considered only when each conventional therapy failed to produce benefits; but that it should be applied as soon as it becomes apparent that conventional

therapies are unable to cause improvement before the lesions become irreversible.

How PEX produces its effects is not completely clear; furthermore therapeutic benefit is unpredictable before trial and patients can show striking improvement after a single session (patient 3) whereas in other patients some symptoms improve and others do not (patient 15). Nonetheless, even if many questions are unsolved, we feel that patients poorly controlled by steroids or with serious complications due to their disease or therapy, should be treated by PEX or CF. At least on the basis of uncontrolled studies these therapies appear to be of benefit to most patients [5–8, 17–23].

References

1. Meltzer, M., Franklin, E. C., Elias, K., McKlusky, M. C., Cooper, N.: Cryoglobulinemia. A study of 29 patients. *Amer. J. Med. 40*, 828 (1966).
2. Lo Spalluto, J., Dorward, B., Miller, W., Ziff, M.: Cryoglobulinemia based on interaction between a gamma macroglobulin and 7S gamma globulin. *Amer. J. Med. 32*, 142 (1962).
3. Riethmuller, C., Meltzer, M., Franklin, E., Miescher, P. A.: Serum complement levels in patients with mixed (IgM-IgC) cryoglobulinemia. *Clin. Exp. Immunol. 1*, 337 (1966).
4. Cream, J. J.: Immunofluorescent studies of the skin in cryoglobulinemic vasculitis. *Brit. J. Derm. 841*, 48 (1971).
5. Kater, L., Schuurman, H. J.: Immunobiology and clinical aspects of cryoglobulinemia. *Plasma Ther. 2*, 83 (1981).
6. Bombardieri, S., Maggiore, Q., L'Abbate, A., Bartolomeo, F., Ferri, C.: Plasma exchange in essential mixed cryoglobulinemia. *Plasma Ther. 2*, 101 (1981).
7. Lockwood, C. M.: Lymphoma, cryoglobulinemia and renal disease. *Kidney Int. 16*, 522 (1979).
8. Geltner, D., Kohn, R. W., Gorevic, P., Franklin, E. C.: The effect of combination therapy (steroids, immunosuppressives, and plasmapheresis) on 5 mixed cryoglobulinemia patients with renal, neurologic, and vascular involvement. *Arthr. and Rheum. 24*, 1121 (1981).
9. Rock, G. A., Blanchette, V. S., McKendry, Kandrish, R.: *Plasmapheresis and cryoglobulinemia: an evaluation of cold precipitation as a method of removing abnormal protein*. Proc. Int. Symp. Plasmapheresis: Therapeutic Application and New Techniques. Cleveland, Ohio April 3–4, 1982.
10. Valbonesi, M., Garelli, S., Zerbi, D., Forlani, G., Cornelio, F., Pelucchetti, D.: Plasma exchange combined with cytotoxic drugs and lymphocytopheresis for myasthenia gravis. *Vox Sang., Basel 43*, 142 (1982).
11. Valbonesi, M., Garelli, S., Montani, F., Cefis, M., Rossi, U.: Management of immune-mediated and paraproteinemic diseases by membrane plasma separation and cascade filtration. *Vox Sang., Basel 43*, 91 (1982).
12. Manca, F., Migliorini, P., Bombardieri, S., Celada, F.: An enzymatically active antigen-antibody probe to measure circulating immune complexes by competition. Use of the E. coli beta-galactosidase in the probe and of bovine congluitin as the complement-binding reagent. *Clin. Immunol. Immunopath. 16*, 131 (1980).
13. Sutton, D. M. C., Cardella, C. J., Uldall, P. R.: *Some observations on the complications of intensive plasma exchange*. Proceedings of the Haemonetics Research Institute Advanced Component Seminar, Boston, MA 1979.
14. Brewer, E. J., Nickeson, R. W. Jr., Rossen, R. D.: Plasma exchange in selected patients with juvenile rheumatoid arthritis. *J. Pediat. 98*, 194 (1981).

15. Lundsgaard-Hansen, P.: Intensive plasmapheresis as a risk factor for atherosclerotic cardiovascular disease? *Vox Sang., Basel 33*, 1 (1977).
16. Aufevre, J. P., Morin-Hertel, F., Cohen. Solal, M., Lefloch, A., Baudelot, J.: *Hazards of plasma exchange: a nationwide study of 3431 exchanges in 592 patiens.* In: *Plasma exchange*. Ed. H. G. Sieberth. Schattauer Verlag, Stuttgart 1980, p. 149.
17. Viguier, E., Quaranta, J. F., Ortonne, J. P., Cassuto, J. P., Duplay, H., Dujardin, P.: *Effect of plasma exchange in the course of cryoglobulinaemias: study of 6 cases.* Proc. Int. Symp. on Plasmapheresis: Therapeutic Applications and New Techniques. Cleveland, Ohio, April 3–4, 1982.
18. Berkman, E. M., Orlin, J. B.: Use of plasmapheresis and partial plasma exchange in the management of patients with cryoglobulinemia. *Transfusion 20*, 172 (1980).
19. Betourne, C. L., Buge, A., Dechy, H., Doria, M., Dournon, E., Rancurel, G.: The treatment of peripheral neuropathies in case of IgA myeloma and one of mixed cryoglobulinemia. *Nouv. Presse méd. 9*, 1369 (1980).
20. Cordonnier, D., Vialtel, P., Chenais, F., Jeannoel, P., Arvieux, J., Couderc, P., Renversez, J. Ch., Alibeu, C.: *Plasma exchange in 3 cases of type II mixed IgM-IgG cryoglobulinemia with severe membrano-proliferative glomerulonephritis.* In: *Plasma exchange*. Ed. H. G. Sieberth, Schattauer Verlag, Stuttgart 1980, p. 213.
21. Vandelli, L., Gaiani, G., Furci, L., Baldini, E., Lusvarghi, E.: *Control of cinical symptoms in mixed essential cryoglobulinemia with plasma exchange alone.* In: *Plasma exchange*. Ed. H. G. Sieberth, Schattauer Verlag, Stuttgart 1980, p. 221.
22. McLeod, B. C., Sassetti, R. J.: Plasmapheresis with return of cryoglobulin depleted autologous plasma (cryoglobulinpheresis) in cryoglobulinemia. *Blood 55*, 866 (1980).
23. Perricone, R., Di Marco, P., Cajozzo, C., Prezioso, A., Caracciolo, C.: *Plasmapheresis in the pathology associated with immune complex disoders. Abstr. Int. Congress ISH-ISBT*, Budapest, August 1–7 (1982) p. 304.

Haematologia 17 (3), pp. 353–357 (1984)

Pure Red Blood Cell Aplasia Associated with Paraproteinemia: in vitro Studies of Erythropoiesis[1]

L. BALDUCCI, CHERYL HARDY, B. DREILING, MEHDI TAVASSOLI, M. H. STEINBERG

Hematology-Oncology Section, VA Medical Center and Department of Medicine, University of Mississipi School of Medicine, Jackson, MS 39216, USA

(Received 13 January 1983; accepted 5 July 1983)

Pure red cell aplasia (PRCA) and monoclonal gammopathy were detected simultaneously in a 57 year old man with severe anemia. While erythroid burst forming units (BFU-E) were absent from blood, his bone marrow contained a high normal number of BFU-E in the absence of morphologically recognizable erythroid precursors. Serum from the patient did not inhibit the growth of BFU-E from normal blood suggesting that his PRCA was not antibody mediated. These studies suggest that in the patient the inability to produce erythrocytes was due to a block in the maturation of BFU-E; however, they do not indicate an etiology for this block. The absence of blood BFU-E and their abundance in the marrow may result from selective trapping of these cells by the marrow-blood barrier.

Keywords: erythropoiesis, monoclonal gammopathy, paraproteinemia, pure red cell aplasia.

*

Pure red cell aplasia (PRCA) has been associated with antibodies which inhibit erythropoiesis [1, 2, 4, 7, 8]. The association of PRCA with monoclonal gammopathy has already been described, but studies of erythropoiesis in vitro have not been done [10, 12]. We have studied a patient with this association and examined the possibility that the paraprotein might have played some role in the inhibition of erythropoiesis.

Case Report

A 57 year old white man was admitted for anemia. Physical examination showed pallor. Hemoglobin concentration and packed cell volume (PCV) were 6.2 g/dl and 17 per cent respectively and the white cell and platelet counts were normal. Evaluation of the gastrointestinal tract by radiologic and proctoscopic examinations was normal. He was transfused with six units of packed red cells and discharged with a PCV of 29 per cent.

Four weeks following discharge he presented again with a PCV of 18 per cent without evidence of blood loss. The red cell indices were normal and the blood film showed normal erythrocyte morphology. The white cell count was

[1] Supported by Research Funds of the Veterans Administration.

5900/μl with 60 per cent neutrophils and the platelet count was 378 000/μl. The reticulocyte count was 0.1 per cent. Serum albumin was 4.1 g/dl and globulin 3.6 g/dl. Serum protein electrophoresis demonstrated a monoclonal peak in the slow gamma range. This was an IgG with kappa light chains. Quantitative immunoglobulins showed IgG of 2640 mg/dl, IgA 125 mg/dl and IgM 50 mg/dl. No lytic lesions or osteoporosis were noted by skeletal X-rays. Concentrated urine was free of light chains and renal function was normal.

Bone marrow aspirate showed normal cellularity. Maturation of myeloid and megakaryocytic cells was normal. There was a total absence of erythroid precursors. Plasma cells constituted 10 per cent of all cells with some atypical forms being present. Computerized tomography of the chest failed to demonstrate thymic enlargement. The spleen was of normal size by scan. Additional studies showed normal serum ferritin, iron, iron binding capacity, B_{12} and folate levels.

A diagnosis of pure red cell aplasia and monoclonal gammopathy, possibly an indolent myeloma was made. Treatment began with prednisone 30 mg daily and cyclophosphamide 100 mg which was later increased to 200 mg. Over the following five months the patient continued to require transfusion of four to six units of packed red cells at approximately four week intervals.

After transfusion of a total of 38 units of packed red cells, splenectomy was done. The mildly enlarged spleen (259 g) showed only congestive changes with an increase in hemosiderin deposits. After surgery, the requirements of transfusion continued, although now at six week intervals. Reticulocyte counts varied between 0 and 0.2 per cent. The IgG level has decreased to 1540 mg/dl.

Methods

The technique for assay of erythroid burst forming cells (BFU-E) in blood and bone marrow has been described [11]. Briefly, venous blood and bone marrow aspirate were obtained in preservative-free heparin dissolved in NCTC 109 (Microbiological Associates, Walkersville, Maryland). Mononuclear cells were separated by density gradient centrifugation on Ficoll–Hypaque [5]. They were then washed and resuspended in Hanks' minimal essential medium, enriched with 2 per cent fetal calf serum, and adjusted to a final concentration of 1.5×10^6/ml of which 0.1 ml (1.5×10^5 cells) was cultured in microtiter wells (Linbro Chemical Co., New Haven, CT).

The culture medium consisted of NCTC 109, 20 per cent heat inactivated fetal calf serum (Sterile Systems, Inc., Logan, Utah), 10 per cent bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri), 10 per cent L-asparagine, 10 per cent citrated bovine plasma (Gibco, Grand Island, New York), 5 per cent thrombin (grade 1, Sigma Chemical Co., St. Louis, Missouri), and 4 units/ml human urinary erythropoietin. The cultures were incubated at 37 °C in 5 per cent CO_2 for 13 days, harvested and stained with benzidine [9]. BFU-E were counted

according to the criteria of Clarke and Housman [3]. The results reported here are the means of at least 4 cultures.

To evaluate the effects of patient serum or immunoglobulins on the development of BFU-E, fetal calf serum was replaced by either patient serum or normal donor serum and heat inactivated at 56 °C. Immunoglobulins were isolated by salt fractionation of the serum, as described by Heide and Schwick [6]: 4 ml of serum was mixed with 2 ml saturated ammonium sulfate, centrifuged and the pellet dissolved in water. This precipitation was repeated twice, and the globulins were then dialysed overnight in the cold against phosphate buffer. The immunoglobulins were sterilized by filtration through a 0.2 μm acrodisc (Gelman, Ann Arbor, Michigan) pretreated with 0.1 per cent bovine serum albumin, and stored at −20 °C until used.

Results

The number of BFU-E in the patient's bone marrow and blood are shown in Fig. 1. No BFU-E were found in the blood while the marrow contained high normal numbers of BFU-E.

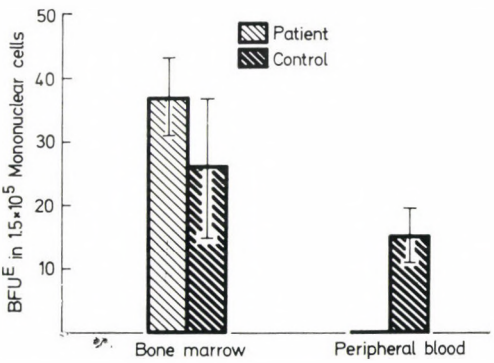


Fig. 1. BFU-E concentrations in patient's bone marrow and peripheral blood (1.5×10^6 mononuclear cells per ml culture) compared with those of 12 normal controls

When the patient's serum or its immunoglobulin (Ig) fraction and normal serum or its immunoglobulin fraction were compared with fetal calf serum for the ability to support the growth of BFU-E from the normal blood, there was no evidence that serum from our patient suppressed BFU-E development. Ig from the normal control, as well as from the patient appeared inhibitory in the cultures while whole serum from the patient showed little inhibition of BFU-E growth (Table 1).

Table 1

Effects of different sera on BFU-E formation from blood of normal donors

	Serum type				
	FCS	MS	AS	MIg	AIg
BFU-E (per 1.5×10^5 mononuclear blood cells)	11.7 ± 5.5	9.5 ± 6.3	2.7 ± 2.1	2.5 ± 1.3	0

FCS: Fetal calf serum
MS: Myeloma serum
AS: Autologous normal serum
MIg: Myeloma immunoglobulin
AIg: Autologous immunoglobulin

Discussion

Immunoglobulins from some patients with PRCA have been reported to interfere with Fe incorporation by erythroid cells in short term suspension cultures, to cause erythroblast cytolysis in the presence of complement, to inhibit the growth of erythroid precursors in semi-solid media or to block the growth stimulating activity of erythropoietin [1]. The role of autoantibodies in the pathogenesis of PRCA is further supported by reversal of the erythroid aplasia by immunosuppressive drugs [1, 7, 8], exchange transfusion and plasmapheresis [10].

Therefore, the coexistence of monoclonal gammopathy and PRCA in the patient raised the possibility that the paraprotein might have a specificity against some components of erythroid tissue. This did not prove to be the case, however, as the serum or its gamma globulin fraction did not suppress the development of BFU-E from normal blood any more than did normal serum or its Ig.

It is of interest that the antibody-mediated PRCA can in some cases be demonstrated only on autologous target cells [4]. In the present case we were unable to study the effects of patient serum or Ig on autologous BFU-E.

Of further interest is that in this patient, the concentration of BFU-E in bone marrow was normal or even increased. A similar finding in PRCA has been reported by others [4]. The presence in the bone marrow of erythroid progenitor cells that can develop into normal bursts in vitro, but not in vivo, indicate the presence of an inhibitory factor in the bone marrow. Such a putative factor could be humoral or cellular. At any rate, it does not appear to abolish the erythroid progenitor cells but prevents their development and maturation. In this respect PRCA appears to be different from aplastic anemia which, in most cases, is essentially a deficiency of the hemopoietic stem cell and therefore amenable to 'replacement therapy' by bone marrow transplantation. No such deficiency of progenitor cells existed in our patient and in the previously reported cases of PRCA.

The absence of BFU-E in the blood of the patient should also be commented upon. BFU-E are normally compartmentalized [13, 14]. A high concentration exists in the bone marrow with a lower concentration in the circulation. The maintenance of this 'gradient' is a function of the bone marrow-blood barrier which may selectively trap the hemopoietic precursor cells [13, 14]. In this patient the absence of BFU-E in the blood and their abundance in the marrow indicated a widened gradient. It is possible that the maintenance of the gradient is regulated by a feedback loop through some by-product of erythropoietic maturation. Such by-products may suppress the selective trapping of BFU-E by the marrow. In the absence of erythropoietic maturation, this postulated by-product would not be produced and the action of the marrow-blood barrier in selective trapping of BFU-E would be heightened resulting in an increased concentration of BFU-E in the marrow and their absence in the blood.

References

1. Abdou, N. I., Verdirame, J. D., Amare, M., Abdou, N. L.: Heterogeneity of pathogenetic mechanisms in aplastic anemia. *Ann. Int. Med. 95*, 43 (1981).
2. Alter, B. P., Potter, N. U., Li, F. P.: Classification and aetiology of the aplastic anaemias. *Clin. Haemat. 7*, 431 (1978).
3. Clarke, B. J., Housman, D.: Characterization of an erythroid percursor cell of high proliferative capacity in normal human peripheral blood. *Proc. Natl. Acad. Sci. USA 74*, 1105 (1977).
4. Cline, M. J., Golde, D. W.: Immunosuppression of hematopoiesis. *Amer. J. Med. 64*, 301 (1978).
5. Heath, D. S., Axelrad, A. A., McLeod, D. L., Shreeve, M. M.: Separation of erythropoietin responsive progenitors, BFU-E and CFU-E in mouse bone marrow by gravity sedimentation. *Blood 47*, 777 (1976).
6. Heide, K., Schwick, H. G.: Salt fractionation of immunoglobulins In: Weiz, D. M.: *Handbook of Experimental Immunology* 6.1–6.11 Blackwell Scientific Publications, Oxford 1973.
7. Krantz, S. B., Zaentz, S. D.: Pure red cell aplasia. In: *The Year in Hematology* 153–190 (1977).
8. Krantz, S. B., Kao, V.: Studies on red cell aplasia. II. Report of a second patient with an antibody to erythroblast nuclei and a remission after immunosuppressive therapy. *Blood 34*, 1 (1969).
9. McLeod, D. L., Shreeve, M. M., Axelrad A. A.: Improved plasma culture system for production of erythrocyte colonies in vitro. Quantitative assay for CFU-E. *Blood 44*, 517 (1974).
10. Messner, H. A., Fauser, A. A., Curtis, J. E., Dotten, D.: Control of antibody-mediated pure red cell aplasia by plasmapheresis. *New Engl. J. Med. 304*, 1334 (1981).
11. Newcomb, M. M., Balducci, L., Coleman, M. B., Steinberg, M. H.: Erythroleukemia: In vitro studies of erythropoiesis. *Amer. J. Hemat. 5*, 291 (1978).
12. Resegotti, L., Dolce, C., Palestro, G., Peschle, C.: Paraproteinemic variety of pure red cell aplasia. *Acta Haemat. (Basel) 60*, 227 (1978).
13. Tavassoli, M.: The marrow-blood barrier. *Brit. J. Haemat. 91*, 297 (1979).
14. Tavassoli, M.: Red cell delivery and the function of marrow-blood barrier. *Exp. Hemat. 6*, 257 (1978).

Haematologia 17 (3), pp. 359—373 (1984)

Release of Microvesicles from Erythrocytes during Storage in Saline-Adenine-Glucose Media*

CH. TRAUTSCH, B. REIMAN, G. GERBER, D. KLATT, A. LADHOFF, D. MARETZKI[1]

Institute of Physiological and Biological Chemistry, Division of Electron Microscopy,
Institute of Pathology, Humboldt University,
Berlin, Hessische Str. 3–4, 1040 Berlin, GDR

(Received 11 March 1983; accepted 30 July 1983)

Storage of human erythrocytes in SAG media results in the release of membrane microvesicles with a diameter of about 190 nm. They can be separated from intact erythrocytes by centrifugation on a dextran barrier solution (density 1.078 g/l). Vesicles prepared from cold stored erythrocyte concentrates are like those released upon ATP depletion by erythrocytes incubated without glucose at 37 °C [15].

The course of vesiculation was followed by measuring acetylcholinesterase during storage of the erythrocyte concentrate for 35 days. Its activity remained constant within the storage units during the preservation period. This enzyme and phospholipids were released continuously in a proportional manner. The release of sialic acid amounted to about half of that of phospholipids.

Owing to depletion of 2,3-bisphosphoglycerate the binding of ATP to haemoglobin increased and the concentration of free ATP declined. Addition of an ion-exchange resin to stored erythrocytes kept the pH constant, retarded the breakdown of 2,3-P_2G and stabilized the concentration of free ATP. That inhibited the rate of irreversible vesiculation. Therefore, maintenance of 2,3-bisphosphoglycerate plus ATP during long-term storage of erythrocytes is a condition of keeping intact their membrane, metabolism and oxygen transport function.

Keywords: acetylcholinesterase, ATP, 2,3-bisphosphoglycerate, blood preservation, erythrocytes, membrane, microvesicles, storage lesion.

Introduction

Liquid-stored erythrocytes undergo various alterations in energy metabolism, membrane composition and shape [26, 36]. A correlation was demonstrated between the depletion of ATP and the loss of viability [6, 22]. During storage of human blood the erythrocytes lose membrane constituents [10, 19]. These cells have to maintain, however, an intact membrane to fulfill their physiological functions within the blood circulation.

Spectrin-free membrane vesicles obtained from human erythrocytes during depletion of ATP were characterized by Lutz et al. [15]. Such microvesicles were also observed after the cold storage of human whole blood in ACD or CPD media

* This paper is dedicated to Prof. Dr. h. c. S. M. Rapoport on the occasion of his 70th birthday.

[1] Senior author.

[16, 28, 30]. The loss of such vesicles should reflect an irreversible damage of the membrane and may lead to storage lesion of the erythrocyte.

In recent years the use of erythrocyte concentrates stored in protein-poor media has become increasingly accepted [12]. Microvesicles were also found in such cell concentrates after storage in SAG medium [13]. Since during storage the concentration of 2,3-bisphosphoglycerate declines, binding sites in haemoglobin may be vacated; therefore, due to an elevated ATP-binding to this protein the concentration of free ATP decreases [2,17]. The concentration of free ATP but not that of total ATP should be decisive for the maintenance of membrane integrity.

In the present study concentrates of erythrocytes were stored for 35 days in saline-adenine-glucose medium; to some storage units an ion-exchange resin was added to keep the pH constant.

The purposes of the investigation were

- characterization of the vesicles
- study of the kinetics of vesicle release in the course of storage
- study of the dependence of vesicle formation on total as well as on free ATP concentration.

The principal result of this investigation is the stabilizing effect of normal levels both of ATP plus 2,3-P_2G on the membrane, based on limitation of vesicle release.

Materials and Methods

Materials

Human whole blood of the group O, Rh^+ collected in ACD-adenine-guanosine medium as an anticoagulant [27] was kindly provided from the local blood bank. Acrylamide and imidazole were purchased from Ferak (West-Berlin); adenine was from Reanal (Budapest); DEAE-cellulose was from Serva (Heidelberg); dextran was obtained from VEB Serumwerk Bernburg, and Glucose was from E. Marck (Darmstadt). The chemicals were commercial products of analytical grade.

Treatment and separation of erythrocytes

The blood samples obtained were centrifuged at 800 g for 20 min, then the plasma and buffy coat were discarded. About 190 ml cell sediment was resuspended with 60 ml SAG-medium containing 150 nM NaCl, 83 mM glucose and 2.1 mM adenine to yield a haematocrit of about 60% [12].

DEAE cellulose packed in a dialysis bag was used as ion-exchange resin for pH-stabilization. The resin was loaded with 1 M Na_2HPO_4 as described by Harmening and Dawson [11] for the Amberlite IR 45 resin. About 4.5 mmol phosphate per g resin were bound. The loaded resin was washed with distilled water for the

removal of unbound phosphate and was equilibrated with the SAG-medium. From the dialysis bag 12 g resin (wet weight) were transferred to 400 ml cell suspension.

The erythrocyte concentrates with and without resin were stored for 35 days at 4 °C in glass bottles without agitation. Samples were drawn weekly from each bottle. Separation of cells according to their density was carried out by the modified [5] procedure of Murphy [21].

Fractions were collected from the top to about 15%, from the middle to 70% and from the bottom to about 15% of the total amount of cells. The fractionated cells were suspended in buffered saline-glucose medium and subjected to analysis of the membrane constituents (10 mM imidazole, 1 mM phosphate, 150 mM NaCl, 5 mM glucose).

Preparation and characterization of microvesicles

Preparation of vesicles was carried out from 4 ml cell suspension which was carefully transferred into 4 ml of isotonic dextran solution (density 1.078 g/ml, m.w. 60,000–90,000, pH = 7.4). By centrifugation at 2500 *g* for 2 hours the erythrocytes were sedimented. The membrane vesicles were found in the dextran layer as well as in the supernatant and were collected with a Pasteur pipette. For removal of dextran the vesicle fractions were washed with isotonic solution and centrifuged at 37,000 *g* for 1 hour. The isotonic solution was also used for the washing of cells. The proteins of the vesicle fractions were analysed by SDS polyacrylamide gel electrophoresis according to Laemmli [14] with 5 to 20% acrylamide gradients.

Electron microscopy was carried out with vesicles layered on carbon coated copper grids. The adsorbed vesicles were stained with 2% phosphotungstic acid pH 7.4 for two minutes. The samples were examined in a Siemens Elmiskop 102, the electron micrographs were evaluated by measuring the outer diameter of the vesicles.

Determination of free intracellular ATP

The erythrocyte concentrate with 60% haematocrit was diluted with one volume of an isotonic saline solution containing 120 mM KCl and 20 mM NaCl and haemolysed by freezing and thawing. The membranes were centrifuged at 20,000 *g* and 4 °C in a Sorvall centrifuge. An aliquot of the supernatant was ultrafiltered for 30 minutes in an Amicon apparatus equipped with a PM-10 membrane. The air pressure was 4×10^5 kPa. Samples of ultrafiltrate and supernatant were precipitated with perchloric acid and used for ATP determinations.

Analytical assays

Analysis of ATP and 2,3-P_2G was performed in neutralized perchloric acid extracts by standard enzymatic procedures [3]. Haemoglobin was determined with

the standardized cyanmethaemoglobin method. The pH read in freeze-thawed haemolysates of packed cells with a glass electrode at 4 °C was taken as the intracellular pH.

The sialic acid content of cells and vesicles was determined by the method of Aminoff [1] after hydrolysis in the presence of sulphuric acid (final concentration 0.1 M) at 80 °C for 1 hour followed by protein removal with zinc acetate potassium ferrocyanide [33].

Phospholipids were extracted as described by Hanahan and Ekholm [9]. Phospholipid phosphorus was measured after digestion with 70% $HClO_4$ at 180 °C according to Taussky and Shorr [32].

Results

Characterization of the vesicles

Vesicles released from erythrocyte concentrates can be isolated by a simple procedure using dextran barrier centrifugation (Fig. 1). The vesicles prepared by this technique appeared in the electron micrographs as electron dense spheres (Fig. 2) with an average diameter of 190 ± 30 nm. They were right-side out since the acetylcholinesterase activity [7] with and without 0.1% Triton X-100 was identical.

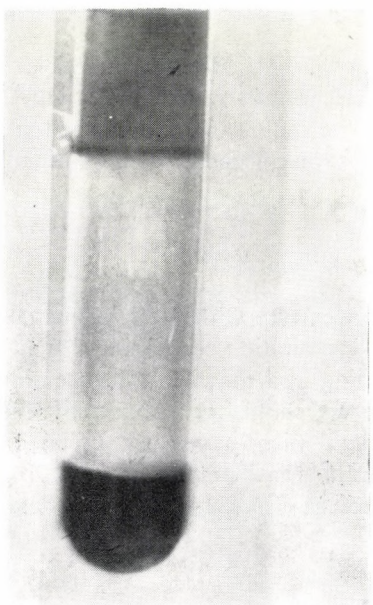


Fig. 1. Separation of vesicles from erythrocytes by the dextron layer technique. Density of dextran = 1.078 g/l. The vesicles were collected from the supernatant and the dextran layer

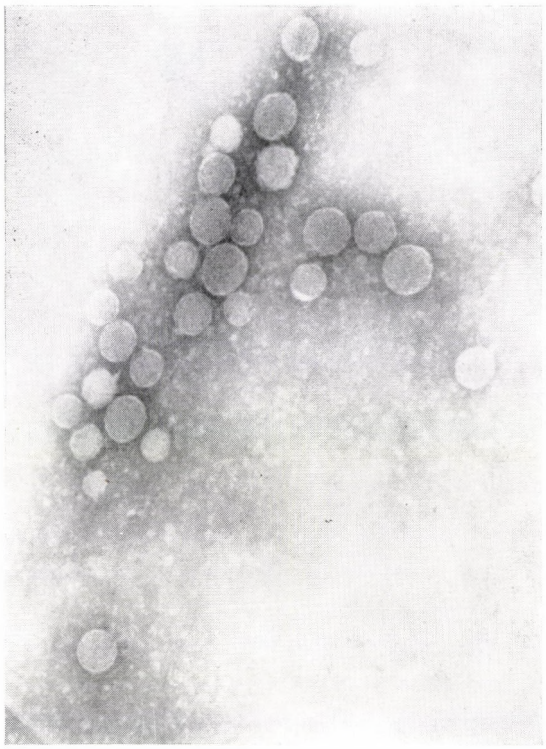


Fig. 2. Electron-micrograph of vesicles isolated from erythrocyte concentrates stored at 4 °C. Magnification, × 50, 000

The vesicles were not completely free of spectrin as shown in the electropherogram of Fig. 3. In contradiction to the reports of Lutz et al. [15] and Stibenz et al. [30] the band 3 protein was not enriched. Several unidentified polypeptides in the regions of band 4 and band 7 were present. The ratio of acetylcholinesterase

Table 1

Averaged ratios for membrane components in erythrocytes and vesicles

	AChE-activity (μmol/min)	μmol sialic acid
	μmol phospholipid phosphorus	μmol phospholipid phosphorus
Intact cells (n = 7)	3.0±0.6	0.20±0.05
Vesicles (n = 5)	4.7±1.7	0.11±0.03

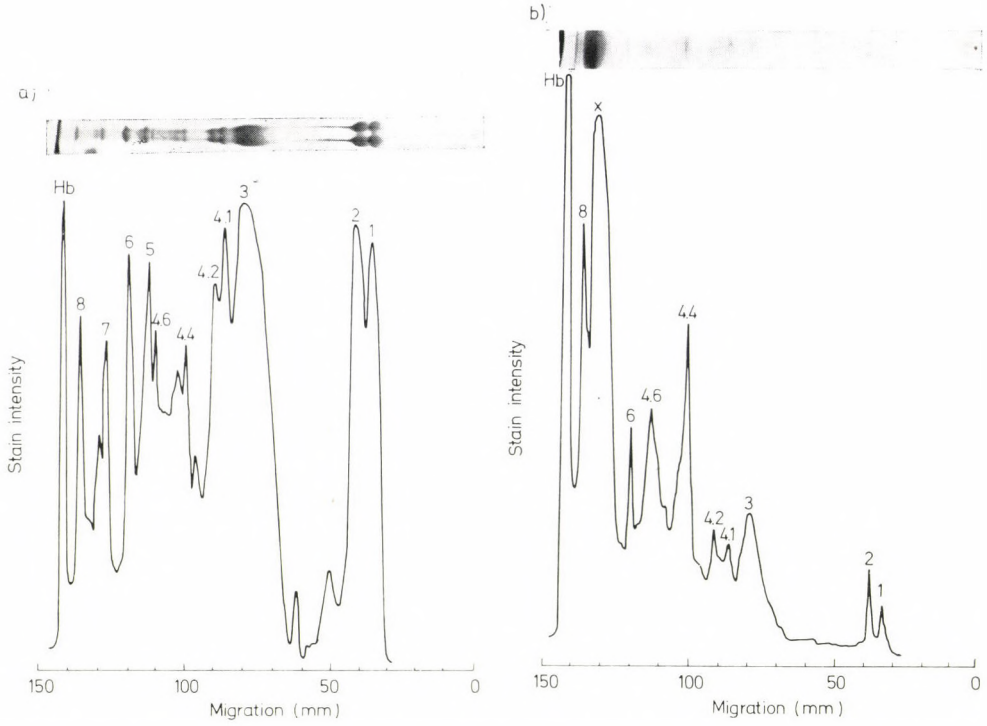


Fig. 3. SDS-polyacrylamide gels from hypotonically prepared membranes [8] (A) and from vesicles (B). Due to the high content of haemoglobin in vesicles acetylcholinesterase activity and protein were determined in the samples before solubilization. A = 57 μg protein, 130 n-mol/min ACHE; B = 190 μg protein, 43 nmol/min ACHE. The gels were scanned at 560 nm using the Hitachi MPF-2 A spectralfluorimeter. The nomenclature of polypeptides is according to that of Steck [29]

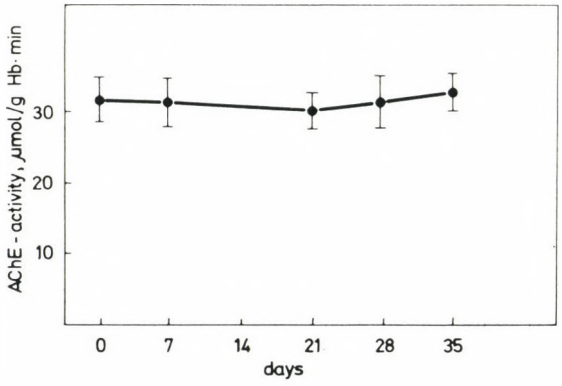


Fig. 4. Total acetylcholinesterase activity during storage of erythrocyte concentrates

activity to the phospholipid phosphorus in the vesicles was the same as in intact cells. On the other hand, the sialic acid content was decreased in the vesicles compared with the cells as indicated by the lower ratio of sialic acid to phospholipid phosphorus (Table 1). Total acetylcholinesterase was estimated in the concentrate of erythrocytes prior to washing. As may be seen in Fig. 4 its activity remained constant during the whole storage period. Therefore the activity of this enzyme was chosen as indicator of vesicle formation.

Extent of vesicle formation

From the erythrocyte resuspensions vesicles were released during the whole storage period (Fig. 5). Surprisingly vesicles were found already in the first week of preservation. There was an almost linear increase in vesicle release which became even more pronounced after two weeks of storage. A disproportional loss of siliac acid into the vesicle was found, about half of that of the released phospholipid. The share of the membrane vesicle fraction attained 16.1 $\pm$ 6.1% of the total cell membrane quantity in SAG-medium after five weeks of storage (Fig. 5). Acetylcholinesterase activity and phospholipid phosphorus were lost proportionally into vesicles in relationship to their membrane content of the intact cells during storage time (Fig. 5).

In the presence of the ion exchange resin (Fig. 6) vesicle release amounted to 4.1 $\pm$ 0.8% in three units of erythrocyte concentrate. From the inhibition of vesiculation one may assume an important metabolic influence on the extent of vesiculation.

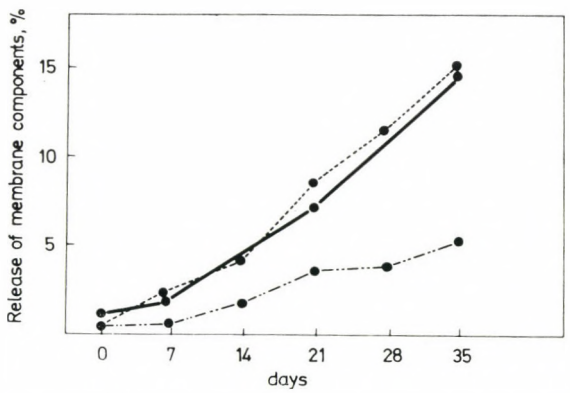


Fig. 5. Time course of vesicle formation analysed by ACHE-activity, phospholipid phosphorus and sialic acid. Results obtained in one storage unit are expressed in relation to the membrane components in the whole cell suspension. ——— phospholipid phosphorus; - - - ACHE activity; —··—··— sialic acid

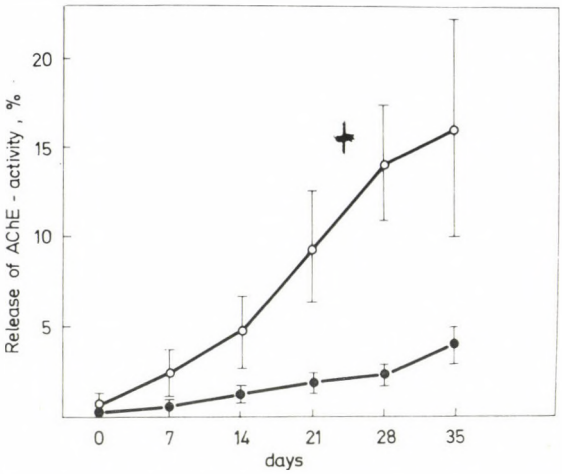


Fig. 6. Vesicle release indicated by ACHE activity in erythrocyte resuspensions
—○— SAG-medium (n = 10); —●— SAG-medium + ion-exchange resin (n = 3)

Vesicle formation in different cell fractions

Whether or not the vesicles are originating from a distinct population of cells was studied in the following way. At first, three units of cell concentrates were stored for five weeks, and every week samples were taken and fractionated according to Murphy [21]. In all of these separation experiments a similar distribution was obtained for the ratio haemoglobin content/ml packed erythrocytes in the different fractions. We could not find a population of damaged cells

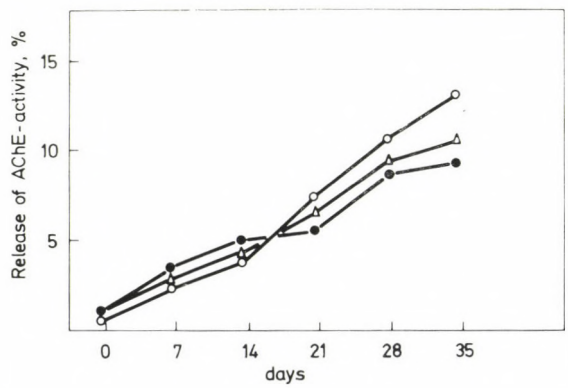


Fig. 7. Vesicle release during storage of young and old erythrocytes. Cells from freshly drawn blood were fractionated according to density and stored in SAG-medium
—○— top fraction (young cells); —●— bottom fraction (old cells);
—△— 1 : 1 mixture of top and bottom cells

as a definite source of the increased vesicle release (Table 2). The mean corpuscular haemoglobin did not change in the course of erythrocyte preservation in SAG medium. From the ratio of MCHC for the bottom 15% fractions versus the top 15% fractions a value of 1.17 was estimated. The acetylcholinesterase activity and the phospholipic-phosphorus content showed a decrease of about 33% from top to bottom cells. Sialic acid residues displayed a decrease of about 15% with increasing cell density. The loss of three membrane components of stored erythrocytes was reflected by the released vesicles. From that it was concluded that the cells lose more phospholipids and acetylcholinesterase than sialic acid.

The extent of vesiculation was followed during storage of young and old cell fractions. Freshly drawn erythrocytes were fractionated and aliquots of the top fraction, of the bottom fraction and of an 1 : 1 mixture of both were stored in SAG-medium for five weeks. It is shown in Fig. 7 that again all fractions released vesicles, but vesiculation was more pronounced in the top fraction after two weeks of preservation. There was an increase in the rate of vesicle formation.

Table 2

Acetylcholinesterase activity, phospholipid phosphorus and sialic acid in erythrocytes during storage in saline-adenine-glucose medium

The data are from three erythrocyte units. Samples of the storage unit were taken weekly and the cells were fractionated. A similar distribution for g haemoglobin per 100 ml cells was found in each sample. The mean ratio of bottom : top values was 1.20

Fraction	MCHC [g Hb/100 ml]	AChE-activity [μmol/g Hb × min]	Phospholipid phosphorus content [μmol/g Hb]	Sialic acid content [μmol/g Hb]
Suspension	29.5 ± 2.5	27.4 ± 2.0	10.6 ± 2.1	1.9 ± 0.1
top (15%)	28.6 ± 1.8	30.8 ± 2.4	13.5 ± 3.4	2.0 ± 0.2
middle (70%)	30.2 ± 1.3	25.4 ± 1.8	9.8 ± 2.0	1.7 ± 0.2
bottom (15%)	34.4 ± 2.9	20.9 ± 3.5	9.1 ± 3.3	1.7 ± 0 4

ATP and 2,3-P_2G concentration in relation to vesiculation

It is known that 2,3-P_2G loss as well as partial ATP depletion occur in stored erythrocytes. In SAG medium the total ATP content decreased from 3.17 ± 0.09 μmol/g Hb on day zero to 2.08 ± 0.11 μmol/g Hb on day 35. The presence of ion-exchange resin had no effect on this drop to about 65% of the initial value (from 3.40 ± 0.20 to 2.18 ± 0.12 μmol/g Hb). The 2,3-P_2G pool was rapidly exhausted, but in the presence of resin 44 ± 4% was still present at the end if storage (Fig. 8). In the SAG-medium the extracellular pH declined from the initial value of 7.25 to 6.65 during preservation. The presence of resin ensured the constancy of pH at

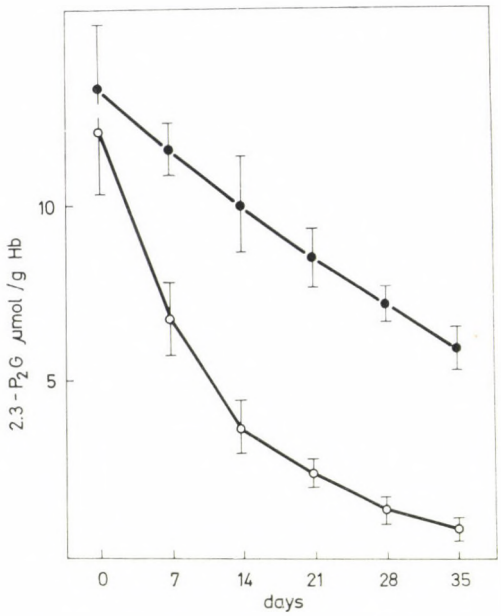


Fig. 8. Effect of ion-exchange resin supplementation to the storage medium on the concentration of 2,3-P_2G
—○— SAG-medium; —●— SAG-medium + ion-exchange resin

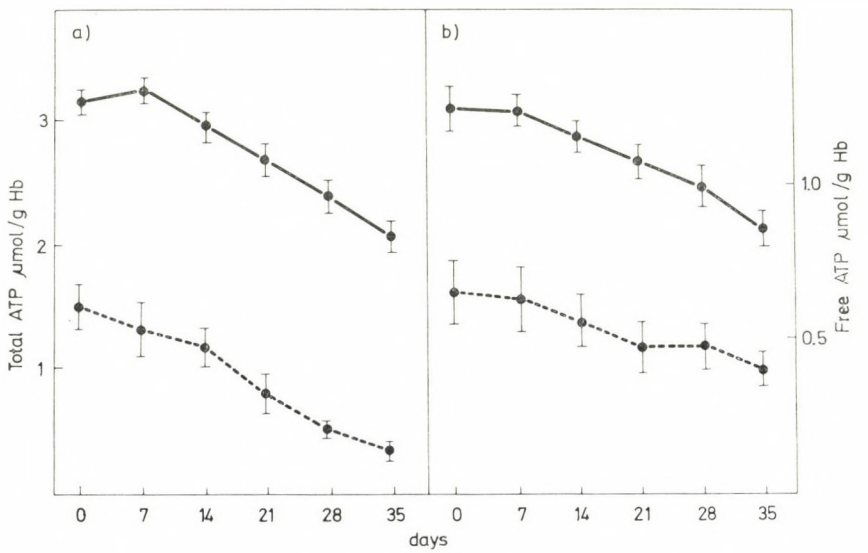


Fig. 9. Concentrations of total and free ATP during storage of erythrocyte resuspensions a) SAG-medium; b) SAG-medium + ion-exchange resin; ——— total ATP; - - - - free ATP

7.1 ± 0.1 during the 35 days. In freeze-thawed haemolysates of such cells the pH remained at values of 6.9 ± 0.1 during storage.

Figure 9 demonstrates the effect of resin addition to the SAG medium on the total and free ATP concentrations. At the beginning of storage about 20% of the total ATP did not bind to haemoglobin. Although there was no difference in the total ATP concentration of both media on day 35, the concentration of free ATP was 0.15 ± 0.2 μmol/g haemoglobin in the absence, and 0.40 ± 0.04 μmol/g haemoglobin in the presence, of resin. From that a relative increase of ATP binding to haemoglobin has been inferred.

Discussion

Vesiculation is the main mode of loss of membrane components during storage of erythrocytes. A simple procedure for the collection of vesicles from erythrocyte concentrates is to centrifuge the cell suspension through a layer of dextran. The cells remain in the supernatant and the dextran layer can be quantitated on the basis of the acetylcholinesterase activity.

The vesicles from erythrocytes stored in SAG medium were morphologically like those released after ATP depletion [15] or after 6 weeks of storage in ACD or CPD [31]. In the electropherogram of the SDS polyacrylamide gels the segregated membrane vesicles showed depletion of spectrin and enrichment of several polypeptide components in the band 4 region. They retained polypeptide band 3 in amounts approximately lower than their share of the lipid phosphorus of the whole membrane. There was an enrichment of an unidentified polypeptide below band 7. It might have been a crosslinked cytosolic protein, because it was not present in hypotonic ghost preparations used as a membrane standard (Fig. 3). Some authors using more purified vesicle fractions reported the formation of complete spectrin-free vesicles after ATP depletion [15, 20], incubation with dimyristoyl phosphatidylcholine without ATP depletion [24] and after storage for several weeks [16, 30]. It cannot be ruled out that some vesicles in our preparations were derived from fragmented membranes segregated by haemolysis, which occurs to about 2% during storage of erythrocyte concentrates in SAG media.

The phospholipid content of the vesicles prepared from ATP depleted erythrocytes has been reported to be identical with that of intact cells except for an elevated phosphatidate content [15]. The acetylcholinesterase activity was enriched twofold in those vesicles.

The vesicles investigated displayed acetylcholinesterase activity identical with that of intact cells on phospholipid basis, but they contained less glycophorin indicated by the sialic acid and less band 3 protein. They were different with respect to vesicles from the ATP depleted cells analysed by Lutz et al. [15].

Our results suggest a preferential release of membrane domains that lack clusters of band 3 protein and glycophorin. This was reflected by the disproportionate loss of sialic acid into the vesicle fraction as well as for density separated

stored erythrocytes which have lost more lipid phosphorus and acetylcholinesterase activity compared to that of sialic acid (Table 2). The acetylcholinesterase activity was enriched in the vesicles relative to both membrane-spanning proteins, band 3 and glycophorin, but not in relation to phospholipid phosphorus content.

The conclusion is that human erythrocytes lose their membrane constituents disproportionately into microvesicles during storage in SAG medium. Earlier we have found a disproportionate loss of membrane acetylcholinesterase (−36%) and sialic acid (−22%) in aged cells using density separation of erythrocytes according to Murphy [21] with 10% fractions of top and bottom cells [34]. Thus the results suggest that during aging in vivo as well as during storage of erythrocyte concentrates in SAG media the essential and irreversible step of erythrocyte senescence is the release of vesicles. Owing to immediate sequestration, the vesicles cannot be isolated from the circulating blood [4].

By freeze fracture analysis only 16% intramembrane particles were found in vesicles prepared from ACD- and CPD-blood in comparison to that in the intact cell membrane [31]. Particles of the fractured vesicle membrane were clustered in contrast to the homogeneous distribution pattern in the erythrocyte membrane. This group has reported also a decrease of the sialic acid content related to the phospholipid phosphorus in membrane vesicles isolated from whole blood after 6 weeks storage. Vesiculation and its biphasic kinetics were more pronounced in young erythrocytes (Fig. 7). Thus a damaged cell membrane is no prerequisite for the phenomenon. It suggests vesicle release as an inherent property of the aging process in vitro as well as in the circulation. Despite the membrane loss with a concomitant decrease of cell surface the shrinkage of the volume must be small because of shape transformation of discocytes to echinocytes and spherocytes.

The surface of the normal discocyte is in the range of 152 μm^2 and that of a spherocyte, 88 μm^2 [23]. Taking into account the vesicle diameter of 190 nm, the vesicle surface as a sphere measures about 0.11 μm^2. After five weeks of erythrocyte storage about 16% of the membrane surface was lost as revealed by acetylcholinesterase and phospholipid phosphorus determinations. Assuming that the vesicles represent the total membrane loss, on the average 200 vesicles per erythrocyte were released during the storage period. A single erythrocyte must lose about 560 vesicles to become a spherocyte as recently estimated by Stibenz et al. [31] for vesicle formation in whole blood.

According to the 24 h posttransfusional survival rate of about 80% in erythrocyte concentrates stored at 4 °C [12] a fraction of cells must be in a critical range of irreversible membrane loss in consequence of vesicle release.

The concentration of ATP was frequently used as an indicator of the post-storage survival rate, but the relationship is rather weak [6, 35, 37]. In the present study a close relation was observed between vesiculation and the decrease of the free ATP concentration.

Our studies suggest that stored erythrocytes release more vesicles when 2,3-P_2G declines, leading to a lower free ATP concentration. There was an increase of vesicle formation after two weeks which might have been due to the metabolic

impairment within the erythrocytes, e.g. the 2,3-P_2G exhaustion and pH decrease. [18,25]. The higher pH value maintained by the addition of ion exchanger improves the 2,3-P_2G content and the free ATP concentration. Optimal levels of both ATP and 2,3-P_2G seem to be a prerequisite for a low rate of vesicle release. The ATP availability in the form of free ATP may affect the ATP consuming reactions of the membrane. Therefore we assume that the storage lesion is due to the decreased free ATP that accompanies the loss of 2,3-P_2G and induces an increased vesicle release to about 16% of the membrane surface during five weeks storage. The improved maintenance of 2,3-P_2G and free ATP concentration resulted in a moderate vesicle release of about 5% of the membrane surface.

Our data do not provide a critical test of the rate of cell survival in the recipient, but they supply an in vitro indicator of the viability of erythrocytes during storage.

It will be necessary to confirm our findings with appropriate in vivo viability studies in relation to vesicle release and possibly also to the free ATP concentration. The improved maintenance of 2,3-P_2G per se has of course the important purpose to warrant the oxygen transport functions of stored erythrocytes and is linked indirectly with free ATP and vesicle release as shown in this study.

The tentative conclusions from our results are that vesicle release occurs continuously in cold stored erythrocytes, but young cells lose relatively more vesicles than old ones. 2,3-P_2G breakdown decreases the concentration of free ATP which may lead in some way to a disturbance of the membrane organization between the network of skeletal proteins and the phospholipid bilayer which is then followed by an increased rate of vesiculation. Finally, the erythrocytes that have lost large amounts of microvesicles should become less deformable. An increased vesicle release may lead to a reduced posttransfusional survival after 3 to 5 weeks of storage.

*

List of abbreviations used

ACD = acid citrate dextrose;
ACHE = acetylcholinesterase;
CPD = citrate phosphate dextrose;
Hb = haemoglobin;
MCHC = mean corpuscular haemoglobin concentration;
2,3-P_2G = 2,3-bisphosphoglycerate;
SAG = saline adenine glucose;
SDS = sodium dodecylsulphate

References

1. Aminoff, D.: Methods for the quantitative estimation on N-acetyl neuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J. 81*, 384 (1961).
2. Berger, H., Jänig, G. R., Gerber, G., Ruckpaul, K., Rapoport, S. M.: Interaction of haemoglobin with ions. Interactions among magnesium, adenosine-5-triphosphate,

2,3-bisphosphoglycerate and oxygenated and deoxygenated human haemoglobin under simulated intracellar conditions. *Eur. J. Biochem. 38*, 535 (1973).
3. Bergmayer, H. U. (Ed.): Methoden der enzymatischen Analyse. Akademie Verlag, Berlin 1970.
4. Bocci, V., Pessina, G. P., Paulesu, L.: Studies of factors regulating the aging of human erythrocytes. III. Metabolism and fate of erythrocytic vesicles. *Int. J. Biochem. 11*, 139 (1980).
5. Cohen, N. S., Ekholm, J. E., Luthra, M. G., Hanahan, D.: Biochemical characterization of density separated human erythrocytes. *Biochim. Biophys. Acta (Amst.) 419*, 229 (1976).
6. Dern, R. J., Brewer, G. J., Wiorkowski, J. J.: Studies on the preservation of human blood. II. The relationship of erythrocyte adenosine triphosphate levels and other in vitro measures to red cell storageability. *J. Lab. clin. Med. 69*, 968 (1967).
7. Ellman, G. L., Courtney, K. D., Andres, V. Jr., Featherstone, R. M.: A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol. 7*, 88 (1961).
8. Farrance, M. L., Vincenzi, F. F.: Enhancement of $(Ca^{2+} + Mg^{2+})$-ATPase activity of human erythrocyte membranes by haemolysis in isoosmotic imidazole buffer. I. General properties of variously prepared membranes and the mechanism of the isoosmotic imidazole effect. *Biochem. Biophys. Acta (Amst.) 471*, 49 (1977).
9. Hanahan, D. J., Ekholm, J. E., Luthra, M. G.: Are lipids lost during the preparation of erythrocyte ghosts? *Biochem. Biophys. Acta (Amst.) 363*, 283 (1974).
10. Haradin, A. R., Weed, R. I., Reed, C. F.: Changes in physical properties of stored erythrocytes. Relationship to survival in vivo. *Transfusion 9*, 229 (1969).
11. Harmening, D. M., Dawson, R. B., Masters, J. M.: The use of ion-exchange resin as a blood preservation system. *Transfusion 19*, 675 (1979).
12. Högmann, C. F., Hedlund, K., Zetterström, H.: Clinical usefulness of red cells preserved in protein-poor media. *New Engl. J. Med. 299*, 1377 (1978).
13. Högmann, C. F., Arro, E., Hedlund, K.: Red blood cell preservation in protein-poor media. 2. Studies of changes in red cell shape during storage. *Haematologica 13*, 135 (1980).
14. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of the bacteriophage T_4. *Nature (Lond.) 227*, 680 (1970).
15. Lutz, H. U., Liu, S. C., Palek, J.: Release of spectrin-free vesicles from human erythrocytes during ATP depletion. I. Characterization of spectrin-free vesicles. *J. Cell. Biol. 75*, 548 (1977).
16. Lutz, H. U.: Vesicles isolated from ATP-depleted erythrocytes and out of thrombocyte-rich plasma. *J. supramolec. Struct. 8*, 375 (1978).
17. Marshall, W. E., Rassaian, N., Greenwald, L. S., Omachi, A.: Ultrafiltrable adenosine triphosphate and 2,3-diphosphoglycerate concentrations in cold-stored human erythrocytes. *Transfusion 17*, 448 (1977).
18. de Mondonca, M., Appel, M., Bidault, J., Masson-Piere, D., Terrier, E.: Maintenance of 2,3. DPG and ATP levels in blood stored for 30 days at a constant pH obtained by variation of CO_2. *Vox Sang. 35*, 184 (1978).
19. Moore, G. L., Antonoff, R. S., Rau, D. C.: Changes in erythrocyte membranes during cold storage of whole blood. *Vox Sang. 22*, 48 (1972).
20. Müller, H., Schmidt, U., Lutz, H. U.: On the mechanism of vesicles release from ATP-depleted human red blood cells. *Biochem. Biophys. Acta 649*, 462 (1981).
21. Murphy, J. R.: Influence of temperature and method of centrifugation on the separation of erythrocytes. *J. Lab. Med. 82*, 334 (1973).
22. Nakao, K. T., Wada, T., Kaniyama, T., Nakao, M., Nagano, K.: A direct relationship between adenosine triphosphate level and in vivo viability of erythrocytes. *Nature (Lond.) 194*, 877 (1962).
23. Nakao, M., Hoshino, K., Nakao, T.: Constancy of cell volume during shape change of

erythrocytes induced by increasing ATP content. *J. Bioenerg. Biomembr. 13*, 307 (1981).
24. Ott, P., Hope, M. J., Verkleij, A. J., Roelofsen, B., Brodbeck, U., van Deenen, L. L. M.: Effect of dimyristoyl phosphatidylcholine on intact erythrocytes. Release of spectrin-free vesicles without ATP depletion. *Biochim. Biophys. Acta 641*, 79 (1981).
25. Rapoport, I., Berger, H., Elsner, R., Rapoport, S. M.: pH-dependent changes of 2,3-bis-phosphoglycerate. *Acta Biol. Med. Germ. 36*, 515 (1977).
26. Rapoport, S.: Dimensional, osmotic, and chemical changes of erythrocytes in stored blood. I. Blood preserved in sodium citrate, neutral, and acid-citrate-glucose (ACD) mixture. *J. Clin. Invest. 26*, 591, (1947).
27. Roigas, H., Strauss, D., Raderecht, H. J.: Verbesserte Blutkonservierung durch Zusätze von Adenin und Guanosin. *Z. Ges. Inn. Med. 24*, 850 (1969).
28. Rumsby, M. G., Trotter, J. A., Allan, D., Michell, R. H.: Recovery of membrane micro-vesicles from human erythrocytes stored for transfusion: A mechanism for the erythro-cyte discocyte-to-spherocyte shape transformation. *Biochem. Soc. Strans. 5*, 126 (1977).
29. Steck, T. L.: The organization of proteins in the human red blood cell membrane. *J. Cell Biol. 62*, 1 (1974).
30. Stibenz, D., Linss, W., Meyer, H. W., Halbhuber, K. J., Geyer, G.: Erythrocyte vesicula-tion. 2. Membrane molecular transformation. *Fol. Haemat. (Lpz.) 108*, 580 (1981).
31. Stibenz, D., Halbhuber, K. J., Linss, W.: Vesiculation von Erythrozyten. 4. Quantifizie-rung von Vesikulationsraten von Standardflüssigkonserven. *Fol. Haemat. (Lpz.)* in press.
32. Taussky, H. H., Shorr, E.: A microcolorimetric method for the determination of in-organic phosphorus. *J. Biol. Chem. 202*, 675 (1953).
33. Tischer, J., Peters, D.: Methode zur Bestimmung des Sialinsäuregehaltes von Erythro-zyten und hämoglobinhaltigen Sialinsäurelösungen. *Zbl. Bakt. 195*, 408 (1965).
34. Trautsch, Ch., Tannert, C., Maretzki, D.: Disproportional loss of membrane constituents in the course of erythrocyte ageing. *Acta Biol. Med. Germ. 40*, 743 (1981).
35. Wood, L., Beutler, E.: The viability of human blood stored in phosphate adenine media. *Transfusion 7*, 401 (1967).
36. Yoshikawa, H., Nakao, M.: Nucleotid metabolism and its relation to functions of pre-served human erythrocyte. *Fol. Haemat. (Lpz.) 78*, 248 (1961).
37. Zuck, T. F., Bensinger, T. A., Peck, C. C., Chillar, R. K., Beutler, E., Button, W. N., McCurdy, P. R., Josephson, A. M., Greenwalt, T. J.: The in vivo survival of red blood cells stored in modified CPD with adenine: Report of a multi-institutional cooperative effort. *Transfusion 17*, 374 (1977).

Haematologia 17 (3), pp. 375–381 (1984)

Micromethod for Colony Growth of Erythroid and Granulocytic Cell Colonies in Glass Capillaries*

E. HEILMANN,[1] P. RIDDERSKAMP, M. WEBER, H. R. MAURER[2]

Medizinische Poliklinik der Universität Münster[1], Domagkstr. 3, D-4400 Münster, and der Freien Universität Berlin[2], FRG

(Received 3 May 1983; accepted 20 May 1983)

Mononuclear cells of peripheral blood were cultured in glass capillary tubes under various conditions. The highest number of granulopoietic colonies was found with 5×10^4 cells per capillary and 10% conditioned medium. The growth of erythroid colonies did not depend on erythropoietin linearly, but a good correlation was found between the number of cells and the erythropoietin concentration respectively when the single erythroid precursor cells were counted on the slides. The highest yield of erythroid cells was noted at 0.5 U erythropoietin at all cell concentrations.

Keywords: glass capillaries, erythroid colonies, granulopoietic colonies, micromethod.

Introduction

Abrams et al. [1] were the first to report on the possibility of culturing haematopoietic colonies by micromethods. The modifications we have described elsewhere [6, 8, 9, 10, 11, 14] enable PHA-stimulated lymphocytes, granulocyte colonies and macrophages from bone marrow cells to be grown in capillary tubes. The culture of haematopoietic progenitor cells from peripheral blood as it has been hitherto performed in Petri dishes [2, 5, 12] is a comparatively large-scale operation. The chance to induce granulopoietic and erythropoietic precursor cells in capillaries to produce colonies makes it desirable to perform such studies with a minimum of test material.

Material and Methods

Mononuclear cells from peripheral blood were separated under sterile conditions in Ficoll-gradient. The required samples of 20 ml venous blood, taken from healthy males, were mixed with 500 I.U. Novo-Heparin®.

For growing the granulopoietic colonies we used conditioned medium from the supernatants of granulocytes from peripheral blood of healthy male subjects (4). The tests were performed using 5%, 10%, 12.5%, 15% and 30% concentrations of conditioned medium.

The erythropoietic colonies were prepared with erythropoietin step III of Connaught Lab., Toronto, Canada [13]. The culture preparation consisted of the

* Supported by LANDESAMT für FORSCHUNG NW
Reprints: Prof. Dr. E. Heilmann, Am Kaiserplatz 3 3280 Bad Pyrmont, FRG

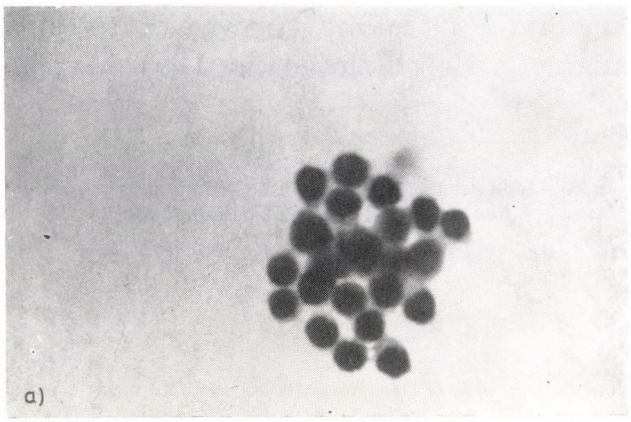


Fig. 1a. Erythropoietic precursors grown in capillaries. Benzidine staining. Magnification 1000 ×

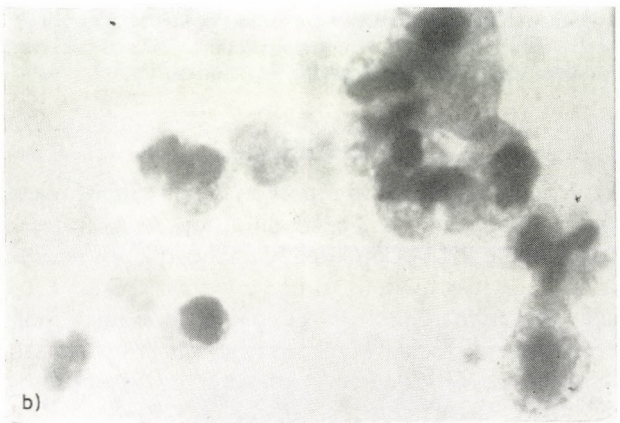


Fig. 1b. Granulopoietic precursors. Giemsa staining. Magnification 1000 ×

following ingredients: 40 µl 20% fetal calf serum of inactivated horse serum (Seromed), 35 µl cell suspension, 100 µl erythropoietin and 65 µl ISCOVE's medium; these were mixed to 60 µl of 1.0% agar boiled and cooled to 50 °C to a total quantity of 300 µl. This suspension was distributed into six glass capillaries (capillettes of 100 µl, 125 mm length, Labora Mannheim), and incubated for 14 days in a CO_2-Incubator (Flow Laboratories) at a temperature of 37 °C. The capillaries were evaluated under a stereo-microscope. Means were calculated for the numbers of colonies grown in the 6 capillaries, then their contents were blown out onto a slide and stained with Giemsa, Luxol and benzidine [3]. Counting of cells was performed at 1000-fold magnification.

Figure 1a shows erythroid precursors, identified by their typical colony formation and special staining with benzidine. Granulopoietic precursors were stained with Giemsa and classified by their nucleus formation (Fig. 1b).

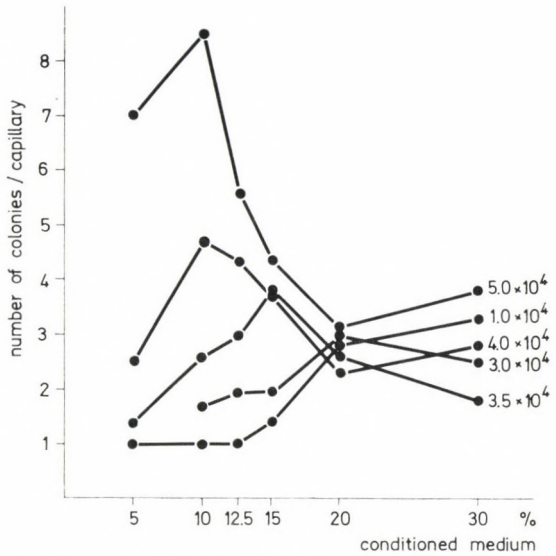


Fig. 2. Mean number of granulopoietic colonies per capillary in relation to mononuclear incubated cells and various concentrations of conditioned medium

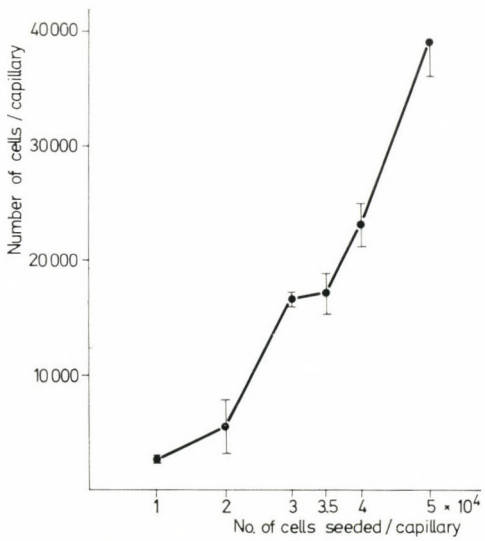


Fig. 3. Correlation between mononuclear incubated cells and granulopoietic cells on slides

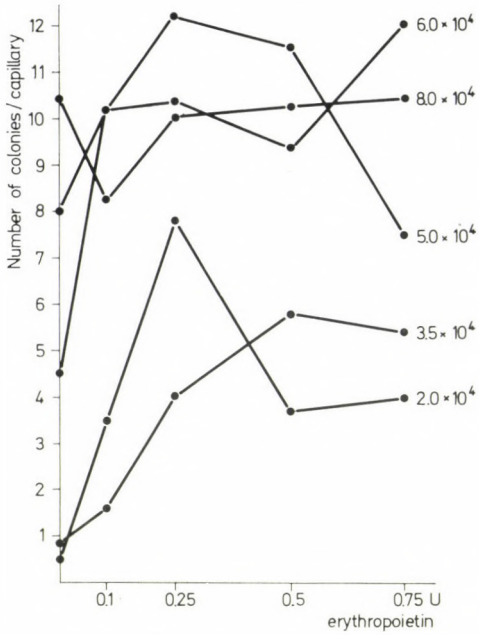


Fig. 4. Mean number of haematopoietic colonies per capillary in relation to mononuclear incubated cells and various concentrations of erythropoietin (step III, Connaught Laboratories, Toronto)

Results

The number of colonies grown per capillary was related to the number of cells injected and the concentration of the conditioned medium as shown in Fig. 2. The highest number of colonies was found in the preparation with 5×10^4 cells per capillary and 10% conditioned medium. An increase of the concentration above 20% caused no additional augmentation of the number of colonies.

The number of cells which could be visualized on the slides at a 10% concentration of the conditioned medium on the 14th day of culturing is shown in Fig. 3. There was a near linear correlation between the injected cell-quantity and the number of granulopoietic cell cultures in the capillaries.

Fig. 4 shows the number of grown colonies in relation to various erythropoietin concentrations and different cell-injections. An increase in the quantity of cells yielded a higher number of colonies. An increase of the erythropoietin concentration resulted in better linearity with lower than with higher quantity of injected cells.

Cells concentrations between 5 and 8×10^4 cells were associated with a marked spontaneous growth of erythroid colonies. There was a more favourable relation in the various preparations between cell concentration, erythropoietin content and number of grown erythropoietic precursor cells when counting single cells on the

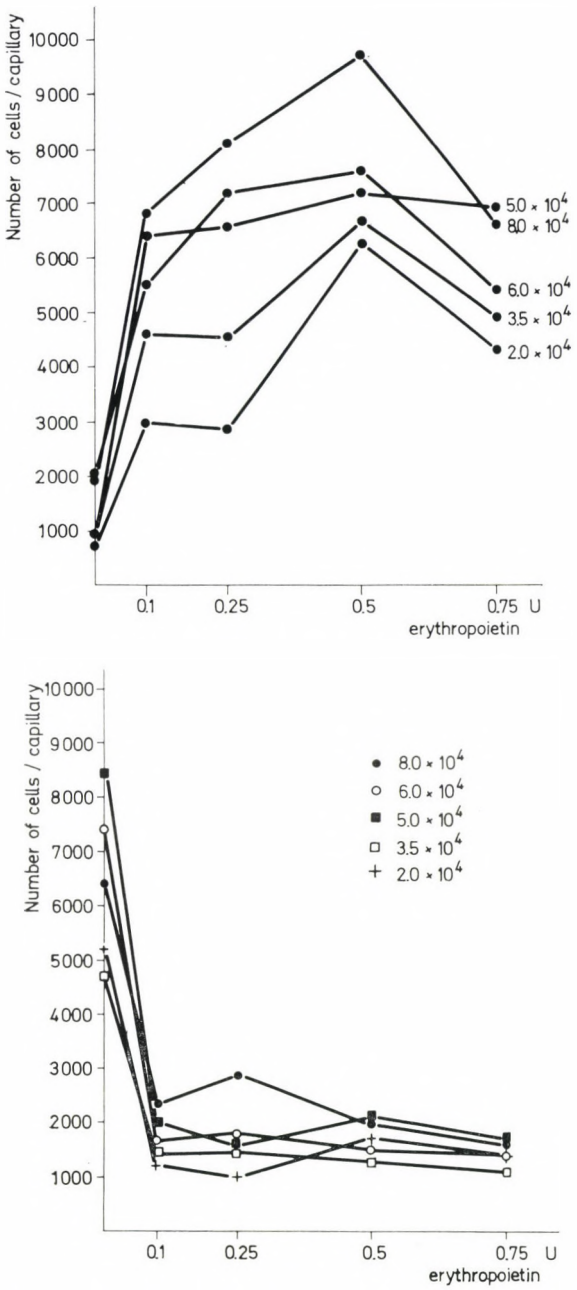


Fig. 5. Mean number of haematopoietic cells per capillary in relation to the number of mononuclear cell cultures and various concentration of erythropoietin: a) erythroid precursor cells b) granulopoietic precursor cells

slides, as shown in Fig. 4. The highest number of erythropoietic cells was found at all cell concentrations at 0.5 U erythropoietin per capillary (Fig. 5). Higher erythropoietin concentrations yielded no increase in cell growth. A nearly mirror-image like situation was shown by the simultaneously counted granulopoietic precursor cells (Fig. 5).

Discussion

The first attempt to grow granulopoietic precursor cells was reported by Abrams et al. [1], who used mouse bone marrow. Later, we succeeded in culturing corresponding colonies from humans [7, 8, 9, 10]. A search of the literature in relation to detection of haematopoietic colonies from peripheral blood mononuclear cells produced references only on the use of Petri dishes [5, 12].

The present paper describes the culture of mononuclear cells in capillaries using a microtechnique. Materials and conditions were similar to those required for the culture of stem cells in Petri dishes, as we have described it elsewhere [6, 7, 8, 9, 10]. Evaluation of the number of colonies grown in capillaries is easier and a more reproducible method.

The statistical variation of the number of counted colonies in the single capillaries is about 7%. In both granulopoiesis and erythropoiesis there was a close relation between the number of injected cells and the number of grown colonies at a given concentration of the conditioned medium or erythropoietin. Haematologic precursor cells, determined by single counting correlated closely in the granulopoiesis series with the number of injected cells. There was a greater variation in the results with respect to erythroblasts. Further studies will have to establish whether it is possible to distinguish erythropoietic from granulopoietic cell formations by stereomicroscopy alone.

References

1. Abrams, L., Carmeci, P., Bull, J. M., Carbone, P. P.: Capillary tube scanning applied to intro mouse marrow granulocyte growth. *J. mat. Cancer Inst. 50*, 267 (1973).
2. Clarke, B. J., Housman, D.: Characterization of an erythroid precursor cell of high proliferative capacity in normal human peripheral blood. *Proc. Nat. Acad. Sci. (Wash.) 74*, 1105 (1977).
3. Dresch, C., Johnson, G. R., Metcalf, D.: Eosinophil colony formation in semisolid cultures of human blood marrow cells. *Blood 49*, 835 (1977).
4. Guilbert, L. J., Iscove, N. N.: Partial replacement of serum by selenite, transferrin, albumin and lecithin in haematopoietic cell cultures. *Nature (Lond.) 263*, 594 (1976).
5. Iscove, N. N., Senn, J. S., Till, J. E., McCullock, E. A.: Colony formation by normal and leukemic human marrow cells in culture, effect of conditioned medium from human leukocytes. *Blood 37*, 1 (1971).
6. Maurer, H. R.: In vitro colony growth of granulocytes, macrophages, T and B lymphocytes in agar capillaries. *Acta Haemat. (Basel) 62*, 322 (1979).

7. Maurer, H. R., Henry, R.: Colony growth of mouse bone marrow cells in agar contained in glass capillaries. *Blut 33*, 11 (1976).
8. Maurer, H. R., Henry, R.: Automated scanning of bone marrow cell colonies in agar containing glass capillaries. *Exp. Cell Res. 103*, 271 (1976).
9. Maurer, H. R., Henry, R.: Growth kinetics by scanning of granulocytic cell colonies in glass capillaries. *Blut 34*, 89 (1977).
10. Maurer, H. R., Maschler, R., Dietrich, R., Goebel, B.: In vitro culture of lymphocyte colonies in agar capillary tubes after PHA-stimulation. *J. Immunol. Methods 18*, 353 (1977).
11. Neumeier, R., Maurer, H. R., Maschler, R., Bombik, B. M.: A micromethod of culture granulocyte colonies from human bone marrow in agar-containing glass capillaries. *Exp. Hemat. 9*, 44 (1981).
12. Ogawa, M., Grush, O. C., O'Dell, R. F., Hara, H., MacEachern, M. D.: Circulating erythropoietic precursors assessed in culture: characterization in normal men and patients with hemoglobinopathies. *Blood 50*, 1081 (1977).
13. Stephenson, J. R., Axelrad, A. A., McLeod, D. L., Shreeve, M. M.: Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. *Proc. Nat. Acad. Sci. (Wash.) 68*, 1542 (1971).
14. Ulmer, A., Maurer, H. R.: The formation of B-lymphocyte colonies in agar contained in glass capillaries. *Immunology 34*, 919 (1978).

Haematologia 17 (3), pp. 383–386 (1984)

Neutrophil Acid Phosphatase Activity in Patients with Gastric or Rectum Carcinoma during Surgical Treatment

KRYSTYNA MERKIEL,[1] J. PROKOPOWICZ[2]

[1]Department of Clinical Diagnostics, Institute of Biochemistry;
[2]Medical Analytics, Medical School, M. Skłodowska-Curie 24A,
15 276 Białystok, Poland

(Received 7 December 1982; accepted 15 February 1983)

In 22 patients with alimentary tract carcinoma granulocyte acid phosphatase activity was determined using the histochemical method of Suzuki. Enzyme activity was determined in peripheral blood 3 times, viz. before surgical intervention, in blood from a vessel draining the tumour before its excision, and in peripheral blood 2–3 weeks after excision of the tumour. In parallel tests, enzyme activity was established in peripheral blood of 22 healthy individuals.

The results showed that acid phosphatase activity of granulocytes is lower than in the controls, especially in granulocytes collected from the vessel draining the tumour. The lower acid phosphatase activity in granulocytes from patients with carcinoma was mainly due to the lower percentage of positive cells. After removal of the tumour acid phosphatase activity of the granulocytes increased in the majority of cases. It is assumed that an enhanced exocytosis of lysosomal enzymes in the course of tumour disease was responsible for the decrease of activity in the granulocytes.

Keywords: neutrophil acid phosphatase, gastric cancer

Correspondence: Prof. J. Prokopowicz

Introduction

Infection is a common complication and the most frequent cause of death in cancer patients [4]. Frequent infections are now ascribed to the presence of the tumour and not only to the immunosuppressive effect of therapy [6, 8]. Attempts to identify polymorphonuclear leukocyte dysfunction as a predisposing factor to infection in these patients have yielded conflicting results [7, 13, 15].

Phagocytosis, the basic biological function of phagocytes depends in a high degree on the functional activity of lysosomal enzymes [5]. According to their biological function the lysosomal enzymes of phagocytes may be divided into three categories: acid hydrolases, enzymes possessing microbicidal activity, and neutral proteinases [5]. Acid hydrolases require a low pH for optimal functioning. They act mainly in the phagolysosome where they participate in the degradation of ingested material [9] at a pH of 4 to 5 [10]. Neutrophilic acid phosphatases may take part in the degradation of ingested material during phagocytosis and in other immunological processes [1].

Previously we have reported [13] that the bactericidal capacity of leukocytes in the blood of patients with neoplasm was decreased in comparison to the leukocytes of normal individuals. The present study has been focussed on the acid phosphatase activity of granulocytes obtained from patients with carcinoma.

Materials and Methods

Blood was collected from 22 patients with tumour of the stomach or colon. These patients were free from infections and had received no treatment before testing.

Blood samples were taken during the surgical procedure from a peripheral vessel, from a blood vessel draining the tumour and 2–3 weeks after removal of the tumour. The control group consisted of the 22 healthy individuals. The blood samples were heparinized with 10 IU/ml and granulocytic acid phosphatase activity was determined using the cytochemical method of Suzuki [14].

The cells with granular reaction products were considered positive and their percentage was counted; 100 positive neutrophilic granulocytes scored from 0 to 3. Results of activity determinations were compared by Student's t test.

Results

In 22 patients with alimentary tract carcinoma mean acid phosphatase activity of granulocytes collected from vessels draining the tumour was 81 with a range of 39 to 115. Mean percentage of positive cells was 77 (Table 1). Mean enzyme activity in leukocytes from a peripheral vessel was 84 with a range of 45 to 110. The mean percentage of positive cells was 80. The difference in acid phosphatase activity between peripheral blood and blood from a tumour efferent vessel was not significant statistically ($0.60 > p > 0.50$) nor was the difference in the percentage of positive cells ($0.60 > p > 0.50$).

Removal of the tumour caused an increase in acid phosphatase activity in granulocytes (mean 99 with a range from 82 to 118) which was significant statistically in comparison to the peripheral blood, $0.005 < p < 0.001$. The mean percentage of positive cells was 94, comparable to the normal value which was 93.

Mean acid phosphatase activity of granulocytes from the controls was 103 with a range from 82 to 119 (Table 1).

The difference in acid phosphatase activity between the patients with carcinoma and the controls was significant statistically ($p < 0.001$) like the difference in the percentage of positive cells ($p < 0.001$).

The difference in acid phosphatase activity of the neutrophils after removal of the tumour and the controls was not significant ($0.40 > p > 0.30$) nor was the difference in percentage of positive cells ($0.20 > p > 0.10$).

Table 1

Neutrophil acid phosphatase activity in patients with carcinoma and normal controls

No.	A		A_1		A_2		B	
	Score of 100 cells	Per cent of positive cells	Score of 100 cells	Per cent of positive cells	Score of 100 cells	Per cent of positive cells	Score of 100 cells	Per cent of positive cells
1	70	65	60	58	82	56	82	74
2	78	72	64	54	97	90	95	90
3	45	44	39	33	94	82	101	92
4	66	54	87	84	107	96	98	93
5	78	75	100	90	118	94	92	81
6	79	70	80	79	—	—	119	97
7	79	70	70	69	118	95	100	94
8	89	85	90	89	104	97	100	90
9	90	86	84	78	118	99	94	92
10	101	97	98	68	85	84	118	91
11	110	98	115	95	101	91	104	98
12	100	96	97	91	104	92	110	97
13	95	93	68	68	91	88	112	100
14	105	98	80	76	110	98	99	99
15	89	80	74	74	94	83	116	100
16	79	79	84	80	86	86	106	98
17	94	92	97	92	101	100	108	100
18	104	99	103	98	97	94	114	98
19	100	99	100	94	—	—	107	100
20	57	57	60	60	84	78	92	91
21	85	80	79	75	102	90	105	100
22	75	72	71	68	106	100	99	90
$\bar{x}$	84	80	81	77	99	94	103	93

A — Peripheral blood before surgical intervention
A_1 — Blood from vessel draining the tumour before its excision
A_2 — Peripheral blood 2–3 weeks after tumour removal
B — Peripheral blood in controls

Discussion

The results showed that the acid phosphatase activity of neutrophils of patients with carcinoma was lower than that of healthy subjects and especially so in the granulocytes collected from the vessel draining a tumour. This was not mirrored by the mean value because in 5 cases the activity was increased. The low activity in the patients with carcinoma was mainly due to the low percentage of positive cells. After removal of the tumour, acid phosphatase activity of the neutrophils increased in the majority of cases.

Earlier we have reported [12] that alkaline phosphatase activity of granulocytes from cancer patients was lower than that of the controls. The same was

reported by other authors too [11]. What explanation can be offered for that finding?

In the view of some authors [8] cancer patients have a factor in their plasma which interferes with phagocytosis and which promotes exocytosis of lysosomal enzymes from the neutrophils. The factor might be synthesized by the tumour itself or by the antigen–antibody complexes fixed on the surface of an intact membrane. In contrast we suggest that in tumour disease the enhanced exocytosis of lysosomal enzymes from the granulocytes may be responsible for the decreased activity of these enzymes.

The present results support the finding of an enzyme deficit or dysfunction in the granulocytes of patients with tumour [11–13]. If we consider the role of these cells in anticancer mechanisms depending on a cytotoxic effect [3] or phagocytosis [2], so the normal function of the granulocytes in these patients must be of great importance. Thus, a dysfunction of the granulocytes may illustrate to some extent their capacity against the spread of tumours.

References

1. Brelińska-Pęczalska, R., Mackiewicz, S., Burchardt, K., Celińska-Szpytko, E.: Cytochemical studies on peripheral blood leukocytes in rheumatoid arthritis. *Rheumatologie 3*, 491 (1973).
2. Chee, D. O., Townsend, C. M., Galbroith, M. A., Eilber, F. R., Morton, D. L.: Selective reduction of human tumor cell populations. *Cancer Res. 38*, 4534 (1978).
3. Clark, R. A., Klebanoff, S. J.: Neutrophil-mediated tumor cell cytotoxicity. Role of the peroxidase system. *J. Exp. Med. 141*, 1442 (1975).
4. Feld, R., Bodey, G. P.: Infections in patients with malignant lymphoma treated with combination chemotherapy. *Cancer 39*, 1018 (1977).
5. Ferenčik, L., Stefanovič, J.: Lysosomal enzymes of phagocytes and the mechanism of their release. *Folia Microbiol. 24*, 503 (1979).
6. Gudewicz, P. W., Saba, T. M.: Inhibition of phagocytosis and glucose metabolism of alveolar macrophages during pulmonary tumour growth. *Brit. J. Cancer 36*, 670 (1977).
7. Hancock, B. W., Bruce, L., Richmond, J.: Neutrophil function in lymphoreticular malignancy. *Brit. J. Cancer 33*, 496 (1976).
8. Henon, P., Gerata, I., Palacios, S.: Functional abnormalities of neutrophils in cancer patients: Inefficient phagocytosis and reverse endocytosis. *Biomedicine 27*, 261 (1977).
9. Hoshing, C. S.: The role of phagocytes. *Aust. Family Phycn. 5*, 21 (1976).
10. Jacques, Y. V., Bainton, D. F.: Changes in pH within the phagocytic vacuoles of human neutrophils and monocytes. *Lab. Invest. 39*, 179 (1978).
11. Lisiewicz, J., Zduńczyk, A.: The role of neutrophils in immunological response against neoplasms. *Pol. Tyg. Lek. 36*, 579 (1981). (In Polish).
12. Merkiel, K., Prokopowicz, J., Krawczuk, J.: Neutrophil alkaline phosphatase activity in patients with neoplasma during surgical treatment. *Neoplasma 28*, 5 (1981).
13. Merkiel, K., Prokopowicz, J., Krawczuk, J.: The depressing effect of tumor on bactericidal capacity of plasma and leukocytes. *Folia Haemat. 105*, 497 (1978).
14. Suzuki, A., Tokohu, J.: Azo dye method for demonstration of leukocyte acid phosphatase activity in blood smears. *J. Exp. Med. 86*, 311 (1963).
15. McRipley, R., Selvara, J. R., Glonsky, M. Sarba, A.: The role of the phagocyte in host-parasite interaction. Phagocyte and bactericidal activities of leukocyte from patients with different neoplastic disorders. *Cancer Res. 27*, 674 (1967).

Haematologia 17 (3), pp. 387—398 (1984)

Identification of Human Platelet Membrane Fibrinogen Receptors by Immunochemical Techniques[1]

ELIZABETH KORNECKI,[2] G. P. TUSZYNSKI, S. NIEWIAROWSKI

Thrombosis Research Center, Temple University Health Sciences Center, 3400 N. Broad St., Philadelphia, PA 19140, USA

(Received 5 February 1983; accepted 15 July 1983)

The fibrinogen receptors of platelets were investigated with the use of three types of anti-platelet membrane antibodies and three types of platelets. We found that antisera raised in rabbits against membranes prepared from human intact, chymotrypsin- or pronase-treated platelets inhibited the fibrinogen-induced aggregations of ADP-stimulated intact platelets, chymotrypsin-treated platelets and pronase-treated platelets. These antisera also blocked the binding of ^{125}I-fibrinogen to ADP-stimulated intact, chymotrypsin-treated, and pronase-treated platelets. These results suggest that all three antisera blocked the interaction of fibrinogen with its receptor on the surface of the three types of platelets studied. Fibrin clot retraction by intact platelets was also inhibited by these three antibodies indicating an important role of platelet membrane proteins in clot retraction. As demonstrated by techniques using ^{125}I-surface labeling, Staphylococcus aureus immunoprecipitation, SDS-polyacrylamide gel electrophoresis and autoradiography, anti-intact platelet membrane antibody immunoprecipitated the membrane glycoproteins GPIIb, GPIII and a protein with an apparent molecular weight of 66 000 from detergent solubilized surface ^{125}I-iodinated chymotrypsin-treated platelets. Anti-chymotrypsin and anti-pronase-treated platelet membrane antisera immunoprecipitated mostly GPIII and the 66 000 molecular weight protein from detergent solubilized, surface ^{125}I-iodinated chymotrypsin-treated platelets. The 66 000 Mr protein was not found on the surface of intact (unstimulated) platelets which do not bind ^{125}I-fibrinogen and are not aggregated by fibrinogen without the prior addition of ADP. The ability of anti-platelet membrane antibodies to block fibrinogen-induced platelet aggregation and fibrinogen binding to platelets correlated with their ability to immunoprecipitate a 66 000 Mr protein from the platelet surface. It is proposed that the 66 000 Mr protein may be the fibrinogen binding domain of GPIII which becomes permanently exposed on the surface of chymotrypsin and pronase-treated platelets following proteolysis and which becomes exposed upon stimulation of intact platelets by agents such as ADP.

Keywords: Anti-platelet membrane antibodies, fibrinogen receptor, platelets.

[1] Supported by NIH grants HL 15226, HL 14217, HL 28149, CA 27458 training grant HL-05976, National Research Service Award HL-06356 and a grant 1538 from the Council for Tobacco Research. E. K. is also a recipient of a Special Investigatorship Award from the American Heart Association, Southeastern Pennsylvania Chapter.

[2] To whom correspondence should be addressed. Present address of Dr Kornecki is Univ Vermont, Dept of Psychiat., Burlington, VT, 05405

Introduction*

Fibrinogen is an essential cofactor for ADP-induced platelet aggregation [1–4] and there is evidence that platelet aggregation to epinephrine [5, 6], thrombin [7, 8] and prostaglandin endoperoxides [9, 10] necessitates the presence of fibrinogen. Radiolabeled fibrinogen does not bind to intact platelets but it does bind to platelets stimulated by ADP [5, 11–13] or treated with chymotrypsin [14, 15] or pronase [15]. It has been demonstrated that ADP and chymotrypsin, acting on the platelet membrane by entirely different mechanisms, expose fibrinogen receptors which are similar in number and binding affinities [15, 16]. We [16] as well as Peerschke et al. [13] have shown that ADP-stimulated normal platelets, expose high and low affinity fibrinogen receptors on their membrane surface. Similarly, two classes of fibrinogen receptor have been demonstrated on chymotrypsin-treated platelets [15]. Platelets of patients with Glanzmann's thrombasthenia, deficient in the two major surface membrane glycoproteins IIb (GPIIb) and glycoprotein IIIa (GPIIIa), contain only the high affinity fibrinogen receptors which can be exposed by chymotrypsin but not by ADP. On the other hand, thrombasthenic platelets appear to lack the low affinity fibrinogen receptors [16].

In all experimental systems described, there is a direct correlation between the rate of platelet aggregation and amount of binding of ^{125}I-fibrinogen to platelets. This evidence favors the idea that it is the occupancy of these fibrinogen receptors by fibrinogen which results in platelet aggregation [12, 13, 15, 16]. These lines of evidence also favor the hypothesis that fibrinogen in the presence of calcium serves to bridge platelets together by linking itself to specific fibrinogen receptors on adjacent platelets [17].

The mechanism by which ADP or chymotrypsin expose fibrinogen receptors on the platelet surface is not clear. A number of agents such as apyrase, an ADPase [18], 5-p-fluorosulfonylbenzoyladenosine, an ADP analog [19], and pyridoxal phosphate (vitamin B_6) [20–22] which have been shown to interfere with the action of ADP, also block the exposure of fibrinogen binding sites on ADP-stimulated platelets. These inhibitors (with the exception of apyrase) appear to act on the platelet ADP receptor since they do not interfere with the binding of ^{125}I-fibrinogen to chymotrypsin-treated platelets nor with the aggregation of these platelets by fibrinogen. EDTA [15] and mepacrine [23] appear to act directly by inhibiting ^{125}I-fibrinogen binding to chymotrypsin-treated platelets. It has been recently suggested that the mechanism by which chymotrypsin removes the ADP requirement for aggregation and fibrinogen binding may be through proteolysis

* The abbreviations used are: SDS (sodium dodecyl sulfate)
PAGE (polyacrylamide gel electrophoresis)
GPIIIa (glycoprotein IIIa)
GPIIb (glycoprotein IIb)
Staph A (Staphylococcus aureus Cowan 1)
HEPES (N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid)
EDTA (ethylene dinitrilo-tetraacetic acid)

of an ADP receptor which results in the uncovering of specific fibrinogen binding sites [19]. The effect of ADP on platelets may be mediated by induction of membrane conformational changes [15, 19].

The purpose of the present studies was to investigate the platelet fibrinogen receptor by characterizing the inhibitory effects of three heterologous anti-human platelet membrane antibodies on the fibrinogen-induced aggregation of ADP-stimulated platelets and on the fibrinogen-induced aggregation of chymotrypsin-treated and pronase-treated platelets. Their effect on the binding of ^{125}I-fibrinogen to intact and proteolytically-treated platelets was also studied. We concurrently studied the inhibitory effects of these antibodies on platelet function with their ability to immunoprecipitate a protein with an apparent molecular weight of 66 000 from the platelet surface. We propose that fibrinogen binds to a 66 000 Mr domain of GPIII resulting in platelet aggregation.

Materials and methods

Human fibrinogen was labeled with ^{125}I and characterized as described previously [15, 16].

Human washed platelets. Platelets from blood freshly collected in the anticoagulant acid-citrate dextrose were washed by the method of Mustard et al. [24] and the washed, intact platelets were suspended in Tyrode solution pH 7.4 containing 0.35 per cent albumin and 2 mmol $CaCl_2$. Chymotrypsin-treated or pronase-treated platelets were prepared as described previously [15]. The platelet count, platelet aggregation and ^{125}I-fibrinogen binding to platelets were determined as previously described [15, 16]. The overall scheme of the methodology is shown in Figure 1.

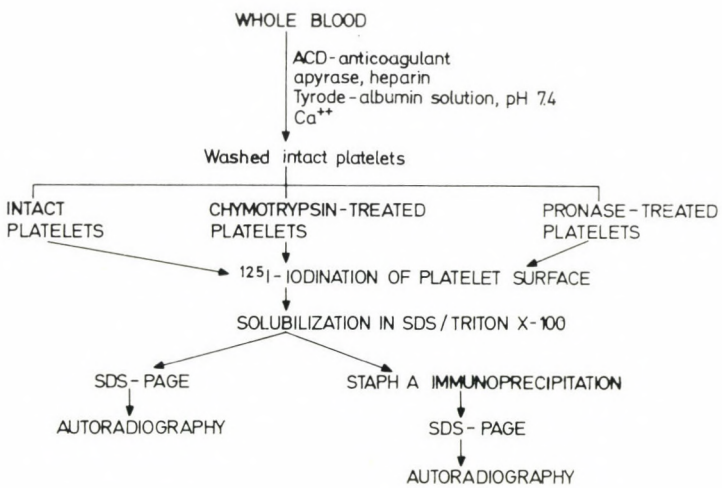


Fig. 1. An overall schema showing the preparation of washed intact platelets, chymotrypsin-treated platelets and pronase-treated platelets

Isolation of platelet membranes. The membranes of washed intact platelets or the membranes of platelets previously treated with chymotrypsin or pronase were isolated by the glycerol lysis technique of Barber and Jamieson [25].

Production of anti-human platelet membrane antisera in rabbits. Isolated platelet membranes were mixed with complete Freund's adjuvant. Antibody production in 3 month old New Zealand rabbits was induced by multiple site injections of 500 μg protein following the procedure of Vaitukaitis et al. [26]. Anti-platelet membrane antiserum to intact, chymotrypsin-treated or to pronase-treated platelet membranes was heated at 56 °C for 30 min to inactivate complement. Pre-immune serum was obtained from rabbits prior to their immunization with platelet antigen. IgG was obtained by initial precipitation with 33 per cent saturation ammonium sulfate followed by two washings with 40 per cent ammonium sulfate. Following dialysis, the samples were chromatographed on a DEAE-cellulose column in 17 mmol phosphate buffer pH 7.0.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli [27] in 5 per cent stacking gels and in 7.5 per cent or 10 per cent polyacrylamide slab gels. The gels were stained for protein with Coomassie Brilliant blue, destained in 10 per cent acetic acid, dried and exposed to Kodak X-Omat R film for approximately 24 hours at -70°C.

^{125}I-iodination of the membrane surface of chymotrypsin-treated platelets was performed by the Iodogen method [28]. A schema describing the ^{125}I-radiolabeling of platelet surface proteins is shown in Figure 2. Iodogen (1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycoluril, Pierce Chem. Co., Rockford, Ill.) was used to iodinate membrane surfaces of intact and chymotrypsin-treated platelets. Na^{125}I was pur-

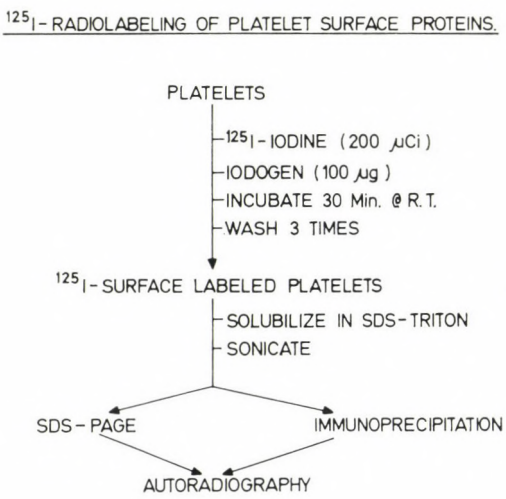


Fig. 2. A schema describing the technique of ^{125}I-iodination of platelet surface proteins of intact, chymotrypsin-treated and pronase-treated platelets

chased from New England Nuclear, Boston, MA. After platelets were incubated with chymotrypsin and washed in the presence of inhibitors [15], 200 μCi of ^{125}I were added to a 1 ml platelet suspension containing 2.5×10^8 platelets/ml. This mixture was then added to a 2 ml glass vial coated with 100 μg of Iodogen and incubated for 30 min at 23 °C. The radiolabeled platelet suspensions were then transferred to septum capped microcentrifuge tubes and washed by centrifugation three times in HEPES buffer containing 0.35 per cent albumin (pH 7.35) to remove unbound iodide. The final platelet pellet was dissolved and sonicated in 100 μl of a solution containing 0.2 per cent SDS, 1 per cent Triton X-100 in 3.8 mmol HEPES, pH 7.35, containing 137 mmol NaCl, 2.7 mM KCl, 1 mmol $MgCl_2$, 0.01 per cent dextrose and 5 mmol DFP (diisopropylfluorophosphate).

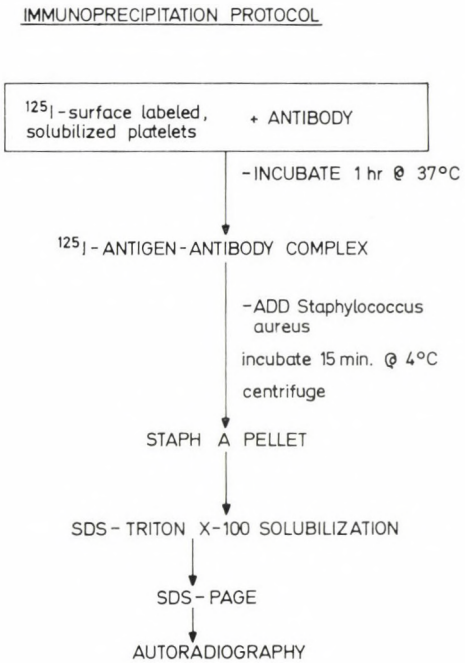


Fig. 3. A schema describing the technique of Staph A immunoprecipitation of ^{125}I-surface labeled platelet proteins by anti-intact, anti-chymotrypsin treated and anti-pronase-treated platelet membrane antibodies

Immunoprecipitation of ^{125}I-surface labeled platelet proteins of chymotrypsin-treated platelets by anti-platelet membrane antibodies. A schema describing the immunoprecipitation protocol is shown in Figure 3. ^{125}I-radiolabeled platelet surface membrane proteins of intact or chymotrypsin-treated platelets were immunoprecipitated basically according to the procedure of Kessler [29]. In brief, 20 μl aliquots of detergent solubilized, ^{125}I-surface labeled chymotrypsin-treated

platelets were incubated with 10 μl of anti-intact, anti-chymotrypsin or anti-pronase-treated platelet membrane anti-serum for 1 h at 37°C. Washed suspensions of Staphylococcus aureus Cowan 1 producing protein A (Staph A) (100 μl of a 10 per cent suspension) were added to these mixtures and after 15 min at 4°C, the antigen–antibody–Staph A complexes were centrifuged and washed three times in a solution containing 0.05 per cent Nonidit P-40 (NP-40) (alkyl phenyl ethoxylate), 5 mmol EDTA and 50 mmol Tris HCl pH 7.4. The pellets containing the Staph A immunoprecipitates were then solubilized by the addition of a solution containing 0.2 per cent SDS and 1 per cent Triton X-100.

Clot retraction. Intact platelets (6×10^8/ml) (700 μl) were incubated for 5 min at 37°C with 200 μl of pre-immune serum or 200 μl of anti-intact, anti-chymotrypsin- or anti-pronase-treated platelet membrane antiserum. Pre-immune or immune IgG (1 mg/ml) was also used. 100 μl or 1 per cent fibrinogen and 100 μl of thrombin (10 units) were then added and the volume of fluid was separated from the clot and that remaining after 45 min was determined.

Results

The inhibitory effects of anti-intact, anti-chymotrypsin and antipronase treated platelet membrane antiserum on platelet aggregation, ^{125}I-fibrinogen binding and clot retraction are shown in Tables 1 and 2. All three antibodies blocked the ^{125}I-fibrinogen binding and the ADP-induced aggregation of intact platelets (Table 1). These three antiplatelet membrane antibodies were also able to block the ^{125}I-fibrinogen binding and the fibrinogen-induced aggregation of chymotrypsin and pronase-treated platelets (Tables 1 and 2). In addition, these three antibodies inhibited the platelet dependent retraction of fibrin clots formed by thrombin (Table 2). Pre-immune sera had no effect on platelet aggregation, ^{125}I-fibrinogen binding, or clot retraction. Our data indicate that the platelet fibrinogen receptor which becomes exposed on the surface of ADP-stimulated intact platelets, chymotrypsin-treated platelets and pronase-treated platelets interacts with all three antibodies since all three of the antibodies could specifically block the interaction of fibrinogen in each type of platelet studied.

In order to determine which of the platelet surface components interacted with these antibodies, the plasma membranes of chymotrypsin-treated platelets were first ^{125}I-iodinated. The total surface ^{125}I-iodination pattern of chymotrypsin-treated platelets is shown in Figure 4E. The plasma membrane of chymotrypsin-treated platelets was found to be deficient in GPIIb as indicated by the slight radiolabeling of the protein band migrating with an apparent molecular weight of 130 000. We did observe radiolabeling of the major glycoprotein, GPIII (96 000 Mr) however it contained much less ^{125}I-radiolabel as compared to the GPIII from intact ^{125}I-iodinated platelets. Most interestingly, we found that a protein of 66 000 Mr was radiolabeled on the surface of chymotrypsin-treated platelets (as indicated by the arrow); this 66 000 Mr protein was not observed to be present on the surface

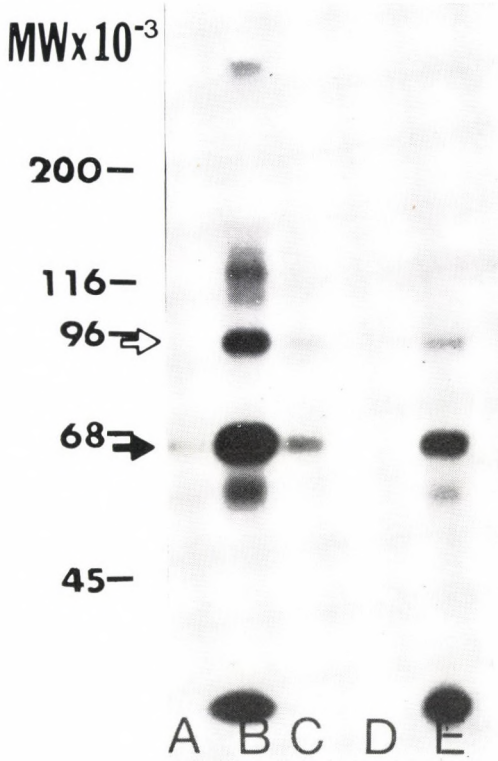


Fig. 4. Autoradiogram of SDS-polyacrylamide gels of surface ^{125}I-radiolabeled chymotrypsin-treated platelets and the immunoprecipitates formed with the use of anti-intact, anti-chymotrypsin and anti-pronase treated platelet membrane antibodies. The surface of chymotrypsin-treated platelets (1000 μg chymotrypsin/10^9 platelets/ml) was radiolabeled with ^{125}I by the Iodogen method. The surface radiolabeling pattern of these platelets is shown in Lane E. The ^{125}I-iodinated platelets were solubilized in 0.2 per cent SDS, 1 per cent Triton X-100 as described in the Methods section. Approximately 1×10^6 cpm each of solubilized ^{125}I-surface-labeled chymotrypsin-treated platelets were immunoprecipitated using the Staph A procedure described in the Methods section with preimmune serum (Lane D) or with anti-intact platelet membrane antiserum (Lane B), anti-chymotrypsin-treated platelet membrane antiserum (Lane C) or anti-pronase treated platelet membrane antiserum (Lane A). These Staph A immunoprecipitates were then solubilized and analyzed on 5 per cent stacking gels, and 7.5 per cent separation gels. The gels were fixed, stained, dried and subjected to autoradiography using intensifying screens. *Lane A*, anti-pronase-treated platelet membrane antibody immunoprecipitate of radiolabeled chymotrypsin-treated platelets; *Lane B*, anti-intact platelet membrane antibody immunoprecipitate of radiolabeled chymotrypsin-treated platelets; *Lane C*, anti-chymotrypsin-treated platelet membrane antibody immunoprecipitate of radiolabeled chymotrypsin-treated platelets; *Lane D*, preimmune serum immunoprecipitate of radiolabeled chymotrypsin-treated platelets; *Lane E*, surface pattern of radiolabeled chymotrypsin-treated platelets, before immunoprecipitation. Molecular weight standards are shown on the left. Molecular weight determinations were made by comparison to Bio Rad's reduced samples of myosin (200 000), phosphorylase b (93 000), bovine serum albumin (69 000), ovalbumin (43 000), carbonic anhydrase (30 000) and soybean trypsin inhibitor (21 000). The open arrow points to GPIII (96 000 Mr) and the dark arrow points to a protein of 66 000 Mr (under reducing conditions) and 60 000 Mr (under nonreducing conditions)

Table 1

Effect of anti-platelet membrane antibodies on ^{125}I-fibrinogen binding and fibrinogen-induced platelet aggregation of intact and chymotrypsin-treated platelets

Addition[b]		Intact platelets		Chymotrypsin-treated platelets	
		% ^{125}I-Fibrinogen bound	% platelet aggregation	% ^{125}I-Fibrinogen bound	% platelet aggregation
Pre-immune serum	(100 μl)	100%[a]	100%	100%	100%
Anti-intact platelet membrane antiserum	(100 μl)	0%	0%	0%	0%
Anti-chymotrypsin-treated platelet membrane antiserum	(100 μl)	10%	7%	8%	0%
Anti-pronase-treated platelet membrane antiserum	(100 μl)	7%	0%	10%	0%

[a] All values for ^{125}I-fibrinogen binding and platelet aggregation which were obtained in the presence of pre-immune serum or pre-immune IgG were set at 100 per cent. ^{125}I-fibrinogen (100 μg/ml final concentration) and ADP (100 μM final concentration) were used to determine the per cent of ^{125}I-fibrinogen specifically bound to intact platelets. The same concentrations of fibrinogen and ADP were used to initiate aggregations of intact platelets. ^{125}I-fibrinogen (100μg/ml final concentration) alone was used to determine the per-cent of ^{125}I-fibrinogen bound to chymotrypsin-treated platelets. The same concentration of fibrinogen was used to initiate the aggregations of chymotrypsin-treated platelets. Nonspecific ^{125}I-fibrinogen binding (subtracted from total ^{125}I-fibrinogen bound) was perfomed in the presence of 10 mmol EDTA and it was not affected by incubating platelets with the antibodies listed above.

[b] Preimmune IgG or immune IgGs of each of the antisera described above (1 mg/ml final concentration) could also be used in place of the serum or antiserum tested.

of intact platelets. Next, the chymotrypsin-treated platelets were solubilized in a 0.2 per cent SDS, 1 per cent Triton buffer solution (as described in the Methods section) and aliquots of these detergent extracts were immunoprecipitated with each of the three antibodies. The immunoprecipitation patterns are shown in Figure 4 (A–D). Anti-intact platelet membrane antisera immunoprecipitated GPIIb (130 000 MW), GPIII (96 000 MW) and a major band of 66 000 molecular weight (Fig. 4B). A radiolabeled band of approximately 50 000 Mr and radiolabeled material on the top and bottom of the gel were also observed. Anti-chymotrypsin-treated platelet membrane antisera (Fig. 4C) immunoprecipitated mostly GPIII (96 000 MW) and the 66 000 MW component. Anti-pronase-treated platelet membrane antisera (Fig. 4A) immunoprecipitated mainly the 66 000 Mr protein, although this antiserum also immunoprecipitated GPIIb/GPIII when the concentration of detergent extracts was increased. Therefore it appears that the 66 000 Mr protein found on the surface of chymotrypsin-treated platelets but not found on the surface of intact (unstimulated) platelets can be immunoprecipitated by all three of the inhibitory antibodies.

Table 2

Effect of anti-platelet membrane antibodies on ^{125}I-fibrinogen binding and fibrinogen-induced platelet of aggregation of pronase-treated platelets and on clot retraction induced by intact platelets

Addition[b]		Pronase-treated platelets[a]		Clot retraction[c]
		% ^{125}I-fibrinogen bound[a]	% platelet aggregation	
Pre-immune serum	(100 μl)	100%	100%	100%
Anti-intact platelet membrane antiserum	(100 μl)	0%	0%	0%
Anti-chymotrypsin-treated platelet membrane antiserum	(100 μl)	0%	0%	25%
Anti-pronase-treated platelet membrane antiserum	(100 μl)	0%	0%	0%

[a] ^{125}I-fibrinogen binding and fibrinogen-induced platelet aggregation were performed as described in the legend in Table I. Control, pre-immune values were set at 100 per cent.

[b] Preimmune IgG or immune IgG fractions (1 mg/ml final concentration) produced the same effects as those shown above.

[c] Clot retraction was performed using washed, intact platelets in the presence of thrombin, fibrinogen and aliquots of the antibodies listed above as described in the Methods section.

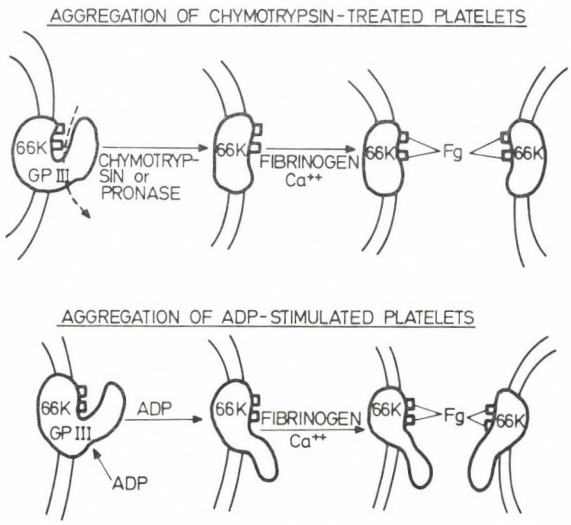


Fig. 5. A hypothetical model describing the mechanisms of the exposure of the 66 000 Mr protein on chymotrypsin-treated platelets (top panel) and on ADP-stimulated intact platelets (bottom panel) and the role of the 66 000 Mr protein in platelet aggregation

Discussion

The data described above clearly demonstrate that three types of heterologous anti-human platelet membrane antibodies block platelet aggregation, ^{125}I-fibrinogen binding and clot retraction (Tables 1 and 2). The three antibodies were able to inhibit the fibrinogen-induced aggregation of ADP-stimulated platelets as well as the fibrinogen-induced aggregations of chymotrypsin-treated or pronase-treated platelets. Binding of ^{125}I-fibrinogen to intact, chymotrypsin-treated and pronase-treated platelets was equally blocked. Therefore, it appears that there was a direct interaction between the heterologous antisera and the platelet membrane fibrinogen receptors. In addition, platelet membrane proteins appear to be involved in clot retraction since these anti-platelet membrane antibodies could interfere with clot retraction.

In an attempt to identify platelet surface components which directly interacted with these three types of anti-platelet membrane antibodies, we conducted experiments which involved the radiolabeling of the membrane components of chymotrypsin-treated platelets followed by immunoprecipitation of these components by these antibodies. Experimental conditions were selected as to give a comparison between these antibodies. Our studies demonstrated that all three anti-platelet membrane antibodies immunoprecipitated a 66 000 MW protein from detergent solubilized, ^{125}I-surface radiolabeled, chymotrypsin-treated platelets. The major glycoproteins GPIIb and GPIII were also immunoprecipitated with anti-intact platelet membrane antisera (Fig. 4B). However, anti-chymotrypsin-treated platelet membrane antisera (Fig. 4C) or anti-pronase-treated platelet membrane antisera (Fig. 4A) were more selective in that they mostly immunoprecipitated a 66 000 MW protein from the surface of ^{125}I-surface labeled, chymotrypsin-treated platelets. The immunoprecipitation of the 66 000 MW protein appears to correlate with the ability of these three anti-platelet membrane antibodies to inhibit ^{125}I-fibrinogen binding and fibrinogen-induced platelet aggregation. We have data which show that the 66 000 Mr protein is derived from GPIII and that this 66 000 Mr protein becomes exposed on the platelet surface following the treatment of platelets with chymotrypsin or pronase. (Kornecki, E., Tuszynski, G. P., and Niewiarowski, S., *J. Biol. Chem.*, *258*, 9349, 1983).

It is well established that the two major platelet membrane glycoproteins, GPIIb and GPIIIa are absent or greatly reduced in the bleeding disorder, Glanzmann's thrombasthenia [30, 31]. It has been proposed that the inability of these thrombasthenic platelets to aggregate upon their exposure to ADP or other aggregating agents may depend on the absence of fibrinogen receptors on thrombasthenic platelets since ^{125}I-fibrinogen does not bind to ADP-stimulated, thrombasthenic platelets [5, 13, 32, 33]. In addition, it is well known that thrombasthenic platelets do not retract fibrin clots. Since these three anti-platelet membrane antibodies induced a thrombasthenia-like effect on platelets, we may conclude that GPIIb, GPIII and the 66 000 Mr protein may play important roles not only in ^{125}I-fibrinogen binding to platelets and fibrinogen-induced platelet aggregation but also in clot retraction.

A hypothetical model describing the mechanism of exposure of the fibrinogen receptor is shown in Figure 5. We believe that the 66 000 MW protein (66K) is part of the GPIII complex which is found on the surface of the platelet plasma membrane. In the presence of chymotrypsin or pronase (Fig. 5, top panel), part of the GPIII molecule is removed resulting in the exposure of the 66 000 Mr fibrinogen binding site. In the presence of calcium, fibrinogen would bind to the 66 000 Mr domain of adjacent platelets resulting in fibrinogen bridging these platelets together. Under 'normal' conditions, i.e. during ADP-induced platelet aggregation (Fig. 5, lower panel), the addition of ADP to intact platelets would result in a conformational change in the GPIII molecule also leading to the exposure of the 66 000 Mr domain. The exposure of this site (in the presence of fibrinogen and calcium) would then result in fibrinogen-induced platelet aggregation.

The authors wish to thank Dr. Linda Knight, Seta Srivastava and Monica Kollman for their invaluable help. We would also like to thank Sheila J. El for preparation of this manuscript.

References

1. Cross, M. J.: Effect of fibrinogen on the aggregation of platelets by adenosine phosphate. *Thromb. Diath. Haemorrh. (Stuttg.) 12*, 524 (1964).
2. McLean, J. R., Maxwell, R. E., Hartler, D.: Fibrinogen and adenosine diphosphate-induced aggregation of platelets. *Nature 202*, 605 (1964).
3. Tangen, P., Berman, H. J., Marphy, P.: Gel filtration. A new technique for separation of blood platelets from plasma. *Thromb. Diath. Haemorrh. (Stuttg.) 25*, 268 (1971).
4. Niewiarowski, S., Regoeczi, E., Mustard, J. F.: Platelet interaction with fibrinogen and fibrin. Comparison of the interaction of platelets with that of fibroblasts, leukocytes, and erythrocytes. *Ann. NY Acad. Sci. 201*, 78 (1972).
5. Bennett, J. S., Vilaire, G. J.: Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J. Clin. Invest. 64*, 1393 (1979).
6. Plow, E. F., Marguerie, G. A.: Induction of the fibrinogen receptor on human platelets by epinephrine and the combination of epinephrine and ADP. *J. Biol. Chem. 255*, 10971 (1980).
7. Tollefson, D. M., Majerus, P. W.: Inhibition of human platelet aggregation by nonvalent antifibrinogen antibody fragments. *J. Clin. Invest. 55*, 1259 (1972).
8. Hawiger, J., Parkinson, S., Timmons, S.: Prostacyclin inhibits mobilisation of fibrinogen-binding sites on human ADP- and thrombin-treated platelets. *Nature 283*, 195 (1980).
9. Morinelli, T. A., Niewiarowski, S., Kornecki, E., Figures, W. R., Colman, R. W., Smith, J. B.: Platelet aggregation and exposure of fibrinogen receptors by prostaglandin endoperoxide analogues. *Blood, 61*, 49 (1983).
10. Bennett, J. S., Vilaire, G., Burch, J. W.: A role for prostaglandins and thromboxanes in the exposure of platelet fibrinogen receptors. *J. Clin. Invest. 68*, 981 (1981).
11. Mustard, J. F., Packham, M. A., Kinlough-Rathbone, R. L., Perry, D. W., Regoeczi, E.: Fibrinogen and ADP-induced platelet aggregation. *Blood 52*, 453 (1978).
12. Marguerie, G. A., Plow, E. F., Edgington, T. S.: Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J. Biol. Chem. 254*, 5357 (1979).
13. Peerschke, E. I., Zucker, M. G., Grant, R. A., Egan, J. J., Johson, M. M.: Correlation between fibrinogen binding to human platelets and platelet aggregability. *Blood 55*, 841 (1980).

14. Greenberg, J. P., Orr, J. L., Packham, M. A., Guccione, M. A., Harfenist, E. J., Kinlough-Rathbone, R. L., Perry, D. W., Mustard, J. F.: The effect of pretreatment of human or rabbit platelets with chymotrypsin on their responses to human fibrinogen and aggregating agents. *Blood 54*, 753 (1979).
15. Niewiarowski, S., Budzynski, A. Z., Morinelli, T. A., Budzynski, T. M., Stewart, G. J.: Exposure of fibrinogen receptor on human platelets by proteolytic enzymes. *J. Biol. Chem. 256*, 917 (1981).
16. Kornecki, E., Niewiarowski, S., Morinelli, T. A., Kloczewiak, M.: Effects of chymotrypsin and adenosine diphosphate on the exposure of fibrinogen receptors on normal human and Glanzmann's thrombasthenic platelets. *J. Biol. Chem. 256*, 5696 (1981).
17. Kornecki, E., Niewiarowski, S.: Requirement of fibrinogen and calcium for inter-platelet bridges in platelet aggregation: a hypothesis. *Thrombos. Haemost. 46(1)*, 951 (1981).
18. Marguerie, G. A., Edgington, T. S., Plow, E. F.: Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. *J. Biol. Chem. 255*, 154 (1980).
19. Figures, W. R., Niewiarowski, S., Morinelli, T. A., Colman, R. F., Colman, R. W.: Affinity labeling of a human platelet membrane-protein with 5'-p-fluorosulfonylbenzoyl adenosine. *J. Biol. Chem. 256*, 7789 (1981).
20. Kornecki, E., Feinberg, H.: Mechanism of inhibition of thrombin-induced platelet aggregation by pyrridoxal phosphate. *Biochem. Biophys. Res. Commun. 90*, 963 (1979).
21. Kornecki, E., Feinberg, H.: Pyrridoxal phosphate inhibition of platelet function. *Amer. J. Physiol. 238*, 54)1979).
22. Lam, S. C. T., Harfenist, E. J., Packham, M. A., Mustard, J. R.: Investigation of possible mechanisms of pyrridoxal 5'-phosphate inhibition of platelet reactions. *Thromb. Res. 20*, 633 (1980).
23. Winocour, P. D., Kinlough-Rathbone, R. L., Mustard, J. F.: The effect of the phospholipase inhibitor mepacrine on platelet aggregation, the platelet release reaction and fibrinogen binding to the platelet surface. *Thromb. Haemost. 45*, 257 (1981).
24. Mustard, J. F., Perry, D. W., Ardlie, N. G., Packham, M. A.: Preparation of suspensions of washed platelets from humans. *Brit. J. Haemat. 22*, 103 (1972).
25. Barber, A. J., Jamieson, G. A.: Isolation and characterization of plasma membranes of human blood platelets. *J. Biol. Chem. 245*, 6357 (1970).
26. Vaitukaitis, J., Robbins, J. B., Nieschlag, E., and Ross, G. T.: A method for producing specific antisera with small doses of immunogen. *J. Clin. Endocr. 33*, 988 (1971).
27. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680 (1970).
28. Tuszynski, G. P., Knight, L., Kornecki, E. and Srivastava, S.: Labeling of platelet surface proteins with [^{125}]-iodine by the Iodogen method. *Anal. Biochem. 130*, 166 (1983).
29. Kessler, S. W.: Rapid isolation of antigens from cells with a Staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with Protein A. *J. Immun. 115*, 1617 (1975).
30. Phillips, D. R., Agin, P. P.: Platelet membrane depects in Glanzmann's thrombasthenia. *J. Clin. Invest. 60*, 535 (1977).
31. Nurden, A. T., Caen, J. P.: The different glycoprotein abnormalities in thrombasthenic and Bernard-Soulier platelets. *Semin. Hemat. 16*, 234 (1979).
32. Mustard, J. F., Kinlough-Rathbone, R. L., Packham, M. A., Perry, D. W., Harfenist, E. J., Pai, K. R. M.: Comparison of fibrinogen association with normal and thrombasthenic platelets on exposure to ADP or chymotrypsin. *Blood 54*, 987 (1979).
33. Lee, H., Nurden, A. T., Thomaidis, A., Caen, J. P.: Relationship between fibrinogen binding and the platelet glycoprotein deficiencies in Glanzmann's thrombasthenia type I and type II. *Brit. J. Haemat. 48*, 47 (1981).

Haematologia 17 (3), pp. 399–403 (1984)

Glucose-6-Phosphate Dehydrogenase Deficiency and Rh Factor

M. TZONEVA, M. MAVRUDIEVA, D. TONCHEVA, S. LALCHEV

Department of Medical Genetics, Medical Academy,
8, Belo more St., 1040 Sofia, Bulgaria

(Received June 30, 1982; accepted October 28, 1982)

The incidence of carriers of the Gd(−) gene and the Rh(+) and Rh(−) alleles was studied in 10 342 Bulgarian individuals living in 306 villages in 5 districts at different altitudes above the sea level.

The results showed that the presence of the Gd(−) gene is considerably less frequent in Rh(−) than in Rh(+) individuals. In populations with a high frequency of G6PD deficiency the frequency of the Rh(−) allele is low.

Keywords: Glucose-6-phosphate dehydrogenase deficiency, natural selection, population genetics, Rh factor.

Introduction

The Rh factor is immunogenetic, creating conditions that give rise to an immunological conflict between mother and offspring. As a result, haemolytic disease develops in the newborn. Glucose-6-phosphate dehydrogenase (G6PD) deficiency brings about in some of its carriers neonatal hyperbilirubinaemia by another mechanism [1].

Studies on the effects of Rh(−) factor and G6PD deficiency in human populations show contrasting results. Bernini et al. (cit. Tarlov et al. [2]) observed that the distribution of the Rh(−) blood group antigen was significantly lower in the coastal areas of Sardinia with a high frequency of the Gd(−) mutant gene in comparison to the mountain areas where G6PD deficiency has a lower frequency. Sonnet and Michaux [3] observed only a 3.3 per cent frequency in Rh(−) subjects among the Bantu tribesmen of Congo where the incidence of hemizygous carriers of the Gd(−) gene is 21 per cent.

Tarlov et al. [2] assumed that the Rh(−)/Gd(−) combination is rare as it gives rise to an increase in mortality from Rh(−) incompatibility erythroblastosis in the neonatal period. Their investigation of 89 hemizygous carriers of the Gd(−) gene and 184 American Negro males with normal G6PD activity suggest an almost identical incidence of Rh(−) in both groups. The number of subjects was, however, too small for this type of study.

The effects of the Rh(−) antigen and Gd(−) gene are of considerable interest

in Bulgaria because of the 4.15 ± 0.17 per cent incidence of G6PD deficiency and the 0.0386 gene frequency [4–6]. The results of such investigations would allow conclusions as to the manifestations of natural selection in the population.

Materials and Methods

The incidence of carriers of the Gd(−) gene and the Rh(+) and Rh(−) alleles was studied in 10 342 subjects from 306 villages of 5 districts of Bulgaria. The villages are at different altitudes above sea level and with respect to the distribution of malaria in the past are: past endemic, mesoendemic and nonendemic districts.

Erythrocyte G6PD activity was determined by the method of Brewer et al. and Tönz and Rossi [7, 8] and the method of fluorescent spots of Beutler and Mitchell [9]. Rh(+) and Rh(−) antigens were studied by conventional methods. Statistical analysis was done using the χ^2 test. The data were analysed along the following lines.

1. Frequency of the Gd(−) gene in Rh(−) and Rh(+) individuals.
2. Frequency of the Gd(−) gene and the Rh(−) allele in the different districts of Bulgaria.
3. Distribution of the Gd(−) gene and Rh(−) allele in the subpopulations of villages at different altitudes above sea level: zone I, 0–199 m; zone II, 200–999 m; and zone III, over 1000 m. They corresponded to the endemic, mesoendemic and non-endemic areas of malaria in the past.

Results

Results are shown in Tables 1 and 2, and Figure 1.

Among the individuals studied, 3.34 per cent were carriers of G6PD deficiency and the frequency of the Rh(−) antigen was 10.73 per cent (Table 1).

Table 1

Distribution of glucose-6-phosphate dehydrogenase deficiency and Rh-allele in five districts of Bulgaria

District	Total No. of individuals tested for G6PD deficiency and Rh⁻ allele	Individuals with G6PD deficiency		Rh⁻ allele	
		No.	%	No.	%
I	1 770	44	2.49	220	12.42
II	1 469	39	2.66	192	13.07
III	3 267	113	3.46	389	11.91
IV	2 475	87	3.52	235	9.49
V	1 361	62	4.56	74	5.44
Total	10 342	345	3.34	1110	10.73

The Gd(−) gene was found in 2 per cent of Rh(−) individuals and in 3.5 per cent of the Rh(+) individuals (Table 2); the difference was significant ($P < 0.001$). There were less subjects with Rh(−)/(Gd(−) than with Rh(+)/Gd(−) in the general population.

Table 2

Distribution of Gd^- gene in individuals with Rh^- and Rh^+ alleles

Total No. of individuals with Rh^- allele	Individuals with Rh^- and Gd^- alleles		Total No. of individuals with Rh^+ allele	Individuals with Rh^+ and Gd^- alleles	
	No.	%		No.	%
1110	22	2.0	9232	323	3.5

Relative risk of Gd^-/Rh^- 0.58
Correlation coefficient 0.026

The same regularity was observed in the districts where a high frequency of the Gd(−) gene combined with a low frequency of Rh(−). In district I with the lowest rate of G6PD deficiency (2.49 per cent), the frequency of Rh(−) was 12.42 per cent, while in the district with the highest rate, 4.56 per cent of G6PD deficiency, the frequency of Rh(−) individuals was lowest, 5.44 per cent (Table 1). In the remaining three districts the relations were similar. These data indicate that a higher incidence of the Gd(−) gene in the population correlated with a lower frequency of the Rh(−) allele. The relative risk of Gd(−)/Rh(−) was 0.58 (correlation coefficient, 0.026).

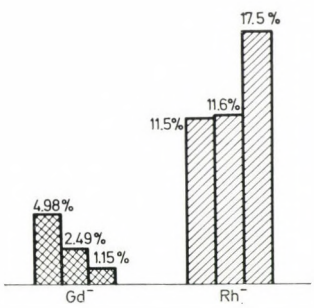


Fig. 1. Distribution of Gd^- gene and Rh^- allele in relation to altitude

The same distribution of the Gd(−) gene and the Rh(−) allele was observed in the subpopulations living at the three altitudes. The higher they live the less is the number of carriers of the Gd(−) gene. In zone I the frequency was 4.98 per cent; in zone II, 2.49 per cent; and in zone III, 1.15 per cent (Fig. 1). This is in accordance with the malaria hypothesis for the distribution of the G6PD deficiency

[10]. Distribution of the Rh(+) and Rh(−) alleles in the same zones illustrates characteristic features: a high frequency of Rh(+) individuals in zone I and a low frequency in zone III, and a low frequency of Rh(−) individuals in zone I and a high frequency in zone III (Fig. 1). The high frequency of the Gd(−) gene in the population is concomitant with a low frequency of the Rh(−) allele.

Discussion

The above results may be interpreted as a manifestation of natural selection in the human population. Natural selection maintains mutant forms of G6PD, but eliminates some of the Rh(−) alleles. G6PD deficiency in the population with a high frequency of the mutant gene is an adaptation of the individual to a strong selective factor such as had been malaria in the past, whereas the distribution of the Rh(−) allele is independent of similar conditions. A decrease in the frequency of the Rh(−) allele was observed especially in the Gd(−) individuals, and similarly in the general population. These facts observed with respect to the correlated dependencies between the Gd(−) gene and the Rh(−) allele have uncovered an interesting area in the study of the thus far little known intergene dependencies and interactions in humans.

Conclusions

The presence of the Gd(−) gene was found to be considerably less frequent in Rh(−) than in Rh(+) individuals. In populations displaying a high frequency of G6PD deficiency a decrease in the frequency of the Rh(−) was observed. These inverse distributions of the Gd(−) gene and the Rh(−) allele are probably due to natural selection in humans.

References

1. Doxiadis, S., Fessas, Ph., Valaes, T.: Glucose-6-phosphate dehydrogenase deficiency. A new aetiological factor of severe neonatal jaundice. *Lancet 1*, 297 (1961).
2. Tarlov, A., Brewer, G., Carson, P., Alving, A.: Primaquine sensitivity. *Arch. Intern. Med. 109*, 209 (1962).
3. Sonnet, J., Michaux, J.: Glucose-6-phosphate dehydrogenase deficiency, haptoglobin groups, blood groups and sickle-cell trait in the Bantus of West Belgian Congo. *Nature 188*, 504 (1960).
4. Tzoneva, M., Mavrudieva, M., Toncheva, D., Lalchev, S., Krasnopolskaya, K., Shatskaya, T.: Incidence and polymorphism of glucose-6-phosphate dehydrogenase deficiency in some Bulgarian districts. *Savrem. Med. 29*, 10–11, 12 (1978). (In Bulgarian.)
5. Tzoneva, M., Mavrudieva, M., Toncheva, D., Novachev, D., Atanasov, D., Petkova, E., Tersijska, L., Sirmina, V.: Incidence of glucose-6-phosphate dehydrogenase deficiency among the newborns from Sofia and Blagoevgrad district, *Pediatria 1*, 54 (1979). (In Bulgarian.)

6. Tzoneva, M., Mavrudieva, M., Proijnova, N., Rachkov, R., Tomaleva, Z., Novachev, D.: Neonatal hyperbilirubinemia and glucose-6-phosphate dehydrogenase deficiency. *Pediatria 4*, 279 (1975). (In Bulgarian.)
7. Brewer, G., Tarlov, A., Alving, A.: Methaemoglobin reduction test. A new simple in vitro test for identifying primaquine sensitivity. *Bull. Wld Hlth Org. 22*, 683 (1960).
8. Tönz, O., Rossi, E.: Morphological demonstration of two red cell population in human females heterozygous for G-6-PD deficiency. *Nature 202*, 606 (1964).
9. Beutler, E., Mitchell, M.: Special modifications of the fluorescent screening method for glucose-6-phosphate dehydrogenase deficiency. *Blood 32*, 816 (1968).
10. Motulsky, A., Vandepitte, J., Fraser, A.: Population genetic studies in the Congo. I. Glucose-6-phosphate dehydrogenase deficiency, hemoglobin S and malaria. *Amer. J. Hum. Genet. 18*, 514 (1966).

ERRATUM

Figure 4 in the article by M. J. Pippard and D. J. Weatherall, entitled "Iron absorption in iron-loading anaemias" (*Haematologia 17*, 1, 1984) has been erroneously printed. We are pleased to re-publish the whole article in this issue of our Journal.

The Editors

Haematologia 17 (3), pp. 407–414 (1984)

Iron Absorption in Non-Transfused Iron Loading Anaemias: Prediction of Risk for Iron Loading, and Response to Iron Chelation Treatment, in β Thalassaemia Intermedia and Congenital Sideroblastic Anaemias

M. J. PIPPARD, D. J. WEATHERALL

Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford, OX3 9DU United Kingdom

(Received 15 February 1983; accepted 15 March 1983)

A variable rate of iron loading, reaching toxic levels in some patients, was seen in a series of non-transfused patients with β thalassaemia intermedia or sideroblastic anaemia. The degree of anaemia was a poor guide to the risk of iron overload. However the extent of erythroid hyperplasia, judged by ferrokinetic studies or more simply by bone marrow aspiration, was useful in predicting both the rate of iron loading and the need for iron chelation therapy.

Keywords: desferrioxamine, ferrokinetics, iron absorption, sideroblastic anaemia, thalassaemia intermedia.

Introduction

In patients with congenital anaemias requiring regular blood transfusions iron overload eventually damages the heart, liver and endocrine organs. This problem is seen most commonly in the major thalassaemia syndromes where, in the absence of measures to remove excess iron, cardiac iron damage can be expected to prove fatal by early adult life [1, 2]. Increased food iron absorption may also contribute to iron overload in these disorders, but is kept to a minimum by transfusion regimens that maintain a high circulating haemoglobin level [3]. Iron loading from the parenteral administration of blood therefore occurs at a predictable rate. By contrast, patients with the clinical picture of thalassaemia intermedia, who maintain a haemoglobin concentration above 5–7 g/dl without regular blood transfusions, show a variable degree of excessive food iron absorption [4] which may lead to serious iron loading by middle life [1, 5, 6]. These patients, in whom the genetic basis of the disease is heterogeneous [1] form a major public health problem in populations in which thalassaemia is common. A similar picture is encountered in other congenital anaemias characterised by ineffective erythropoiesis and massive erythroid expansion, for example the sideroblastic disorders [7, 8]. Because the increase in iron absorption is so variable and the mechanism by which this is maintained in the face of increased body iron stores is poorly understood, it has been difficult to predict the risk of serious iron-loading in individual non-transfused patients. This study examines the factors influencing

the risk in a series of non-transfused patients with either β thalassaemia intermedia or congenital sideroblastic anaemia. In addition, the potential role of iron chelating agents in reducing iron absorption and/or removing established iron overload is discussed.

Patients and Methods

Patients

Seven patients had sideroblastic anaemia with haemoglobin levels ranging from 5.6 to 12.8 g/dl. Five cases were clearly familial and in two, disease had been present at least since childhood. No patient had received blood transfusions. Further details of these patients are to be published elsewhere [9]. Of 25 patients with β thalassaemia intermedia, 17 had received no, or only very occasional, transfusions in the past. In this group, haemoglobin levels ranged from 5.1 to 11.5 g/dl and the haematological features were characteristic of homozygous β thalassaemia.

Methods

Haemoglobin concentrations and red cell indices were measured using a Coulter S counter. Standard methods were used to determine the plasma iron and iron binding capacity [10, 11]. Plasma ferritin was determined by immunoradiometric assay, by courtesy of Dr. Mark Worwood, University Hospital of Wales. Radioiron absorption was measured as the total body retention at 14 days of a test dose of 5 mg of oral iron ($^{59}FeSO_4$ with 50 mg ascorbate) [12]. Plasma iron turnover (PIT) and erythroid iron turnover (EIT) were determined after i.v. injection of 2 μCi of ^{59}Fe in 5 ml plasma [13, 14]. Since many of the patients had a saturation of the plasma iron binding capacity that was greater than 80 per cent, plasma from a normal human donor was labelled *in vitro* using ^{59}Fe citrate (The Radiochemical Centre, Amersham). The ^{59}Fe dose was diluted to 1 ml with pH 2 saline and added to the plasma over 5 minutes with constant mixing. The plasma was then incubated at 37 °C for 30 minutes, and brought to 90 per cent iron saturation with ferrous ammonium sulphate before injection. Bone marrow erythroid/myeloid (E:M) ratios were obtained from a 1000 cell count on bone marrow aspirate smears. Erythroid expansion was calculated from ferrokinetic or morphological data as a multiple of normal erythroid activity, the latter being taken as an EIT of 100 μmol/litre blood/24 hours or an E:M ratio of 0.3.

Results

Absorption of radioiron

The mean value for the retention of the test dose of ^{59}Fe was 55 per cent (range 20 to 89 per cent) in 20 patients with β thalassaemia intermedia, and 31 per cent (range 5 to 71 per cent) in 7 patients with congenital sideroblastic anaemias (Fig. 1). Despite the increased iron stores present in many of these

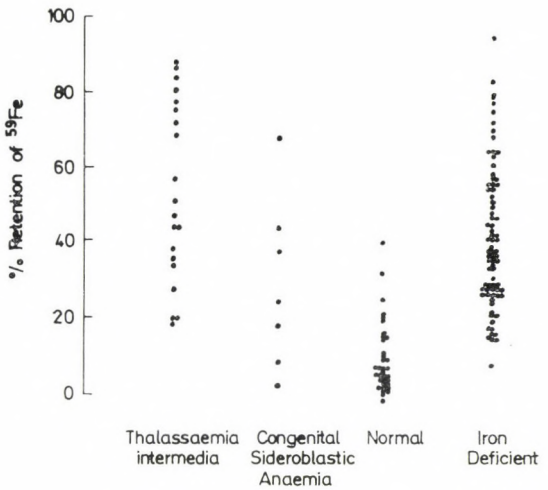


Fig. 1. Iron absorption in patients receiving a test dose of 5 mg oral iron ($^{59}FeSO_4$)

patients (see Fig. 2), iron absorption was closer to that of an iron deficient group (mean 45.5 per cent, n = 90) than that of iron replete normal controls (mean 14.7 per cent, n = 35).

Rate of iron loading

The plasma ferritin level was directly related to liver iron concentration in 10 patients in whom liver biopsies were obtained (data not shown). The amount of iron overload increased with age in both β thalassaemia intermedia and sideroblastic anaemia (Fig. 2). However, the rate of iron loading varied widely between patients and additional risk factors for iron loading are clearly involved.

Effects of anaemia and erythroid expansion

The degree of anaemia and the extent of erythroid expansion were poorly correlated with each other (Fig. 3) and their effects were therefore examined separately. The haemoglobin deficit (taken as 14 g/dl minus the patient's circulating haemoglobin level) or the eryhtroid expansion (expressed as a multiple of

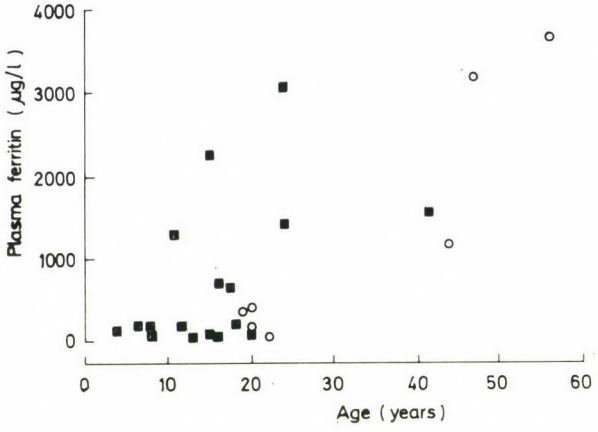


Fig. 2. Plasma ferritin levels as a function of patient age in β thalassaemia intermedia (■) or sideroblastic anaemia (○)

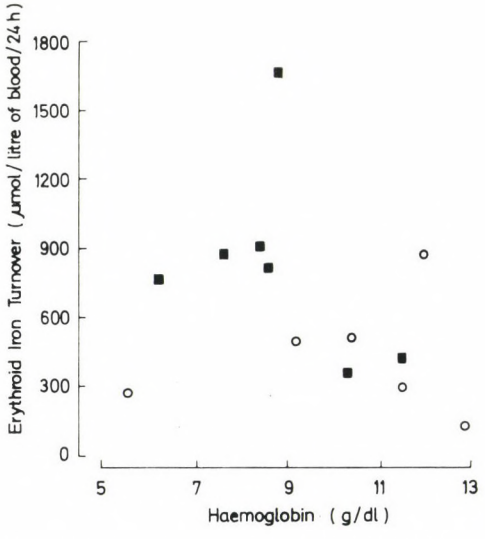


Fig. 3. Relationship between haemoglobin level and erythroid iron turnover in patients with β thalassaemia intermedia (■) or sideroblastic anaemia (○)

normal erythroid activity) was multiplied by the patient's age (representing the duration of exposure to each potential risk factor). In the 14 patients in whom a comparison of these two measurements could be made the product of haemoglobin deficit and age showed a poor correlation with the level of iron loading (Fig. 4a), whereas the product of erythroid expansion and age was a much better guide (Fig. 4b). In addition, in those patients in whom erythroid expansion was assessed by both ferrokinetic and morphological means, a good correlation between the two measurements was obtained (Fig. 4b). This suggests that assess-

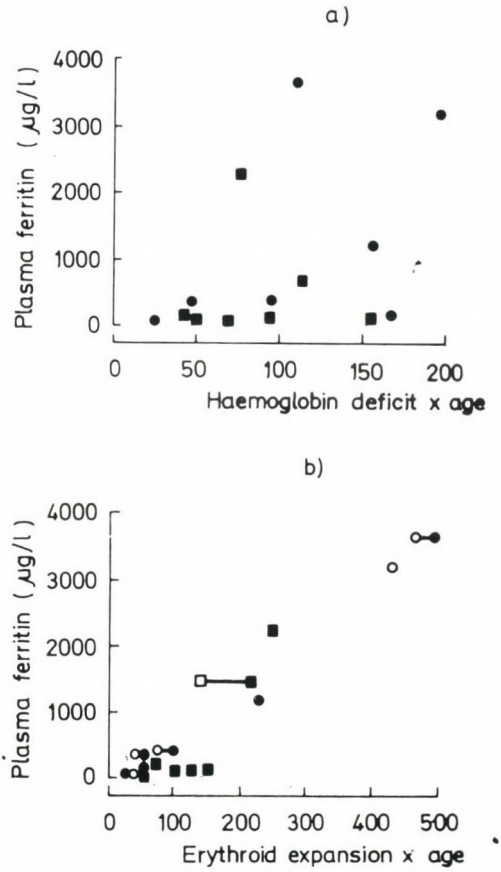


Fig. 4. Relationship between plasma ferritin and (a) degree of anaemia or (b) erythroid expansion, in patients with β thalassaemia intermedia (■) or sideroblastic anaemia (●). The degree of anaemia was determined as (14 minus the patient's circulating haemoglobin level in g/dl), and the erythroid expansion (times normal) from ferrokinetic studies (closed symbols) or marrow E : M ratios (open symbols). In 5 subjects paired values for the two measures of erythroid expansion are shown

ment of erythroid expansion from simple marrow examination may be as good as the more detailed ferrokinetic measurements in predicting the risk of iron loading.

Effect of iron chelation treatment

In four adult patients with β thalassaemia intermedia, control metabolic iron balance studies confirmed a positive iron balance (mean +2.9 mg/day, range +1.2 to 3.9 mg). Oral DF (1 g taken with meals) converted this to a small negative iron balance (mean −1.2 mg/day, range −0.2 to 2 mg). However, a single subcutaneous infusion of 150 mg DF/kg/24 hours produced a much more substantial net iron loss over a balance period of equivalent length (Fig. 5).

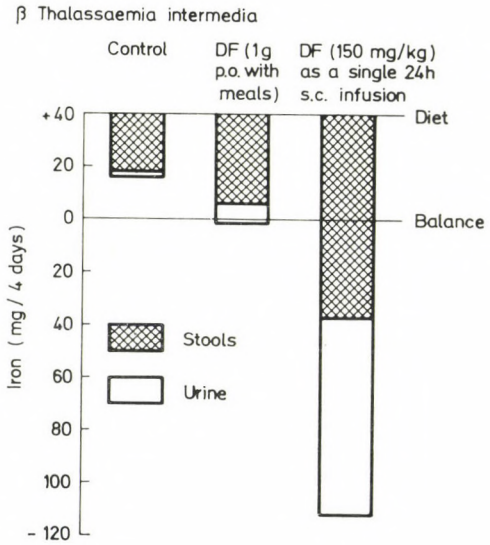


Fig. 5. Metabolic balance study in a patient with β thalassaemia intermedia. A constant diet (10 mg iron/day) was taken during three consecutive 4-day periods. During a control period the total iron intake (40 mg) was not matched by the total iron excretion in stools and urine, and a net iron gain (positive iron balance) resulted. The positive iron balance was abolished by oral DF (1 g in 10 ml water t.d.s.), and converted to substantial net iron loss by a single 24 h subcutaneous infusion of 8 g DF (150 mg/kg)

Discussion

These studies have shown that in non-transfused patients with sideroblastic anaemia or with β thalassaemia intermedia, the degree of anaemia is a poor guide to the amount of increased gastrointestinal iron absorption and the rate of iron loading. On the other hand, the magnitude of the erythroid expansion

correlated much better with the risk of developing iron overload. This is consistent with the hypothesis [15] that excess food iron absorption is directly related to increased plasma iron turnover. The correlation with erythroid expansion held good despite the limitations inherent in a single measurement taken during the course of a disease which may evolve with time, and despite other obvious potential causes of variation (e.g. differences in the amount of available iron in the patients' usual diets, and differences in iron balance between men and women). It should be emphasised that the degree of anaemia is clearly not the only determinant of the magnitude of erythroid expansion. Other factors may include both the nature of the underlying disease and the degree of displacement of the haemoglobin oxygen-dissociation curve (e.g. with different amounts of circulating Hb F in β thalassaemia intermedia) [6]. Clearly, gross erythroid expansion may be present at a near normal haemoglobin level, and the possibility that otherwise asymptomatic patients may be at risk of iron overload will be missed if erythroid expansion is not assessed. In this study a simple determination of the erythroid/myeloid ratio from bone marrow smears appeared as good as ferrokinetic measurements of erythroid expansion in predicting the risk of iron loading. This is important, both because it avoids the use of radioisotopes and because the more detailed investigations are time consuming and are unlikely to be available in many parts of the world where β thalassaemia intermedia is common.

The price of neglecting the risk of iron loading in these patients is the development of tissue iron toxicity. Indeed several of our older patients have evidence of cardiac damage, hepatic cirrhosis, or endocrine failue [6, 9]. Reduction of iron absorption by dietary modification and/or the use of oral iron chelating agents to reduce the amount of available food iron [16] might offer these patients the hope of a life free from these complications of iron overload. Although our metabolic iron balance studies have indicated that the net dietary iron gain can be negated by the use of oral desferrioxamine (DF), subcutaneous infusions of the drug both reduce radioiron absorption [17] and give a much more marked negative iron balance. Further studies have shown that even in subjects with no evidence of excess iron stores, single infusions of 150 mg DF/kg/24 hours gave total (faeces plus urine) iron excretions that ranged from 41 to 92 mg (unpublished data). This raises the possibility that in patients with gross ineffective erythropoiesis who are shown to be at risk of iron loading, subcutaneous DF used only occasionally (e.g. a single 24 hour infusion every 3 to 4 weeks) may be sufficient to prevent iron accumulation completely and thus potentially to avoid the complications of iron overload. Such an approach is likely to be more practical and acceptable to patients than the inefficient and very expensive use of prophylactic oral DF.

We are grateful to Dr. S. T. Callender and Dr. G. Warner. Nuffield Department of Clinical Medicine, Oxford, for allowing us to use their data on radioiron absorption in normal and iron deficient subjects, and to Miss Hesma Goodburn who provided excellent technical assistance. This work was supported by the Wellcome Trust, London and a Wellcome Research Travel Grant made it possible for M. J. P. to present these data at the 19th Congress of the International Society of Haematology in Budapest, 1982.

References

1. Weatherall, D. J., Clegg, J. B.: *The Thalassaemia Syndromes* (3rd Edition). Blackwell Scientific Publications, Oxford 1981.
2. Engle, M. A.: Cardiac involvement in Cooley's anemia. *Ann. N. Y. Acad. Sci. 119*, 694 (1964).
3. Heinrich, H. C., Gabbe, E. E., Oppitz, K. H., Whang, D. H., Bender-Gotze, Ch. Schafer, K. H., Schroter, W., Pfau, A. A.: Absorption of inorganic and food iron in children with heterozygous and homozygous β-thalassemia, *Z. Kinderheilk, 115*, 1 (1973).
4. Pippard, M. J., Callender, S. T., Warner, G. T., Weatherall, D. J.: Iron absorption and loading in β-thalassaemia intermedia, *Lancet ii*, 819 (1979).
5. Bannerman, R. M., Keusch, G., Kreimer-Birnbaum, M., Vance, V. K., Vaughan, S.: Thalassemia intermedia, with iron overload, cardiac failure, diabetes mellitus, hypopituitarism and porphyrinuria, *Am. J. Med. 42*, 476 (1967).
6. Pippard, M. J., Rajagopalan, B., Callender, S. T., Weatherall, D. J.: Iron loading, chronic anaemia, and erythroid hyperplasia as determinants of the clinical features of β-thalassaemia intermedia. In: *Advances in Red Blood Cell Biology*. D. J. Weatherall, G. Fiorelli, S. Gorini (eds). Raven Press, New York 1982, p. 103.
7. Soloman, L. R., Hillman, R. S., Finch, C. A.: Serum ferritin in refractory anemias. *Acta Haemat. 66*, 1 (1981).
8. Brain, M. C., Herdan, A.: Tissue iron stores in sideroblastic anaemia. *Brit. J. Haemat. 11*, 107 (1965).
9. Peto, T. E. A., Pippard, M. J., Weatherall, D. J.: Iron overload in mild sideroblastic anaemias. *Lancet i*, 375–378 (1983).
10. International Committee for Standardization in Haematology: Recommendations for measurement of serum iron in human blood. *Brit. J. Haemat. 38*, 291 (1978).
11. International Committee for Standardization in Haematology: The measurement of total and unsaturated iron-binding capacity in serum. *Brit. J. Haemat. 38*, 281 (1978).
12. Callender, S. T., Witts, L. J., Warner, G. T., Oliver, R.: The use of a simple whole-body counter for haematological investigations. *Brit. J. Haemat. 12*, 276 (1966).
13. Cook, J. D., Marsaglia, G., Eschbach, J. W., Funk, D. D., Finch, C. A.: Ferrokinetics: a biologic model for plasma iron exchange in man. *J. Clin Invest. 49*, 197 (1970).
14. Cook, J. D., Finch, C. A.: Ferrokinetic measurements. In: *Iron* (Methods in Haematology, Vol. 1). J. D. Cook (ed.). Churchill Livingstone, Edinburgh 1980, p. 134.
15. Cavill, I., Worwood, M. and Jacobs, A.: Internal regulation of iron absorption. *Nature 256*, 328 (1975).
16. De Alarçon, P. A., Donovan, M., Forbes, G. B., Landaw, S., Stockman, J. A.: Iron absorption in the thalassemia syndromes and its inhibition by tea. *New Engl. J. Med. 300*, 5 (1979).
17. Pippard, M. J., Callender, S. T., Warner, G. T., Weatherall, D. J.: Iron absorption in iron-loading anaemias: Effect of subcutaneous desferrioxamine infusions. *Lancet ii*, 737 (1977).

ANNOUNCEMENT

The French Association of Haemophiliacs International Prize of FF 15.000, whose aim is to encourage medical research into the disease will be awarded for the sixth time in July 1985.

The previous winners have been:

- 1975 Dr J. P. ALLAIN (France)
- 1977 Dr R. H. WAGNER (Chapel Hill, N.C. U.S.A.)
- 1979 Prs BLOOM and PEAKE (Cardiff England)
- 1981 Pr MANNUCI (Milan – Italy)
- 1983 Prs EARL DAVIE and KOTOKU KURACHI (Washington Seattle)

and two medals awarded to Pr HOYER (Connecticut) and Pr TUDDENHAM (London) for their interesting researches.

The regulations in French and English will be forwarded on request by the Secretariat of the selection committee (Association Française des Hémophiles – C.N.T.S. – 6, rue Alexandre Cabanel – 75015 – PARIS).

The work submitted for the Prize must reach the Secretariat of the Selection committee by March 15th 1985 at the latest.

OBITUARY

Dr. Alfredo PAVLOVSKY passed away at the age of 76, on April 25, 1984.

He had been in good health with minor rheumatoid disabilities until his death. He resigned as Director of the "Instituto de Investigaciones Hematológicas" (Buenos Aires) only two years ago, after 25 years. He had kept his interest in the academic activities and remained a Honorary Director until his death.

Son of a Russian father and Argentine mother, he was born in 1907, in Buenos Aires, Argentina. He studied at the University of Buenos Aires, and obtained his Medical degree in 1931. In 1934, he published his Doctoral Thesis on "Lymph Nodes Punction" that received the Pascual Palma Award (best thesis) at the University of Buenos Aires, in 1935. In the same year his thesis was reproduced in extense in the classical book "Hematología" (Ferrata, Italy).

His two main lines of research had been on Lymphnodes Pathology where he is considered a pioneer in the field of needle biopsy cytology, and in Hemophilia where he described for the first time in 1937 the existence of two different types of hemophilia. In 1944, he founded the first Hemophilia Foundation in the world, and in 1957 the Fundaleu (Leukemia Foundation). Both organizations have since been considerably promoting the research and better care of such patients.

During his 53 years in medicine, as a researcher and leader, he made many original contributions in different fields of hematology, concentrating his efforts on Hemophilia and Lymphopathies.

His activity in scientific societies led him to head the International Society of Hematology, Argentine Society of Hematology, Argentine Society of Internal Medicine, Argentine Society of Clinical Investigation.

He has participated for many years in two international societies as member of the board, president or chairman of congresses and symposia, e.g. the International Society of Hematology; International Committee (now: Society) on Hemostasis and Thrombosis.

Dr. A. Pavlovsky was a member of the Editorial Board of a number of scientific journals: Medicina, Blood, Acta Hematologica, Sangre, Thrombosis et

Diathesis Haemorrhagica Hemostase, Nouvelle Revue Française d'Hematologie, and Coagulation.

But his interest in life has not only been towards Hematology: an enthusiastic sportman, he had won Argentine championship in rugby, athletics, swimming, basketball and waterpolo. After leaving active sports (he continued with golf and ski till his 70s), he found time to teach and coach young men.

He has been a good husband and father of four sons and a daughter. Two of the sons are Medical Doctors and Hematologists. One, Miguel, is at present Secretary General of the Interamerican Division of the International Society of Hematology, while Santiago is Regional Advisor, Cancer Treatment Programme at the Pan American Health Organization in Washington.

Susan R. Hollán

INSTRUCTIONS TO CONTRIBUTORS

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

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24
H-1113 Budapest, Hungary

Three copies of the manuscript should be submitted. They should be typed double-spaced on one side of a good quality paper with proper margins. The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which the work has been carried out; (4) name and full postal address of the author to whom communications regarding the manuscript should be directed; (5) a short title not to exceed 40 characters (including space) to be used as a running head. The second page should contain an **abstract** of 50—100 words, summarizing the reasons for the study, the methods used, the results, and the major conclusions. This page should also contain 4—8 **keywords** placed in alphabetical order. Original papers should not exceed 15 printed pages including tables, figures, and references. Case reports should not be more than four, technical notes and Letters to the Editor not more than two printed pages in length. In the manuscripts the approximate location of tables and figures should be indicated in the margin. The manuscript of original papers should be divided into summary, introduction, materials and methods, results, discussion, acknowledgements and references. Review articles should also be appropriately divided. SI units should be used in the manuscript, except that, for the time being, litre (l) may be used as a unit of volume.

References: Only papers closely related to the author's work should be referred to. In the text citations should include the name of the authors and/or the reference number in parentheses. A list of references, numbered according to the order of their occurrence in the text, should be compiled at the end of the paper. It should be typewritten, double-spaced, on a separate sheet.

References to periodicals should be typed double-spaced and mention: (1) name(s) and initials of all the authors; (2) title of the paper; (3) international abbreviation of the periodical; (4) volume; (5) first page number; (6) year of publication in parentheses. Thus: 4. Liebermann, J. E., Gordon-Smith, E. C.: Red cell pyrimidine 5'-nucleotidase and glutathione in myeloproliferative and lymphoproliferative disorders. *Brit. J. Haemat. 44*, 425 (1980).

References to books should include: (1) author(s)' name(s); (2) title; (3) editor(s); (4) publisher; (5) place and year of publication. Thus: 5. Guyton, A. C., Taylor, A. E., Granger, H. J.: *Circulatory Physiology. II. Dynamics and Control of Body Fluids.* Saunders, Philadelphia 1975. Or: 6. Rappaport, J. M.: Granulocyte transfusion. In: *Haematology of Infancy and Childhood.* D. G. Nathan, F. A. Oski (eds). Saunders, Philadelphia 1974, p. 875.

Illustrations must be cited and numbered in the order they appear in the text. All line drawings should be submitted as clear, glossy, black and white originals or good quality photographs. The author's name and the title of the paper together with the serial number of the figure should be written on the back of each print. Legends for illustrations should be typewritten, double-spaced, on a separate sheet, and included at the end of the manuscript. Tables, each bearing a title, should be self-explanatory and numbered consecutively.

Authors will receive one set of proofs which must be corrected and returned to the Editor-in-Chief *within three days of receipt*. Major alterations of the text cannot be accepted.

Authors are entitled to 50 reprints free of charge.

HAEMATOLOGIA
VOLUME 17 · NUMBER 3 19

Contents

Index: 26861

VOLUME 17 • NUMBER 4 • 1984

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

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.

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

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24.
H-1113 Budapest
Hungary

Technical Editor:
R. J. Valenti

Distributor for

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

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

For all other countries subscriptions should be sent to

VNU SCIENCE PRESS
P.O.B. 2073
3500 GB Utrecht, The Netherlands

Subscription price for volume 17

DM 279 incl. surface mail
DM 305 incl. air mail

VOLUME 17 · NUMBER 4

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

VNU SCIENCE PRESS, UTRECHT, THE NETHERLANDS

AKADÉMIAI KIADÓ, BUDAPEST

1984

Printed in Hungary
Akadémiai Kiadó és Nyomda, Budapest

Haematologia 17 (4), pp. 419—425 (1984)

Sterile, Medium Scale Age Fractionation of Human Red Blood Cells

A. J. P. F. LOMBARTS*, J. K. JAGDEWSING, ALICE G. M. BOT, B. LEIJNSE

Department of Clinical Chemistry and Haematology, Leyenburg Hospital, PO Box 40551 2504 LN The Hague, The Netherlands*, Regional Bloodbank 'S-Gravenhage, Leyweg 275, The Hague, and Department of Chemical Pathology, Erasmus University/Department of Clinical Chemistry, Dijkzigt Hospital, Rotterdam, The Netherlands

(Received 3 August 1983; accepted 23 September 1983)

After sterile removal of white blood cells and the majority of platelets from blood in a regular transfusion bag, the remaining red blood cells were separated under sterile conditions into a relatively young and a relatively old fraction. Making use of the different densities of old and young red blood cells, the separation occurs in a double transfusion bag by centrifugation, after the cells have been carefully layered on top of Percoll® of "tailor-made" density.

The successful results of this simple and inexpensive technique are assessed by comparing the creatine concentration and the acetylcholinesterase activity in both fractions.

Thus, this medium scale fractionation technique yields hundreds of ml of sterile, young red blood cells.

Keywords: Medium scale fractionation; Percoll®, red blood cell fractionation; sterile fractionation.

Introduction

In the course of investigations into the laboratory preparation of quality control materials for flow haemocytometry [1], we were in need of a method for the sterile separation of hundreds of ml of young red blood cells (rbc). Perusal of the literature revealed only one medium or large scale method (hundreds or thousands of ml) for rbc fractionation, using the recently introduced blood cell separators [2–4]. These cell processors are, however, expensive, not universally present and primarily developed and in use for in vivo apheresis techniques [2–4].

The objective of this paper is to describe our development and evaluation of an alternative method for medium scale fractionation of rbc, employing simple means. After separation of the buffy-coat in a plasma extractor [5], white blood cells (wbc) and the majority of platelets (plt) are removed by the well-established routine method of sterile cotton-wool filtration [6]. Making use of the different densities of old and young rbc [7, 8], rbc fractionation is performed in a double transfusion bag by centrifugation, after blood has been carefully layered on top of Percoll® of a "tailor-made" density.

Materials and Methods

Preparation of red blood cell suspension for fractionation from citrated whole blood

In this paper we shall describe the whole blood processing procedure for *one* normal 500 ml ACD or CPD blood unit. It will be evident that scaling up can be achieved by simultaneously processing several blood units.

After routine centrifugation of the regular double blood bag and pressing out the plasma and buffy-coat in a plasma extractor [5], the remainder of wbc and plt are removed by filtration through a sterile cotton-wool column. We used the Cellselect leukocyte filterset D from the NPBI (Nederlands Produktielaboratorium voor Bloedtransfusie-apparatuur en Infusievloeistoffen), P. O. Box 9148, 1006 AC Amsterdam (Order number 1004). This routine technique has been shown [6] to be the method of choice for the preparation of wbc-free and plt-poor rbc suspensions.

After centrifugation and removal of the saline via the satellite bag in a plasma extractor [5], a representative blood cell sample is drawn without using the ports of the primary bag. This is done by entirely clamping the empty satellite bag at about one tenth from the top, leaving the headspace in connection with the primary bag via the transfer tubes and by carefully homogenizing the suspension in these compartments. The sample, obtained through one of the ports of the satellite bag, is to be used to determine the exact Percoll density needed for a successful fractionation (see below).

Preparation of Percoll®

Percoll®, a colloidal silica coated with polyvinylpyrrolidone, was purchased from Pharmacia Fine Chemicals, Sweden, in a sterile 1 L unit (cost about $ 70). Percoll was divided into quantities sufficient for 1 blood bag by filling out 225 ml into four 300-ml glass injection bottles each. After the addition of 17 ml of distilled water the Percoll was sterilized for 20 min at 120 °C. Since Percoll has a low osmolality (20 mOsm/kg) and autoclaving with salts causes gelation, sterile saline solutions must be added after sterilization. To minimize the volume of salt solutions to be added afterwards, 17 ml of distilled water was added before sterilization and 8.3 ml of 27% sterile sodium chloride solution was added after sterilization, yielding an osmolality of about 300 mOsm/kg. These solutions are stable at room temperature. To achieve the approximate density of rbc (1.080–1.115) [9] some 38 ml of sterile 0.9% saline are supplemented yielding a working Percoll solution with a density of about 1.105. This density can easily be determined by refractometry, as indicated in the Percoll package insert. The exact density needed is dependent on (1) the density of the rbc to be fractionated and (2) the amount of remaining saline after the majority of the saline had been removed after filtration of the blood through the cotton-wool column, as well as on (3) the desired ratio of the fractions of relatively old and relatively young rbc.

Assessment of the desired Percoll density

By the addition of increasing, exactly known amounts of extra saline, a series of test tubes of decreasing Percoll densities is prepared. After carefully layering aliquots of the sample rbc suspension (see above) on top of the Percoll solutions and after centrifugation, preferably under the same conditions as the actual large-scale centrifugation, the desired density can be assessed. Thus, the calculated amount of extra saline can be added to the working Percoll solution (generally about 15 ml).

Age fractionation

By using 2 large, long-pronged clamps and 2 additional ordinary Kocher clamps, the red cell suspension is pressed into the bottom compartment of the primary blood bag, as shown in Fig. 1. Via one of the ports of the satellite bag, Percoll of the appropriate density can be layered on top of the rbc without using the

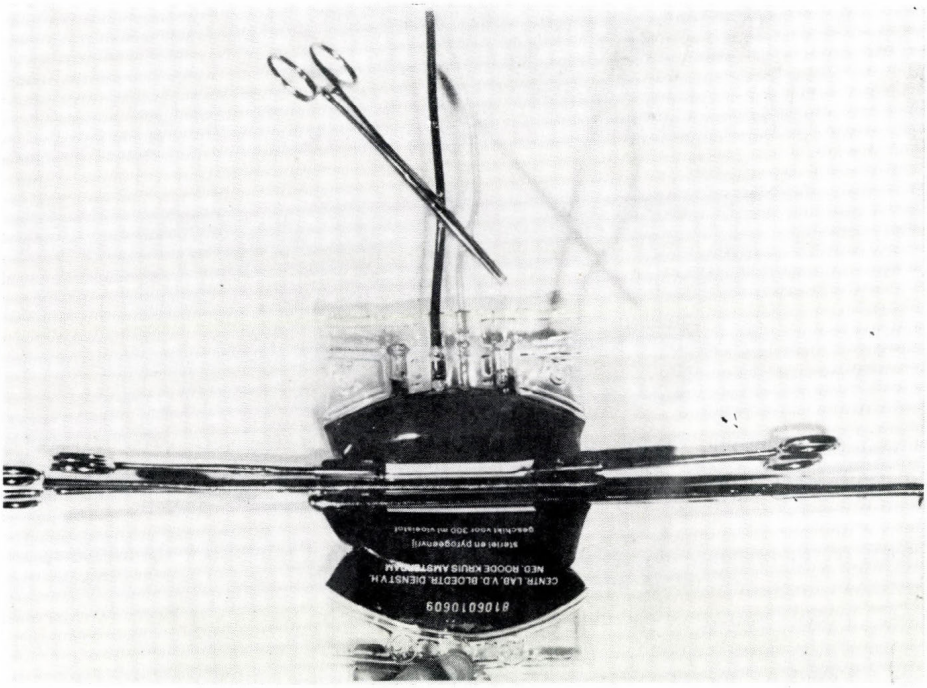


Fig. 1. Careful layering of Percoll® of appropriate density on top of red blood cells, using several large, long-pronged clamps. The Percoll was added via the satellite bag and one of the connecting tubes (light colour), obviating the need for using the injection ports. Since the subsequent centrifugation occurs with the ports in downward position, they may not be used before centrifugation, to prevent leakage. The other connecting tube shown (dark colour) was used to take a representative red blood cell-saline sample for the preliminary accurate density assessment

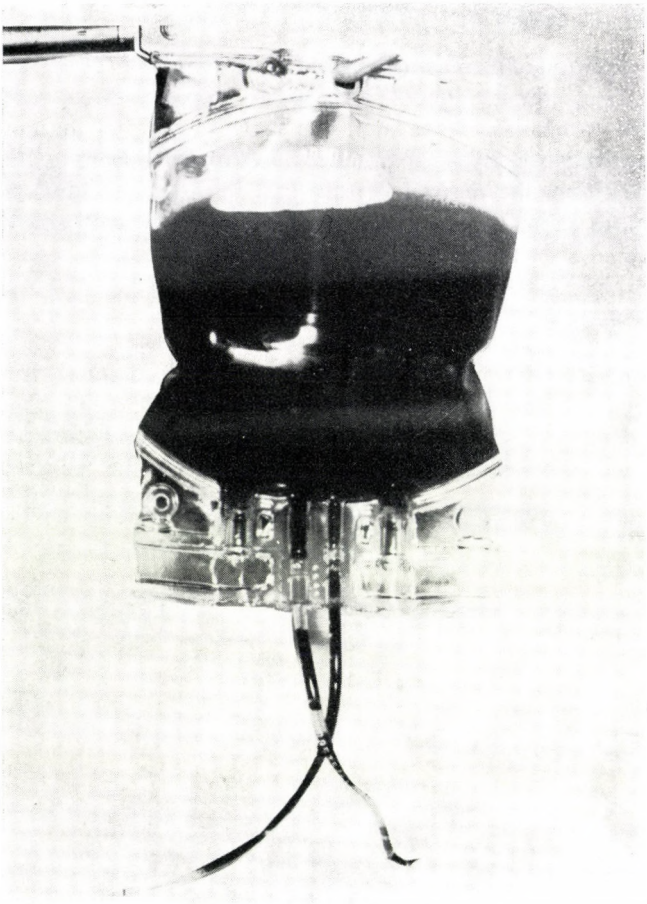


Fig. 2. Typical result of the fractionation after centrifugation. By chemical means, as described in the text, the lower red blood cell (rbc) layer is shown to consist of comparatively old rbc, the upper layer of the comparatively young rbc

ports of the primary bag. After sealing the connecting tubes (not too close to the primary bag) the satellite bag can be discarded. After inversion of the primary bag and careful removal of the clamps, the rbc are now on top of the Percoll. The bag is carefully placed upside down in a centrifuge beaker, that subsequently is stuffed up with rubber and plastic pads to prop the bag during centrifugation. This spinning occurs at routine bloodbank conditions, leakage being impossible because the ports are intact. Figure 2 shows 2 distinct (dark) cell layers after centrifugation, the bottom layer consisting of the relatively old fraction.

Through one of the ports of the primary bag, the layers can consecutively be harvested and washed with saline in separate bags. Since the density of the Percoll is greatly decreased by the addition of saline, the washing should be performed with

the bags in the normal upright position, the cells being in the bottom layer after centrifugation. The saline can be pressed out in a plasma extractor and washing can be repeated.

Tests of age fractionation

The content or activity of several substances in the rbc decrease with increasing rbc age [7–8]. Of the many age indicators described we chose creatine and acetylcholinesterase (ACh-ase) (EC 3.1.1.7) because of their stability and discriminating power [7, 10].

There is a non-linear relationship between rbc age and the creatine content [10]. The creatine concentration in young rbc is considerably higher than in older rbc. Creatine was assayed by the colorimetric method of Griffiths [11] as modified by Li et al. [12]. The intra-assay coefficient of variation (CV) (n = 10) was 3.4%.

ACh-ase activity in the rbc membrane decreases with age. The activity was determined according to the slightly modified method of Michel [13, 14]. In this method the pH decrease due to acetic acid formation from the substrate acetylcholine is a measure of ACh-ase activity. The modification consists of accurately registering the pH decrease in an ABL-2 blood gas instrument (Radiometer Copenhagen). The inter-assay CV (n = 5) was found to be 4.1% against a stated CV of 16% in the original method [13].

Results

Figure 2 visualizes the result of fractionation, the bottom dark layer consisting of relatively old rbc. Table 1 compiles the creatine contents and ACh-ase activities in washed samples of both fractions (blood No. 1) as well as those in the fractions of three other blood units. Table 1 shows highly significant differences in both age markers between the two fractions of the four blood units.

Table 1

Typical creatine contents and acetylcholinesterase activities of young and old fractions of four blood units

	Creatine (μg/ml rbc*)		ACh-ase** (ΔpH/h)	
	young	old	young	old
Blood No. 1	31.3	24.2	1.028	0.896
Blood No. 2	38.8	25.7	1.985	1.744
Blood No. 3	39.8	17.7	2.120	1.696
Blood No. 4	35.5	20.1	1.847	1.744

* red blood cells
** acetylcholinesterase (EC 3.1.1.7)

Discussion

All existing rbc separation techniques are based on the fact that rbc density increases with cell age. Routine bloodbank centrifugation does not lead to cell separation [15]. Angle-head centrifugation (to enhance the internal circulation) at 30 °C (to improve the flow properties of rbc) at $39{,}000 \times g$ for 1 hour leads to reasonable cell separation. However, this method requires a special angle-head rotor, is rather cumbersome and can handle only quantities of 80 ml blood [15]. Percoll has been successfully used before, but only on a small scale basis [7, 8]. The proposed method has been shown to separate rbc into a comparatively old and young fraction. The ratio of these fractions can be chosen by carefully assessing the proper Percoll density. Likewise, the 2 fractions can possibly be separated further into subfractions.

The method can be scaled up by simultaneously processing several bags. After removal of the plasma from various blood units, rbc with different antigenic make-up can be mixed with no apparent damage to the cell membranes [16], offering the possibility of preparing hundreds to thousands of ml of relatively young rbc. Scaling up is, however, rather time-consuming and could probably be done much easier with modern blood cell separators [2–4]. Our method seems to be most rewarding for medium scale purposes.

Unfortunately, Pharmacia Sweden cannot guarantee the safe usage of Percoll® for in vivo purposes. Consequently, rbc fractionated with Percoll can only be used for in vitro goals.

The authors wish to thank Messrs. C. A. van Beek and A. van Tiel, department of medical photography Leyenburg-Eye-Hospital, for making the photographs. They are much indebted to Dr. P. Draper, National Institute for Medical Research, London, for most valuable discussions.

References

1. Lombarts, A. J. P. F., Leijnse, B.: Preparation and evaluation of a 7-parameter intralaboratory control blood of 4 month stability. *Ann. Clin. Biochem. 20*, 302 (1983).
2. Kurtz, S. R., McMican, A., Carciero, R., Melaragno, A., Abdu, W., Katchis, R., Valeri, C. R.: Platelet pheresis experience with the Haemonetics Blood Processor 30, the IBM Blood Processor 2997, and the Fenwal CS-3000 Blood Processor. *Vox Sang. 41*, 212 (1981).
3. Graziano, J. H., Piomelli, S., Seaman, C., Wang, Th., Cohen, A. R., Kelleher, J. F., Schwartz, E.: A simple technique for preparation of young red cells for transfusion from ordinary blood units. *Blood 59*, 865 (1982).
4. Corash, L., Klein, H., Deisseroth, A., Shafer, B., Rosen, S., Beman, J., Griffith, P., Nienhuis, A.: Selective isolation of young erythrocytes for transfusion support of thalassemia major patients. *Blood 57*, 599 (1981).
5. Fenwal products, systems and methods. Travenol Laboratories, Inc., Deerfield, Ill. 60015 USA/Locatellikade 1, 1076 AZ Amsterdam
6. Reesink, H. W., Veldman, H., Henrichs, H. J., Prins, H. K., Loos, J. A.: Removal of leukocytes from blood by fibre filtration. A comparison study on the performance of two commercially available filters. *Vox Sang. 42*, 281 (1982).

7. Rennie, C. M., Thompson, S., Parker, A. C., Maddy, A.: Human erythrocyte fractionation in "Percoll" density gradients. *Clin. Chim. Acta 98*, 119 (1979).
8. Salvo, G., Caprari, P., Samoggia, P., Mariani, G., Salvati, A. M.: Human erythrocyte separation according to age on a discontinuous "Percoll" density gradient. *Clin. Chim. Acta 122*, 293 (1982).
9. Spooner, R. J., Percy, R. A., Rumley, A. G.: The effect of erythrocyte ageing on some vitamin and mineral dependent enzymes. *Clin. Biochem. 12*, 289 (1979).
10. Syllm-Rapoport, I., Daniel, A., Starck, H., Hartwig, A., Gross, J.: Creatine in density-fractionated red cells, a useful indicator of erythropoietic dynamics and of hypoxia past and present. *Acta Haemat. 66*, 86 (1981).
11. Griffiths, W. J.: Estimation of creatine in red cells. *J. Clin. Pathol. 21*, 412 (1968).
12. Li, P. K., Lee, J. T., Li, C., Deshpande, G.: Improved method for determining erythrocyte creatine by the diacetyl-α-naphthol reaction: Elimination of endogenous glutathione interference. *Clin. Chem. 28*, 92 (1982).
13. Michel, H. O.: Cholinesterase in human red blood cells and plasma. In: *Standard methods of clinical chemistry*. D. Seligson, (ed). Academic Press, New York 1961, p. 93.
14. Lewis, P. J., Lowing, R. K., Gompertz, D.: Automated discrete kinetic method for erythrocyte acetylcholinesterase and plasma cholinesterase. *Clin. Chem. 27*, 926 (1981).
15. Murphy, J. R.: Influence of temperature and method of centrifugation on the separation of erythrocytes. *J. Lab. Clin. Med. 82*, 334 (1973).
16. Anido, G.: Preparation of quality control materials in Clinical Chemistry and Haematology. *Proc. Roy. Soc. Med. 68*, 624 (1975).

Haematologia 17 (4), pp. 427–432 (1984)

Rosetting Index (E Rosettes/Mouse Rosettes): A Simple Diagnostic and Prognostic Value in CLL

A. BERREBI, M. TALMOR, Y. KLUGER, M. SHTALRID, J. VORST

Hematology Unit, Kaplan Hospital, Rehovot 76100, Israel

(Received 14 December 1982; revised 28 May 1983; accepted 1 June 1983)

The rosetting index (E rosettes/mouse rosettes) was evaluated in 34 controls, 4 chronic benign lymphocytosis and 28 CLL patients, grouped according to the Rai classification: 1) 6 asymptomatic stage 0 patients (lymphocytosis less than 10,000/mm^3) with a rosetting index of 0.35 ± 0.2; 2) 11 asymptomatic stage 1–2 patients (lymphocytosis 10,000–40,000/mm^3) with a rosetting index of 0.19 ± 0.11; 3) 9 symptomatic stage 3–4 patients (lymphocytosis greater than 40,000/mm^3) with a rosetting index of 0.09 ± 0.09 and 4) 2 T-CLL patients post splenectomy and 4 chronic benign lymphocytosis patients with a rosetting index of 25 ± 15. The average rosetting index for the controls was 10.0 (range 3.5–54). Student's t test was $p < 0.0001$ for groups 1, 2 and 3 and $p < 0.02$ for group 4. These results show that 1) the rosetting index is helpful for the characterization of B-CLL in all stages, 2) the rosetting index decreases with the proliferative activity of the disease and could be used as a prognostic factor, and 3) the rosetting index is significantly increased in T-CLL and chronic benign T lymphocytosis thus being useful in differentiating these conditions from the early stage of CLL, when the SmIg is not conclusive of a monoclonal proliferation.

Keywords: Mouse rosettes, E rosettes, CLL, lymphocytosis

Introduction

It has been established that a proportion of normal lymphocytes form spontaneous rosettes with mouse red blood cells (MRBC) [4]. These lymphocytes are immunoglobulin bearing and are considered to be a subset of the B-cell population. The percentage of MRBC rosettes is significantly increased in B-CLL, which is also characterized by low E rosettes and weak surface immunoglobulins (SmIg). It has been suggested that the absolute increase in MRBC rosettes and the corresponding decrease in E rosettes may be a useful diagnostic tool in CLL [5, 6, 10, 13] and may indeed correlate with the severity of the disease [1, 2].

The ratio between the E and M rosettes, which we call the rosetting index (RI) may be an interesting new parameter to use diagnostically and prognostically in CLL and related disorders. We evaluated this index in 26 B-CLL, 2 treated T-CLL and 4 chronic benign lymphocytosis cases along with 34 normal controls.

Materials and Methods

Patients. Twenty-six B-CLL, 2 T-CLL and 4 chronic benign lymphocytosis patients along with 34 normal controls were studied.

The B-CLL patients were divided into 3 groups according to the Rai classification: stage 0 (6 asymptomatic patients, less than 10,000 lymphocytes/mm^3), stage 1–2 (11 asymptomatic patients, 10,000–40,000 lymphocytes/mm^3) and stage 3–4 (9 symptomatic patients (over 40,000 lymphocytes/mm^3). Of these, only 3 patients (Nos 22, 23 and 24 in stage 3–4) had previously received chemotherapy, but had been therapy-free for at least 3 months prior to this study. The remaining 23 patients were untreated. The 2 T-CLL patients had undergone splenectomy: patient No. 27 developed Richter syndrome (histiocytic lymphoma involving the spleen), received Chop-Bleo chemotherapy, entered remission, and when tested for this study had absolute lymphocytosis with no treatment. Patient No. 28 was treated with Leukeran (chlorambucil) after splenectomy and was receiving prednisone only when tested. The patients with chronic benign lymphocytosis were not treated.

Methods. Lymphocyte counts were obtained from finger-prick samples. Rosettes and surface membrane immunoglobulins were performed on lymphocytes separated from heparinized blood on a Ficoll-metrizoate density gradient [4] as follows:

E-rosettes [3]. 1×10^6 lymphocytes in PBS were incubated with an equal volume of a 2% saline suspension of sheep red blood cells at 37 °C for 10 minutes, centrifuged briefly at $400 \times g$ and incubated at 4 °C for at least one hour before counting.

Mouse rosettes [7]. 1×10^6 lymphocytes in PBS were incubated with 25 µl fetal calf serum and a 2 ½% suspension of MRBC in M-199 culture medium (Balb C mice, freshly bled) and treated in the same manner as for the E-rosettes.

SmIg [3]. 50 µl lymphocytes (20×10^6/ml) were incubated with 100 µl rhodamine labelled antisera for mu, delta, alpha and gamma heavy chains and kappa and lambda light chains (M, D, A, G, K, L) according to the manufacturer's (Kallestad) instructions and examined with a Leitz fluorescent microscope.

Student's *t* test was used for statistical analysis.

Results

The results for E and M rosettes, SmIg, rosetting index and lymphocyte counts for all the patients are shown in Tables 1–4. The SmIg values represent the total count and the type is reported when a monoclonal proliferation was found. As is reflected in Tables 1–4 not all of the leukaemic patients had a monoclonal proliferation of immunoglobulin bearing lymphocytes, but for those that did, it was usually of the IgM, K type. Figure 1 illustrates the distribution of RI values for

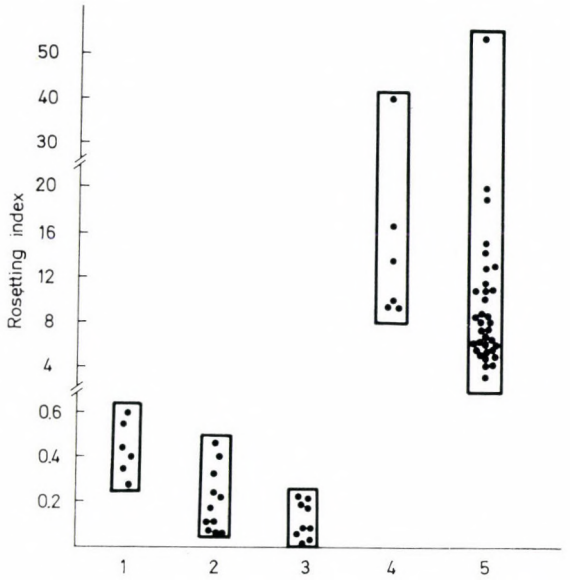


Fig. 1. Rosetting index of patients and controls. 1—stage 0; 2—stage 1–2; 3—stage 3–4; 4 T-CLL and chronic benign lymphocytosis; 5—control

the patients and the controls. The average RI values were as follows: stage 0 B-CLL, RI 0.35 (range 0.28–0.60); stage 1–2 B-CLL, RI 0.19 (range 0.06–0.47); stage 3–4 B-CLL, RI 0.09 (range 0–0.19); T-CLL and chronic bening lymphocytosis, RI 25 (range 9.7–40) and normal controls RI 10.0 (range 3.5–54.6). The *t* test was $p < 0.0001$ for groups 1, 2 and 3 and $p < 0.02$ for group 4.

Table 1

Stage 0 CLL

Patient No.	Lymphocyte count (per mm³)	SmIg (%)*	E-ros. (%)	M-ros. (%)	RI (E/M)
1	7000	19	25	56	0.44
2	9000	6	28	51	0.55
3	9600	24	18	52	0.35
4	9050	6	19	76	0.28
5	8500	25(MK)	31	48	0.60
6	8300	6	20	50	0.40

* The SmIg values represent the total count and the specificity is reported ( ) when a monoclonal proliferation was found.

Table 2
Stage 1–2 CLL

Patient No.	Lymphocyte count (per mm³)	SmIg (%)	E-ros. (%)	M-ros. (%)	RI (E/M)
7	13 200	7	14	79	0.18
8	25 600	65(ML)	7	64	0.11
9	19 600	14	22	55	0.40
10	19 200	50(MK)	3	51	0.06
11	19 000	0[i]	23	49	0.47
12	22 300	37(MK)	15	62	0.24
13	21 500	8	8.5	68	0.12
14	13 260	37(MK)	5	59.5	0.08
15	31 140	51(MDK)	15	45	0.30
16	27 000	41(MK)	4.5	64	0.07
17	27 700	22(MDK)	7.5	61	0.12

i — Intracytoplasmic Ig negative

Table 3
Stage 3–4 CLL

Patient No.	Lymphocyte count (per mm³)	SmIg (%)	E-ros. (%)	M-ros. (%)	RI (E/M)
18	40 000	29(G)	5	27	0.19
19	43 350	26(MK)	4.5	60	0.07
20	43 400	13	2.5	63	0.03
21	56 000	36(MK)	4	43	0.09
22[i]	89 000	30(ML)[ii]	8.5	67	0.13
23[i]	80 000	61(MK)	6.5	53	0.12
24[i]	200 000	70(M)[ii]	4	50	0.08
25	60 000	28(M)	6.5	35	0.18
26	80 000	n.t.	0	80	0

i — Treated previously, but not receiving treatment when tested
ii — Intracytoplasmic
n.t. — Not tested

Table 4
T-CLL (Nos 27, 28) and chronic benign lymphocytosis (Nos 29–32)

Patient No.	Lymphocyte count (per mm³)	SmIg (%)	E.-ros. (%)	M.-ros. (%)	RI (E/M)
27	11 900	0	81	2	40
28	8 500	17	67	5	13.4
29	8 500	15	71	7	10.1
30	7 600	8	58.5	6	9.7
31	5 000	9	73	7.5	9.7
32	5 100	6	50	3	16 6

Discussion

Chronic lymphocytic leukemia is a disorder characterized by a monoclonal proliferation of B lymphocytes, with an associated immunoglobulin usually of the M, K type. Proliferation of T lymphocytes may occur, but cases of true T-CLL are rare. A characteristic finding in CLL is a low percentage of E rosettes, but this value is often normal or slightly below normal, and it is not limited to this disorder. Surface membrane immunoglobulin is sometimes difficult to demonstrate and it is by no means a consistent finding in CLL, nor is it limited to this disorder [6, 9, 12].

Since the finding of Stathopoulos and Elliott [14] that a certain portion of normal B lymphocytes and the majority of CLL lymphocytes form rosettes with MRBC, it has repeatedly been shown that an increase in mouse rosettes is a consistent and almost exclusive property of B-CLL lymphocytes, and of more diagnostic value than E rosettes and SmIg alone for this disorder [6, 8, 10, 11, 13]. It has also been shown that M rosette forming cells are Ig bearing, but not all Ig bearing cells form mouse rosettes [7]. In view of our results (see Table 1), the mouse rosette marker is of even greater significance in cases of early CLL (stage 0 according to the Rai classification) when the lymphocyte proliferation is slight, SmIg, if present, is weak and sometimes not detectable and there are no clinical symptoms. Moreover, an increase in M rosettes in these cases distinguishes categorically between early CLL and chronic benign T lymphocytosis, two conditions otherwise indistinguishable by routine laboratory tests.

The rosetting index (RI), defined as the ratio between the E and M rosettes is an additional parameter useful in the diagnosis and evaluation of CLL, as well as for distinguishing between early CLL and benign lymphocytosis. To the best of our knowledge this parameter had been previously mentioned only twice in the literature as an interesting value relating to the effect of treatment on CLL patients [1, 2, 11]. Beaumariage and Focan [2] studied 35 CLL cases and showed decreased E rosettes, with highly increased percentage and absolute number of lymphocytes and M rosettes. In all their cases the E rosettes/M rosettes ratio was decreased (median value 0.15). In their study, patients were not grouped according to the severity of the disease and therefore a prognostic application of the method was not done. Our results show a clear distinction between B-CLL patients (RI 0–0.6) and normal, T-CLL and benign lymphocytosis patients (RI 3.5–55). Also, they show that the RI decreases with an increase in the severity of the disease, a fact indirectly confirmed by others [1, 5]. The overlap of RI values between the different stages of CLL as shown in Fig. 1 is probably due to the fact that the Rai classification is a crude classification, and it may be that with a slightly different criteria the RI may be used as an even more accurate indicator of the disease. Still, there is no overlap between stage 0 and stage 3–4 CLL cases.

Since most of our B-CLL patients were untreated, no attempt is made here to relate the RI to chemotherapy, but others have observed that although treatment reduces the total number of lymphocytes and may reduce the M rosettes as well, the M rosette count remains higher than normal [10, 11]. The findings of

Beaumariage and Focan [1] that treatment restores a normal RI have not been confirmed either by us or by others [11]. When the RI was recalculated for 3 of our stage 4 patients who received chemotherapy after this study, we found the same significantly decreased RI. It is worth noting that in the 2 T-CLL patients, T-lymphocytosis was very high in spite of previous treatment, and this is reflected in the high RI values.

In conclusion, we wish to emphasize the simplicity of determining the rosetting index for patients with lymphocytosis, no matter how mild. We feel that 1) the RI is helpful in the characterization of B-CLL in all stages, 2) this parameter decreases with the proliferative activity of the disease and could be used as a prognostic factor and 3) the RI is significantly increased in T-CLL and chronic benign T-lymphocytosis, thus being useful in differentiating these conditions from early B-CLL, when the SmIg is not conclusive of a monoclonal proliferation.

References

1. Beaumariage, M. L. and Focan, C. (1975) Immunocytoadherence of human lymphocytes in lymphoproliferative disorders. *IRCS Med. Sci.: Cancer; Cell and Membrane Biol.; Hematol. Immunol. and Allergy 3*, 476.
2. Beaumariage, M. L. and Focan, C. (1978) Double test of spontaneous rosettes with sheep and mouse erythrocytes. *Acta Haemat. (Basel) 59*, 321—331.
3. Bentwich, Z. and Kunkel, H. G. (1973) Specific properties of human B and T lymphocytes and alterations in disease. *Transplant. Rev. 16*, 29.
4. Boyum, A. (1968) Separation of leucocytes from blood and bone marrow. *Scand. J. Clin.. Lab. Int. Suppl. 21*, 97.
5. Catovsky, D., Milliani, E., Okos, A. and Galton, D. A. G. (1974) Clinical significance of T cells in chronic lymphocytic leukemia. *Lancet ii, 751.*
6. Catovsky, D., Cherchi, M., Okos, A., Hedge, U. U. and Galton, D. A. G. (1976) Mouse red cell rosettes in B-lymphoproliferative disorders. *Brit. J. Haemat. 33*, 173.
7. Gupta, S., Good, R. A. and Siegal, F. P. (1976) Rosette formation with mouse erythrocytes *Clin. Exp. Immunol. 25*, 319.
8. McGraw, D. J., Kurec, A. S. and Davey, F. R. (1981) Mouse erythrocyte rosette formation. A marker for resting B lymphocytes. *Am. J. Clin. Pathol. 76*, 177.
9. McLaughlin, H., Wetherly-Mein, G., Pitcher, C. and Hobbs, J. R. (1973) Non-immunoglobulin bearing "B" lymphocytes in chronic lymphatic leukemia? *Brit. J. Haemat. 25*, 7.
10. Millard, R. E. (1976) Mouse red-cell rosetting test in chronic lymphocytic leukemia. *Lancet ii*, 1204.
11. Pegrum, G. D. and Evans C. A. (1978) Significance of mouse red cell rosette forming lymphocytes in chronic lymphocytic leukemia. *Acta Haemat. (Basel) 60*, 36.
12. Rudders, R. A. and Howard, J. P. (1978) Clinical and cell surface marker characterization of the early phase of CLL. *Blood 52*, 25.
13. Stathopoulos, G. and Davies, A. J. S. (1976) Human lymphocytes and mouse red cells. *Lancet i*, 1078.
14. Stathopoulos, G. and Elliott, E. V. (1974) Formation of mouse or sheep red-blood-cell rosettes by lymphocytes of normal and leukemic individuals. *Lancet i*, 600.

Haematologia 17 (4), pp. 433–438 (1984)

Stability of Serum Ferritin in Healthy Subjects

R. J. ULVIK

Laboratory of Clinical Biochemistry, The University of Bergen, Haukeland sykehus, 5016 Bergen, Norway

(Received 3 May 1983; accepted 30 May 1983)

Recognition of chronobiological rhythms is important for the optimum use of blood parameters in clinical practice. The variation in serum ferritin, serum iron and total iron binding capacity was studied on two consecutive days in 16 healthy subjects. While serum iron changed in a typical circadian rhythm, no significant rhythm was found for serum ferritin or the total iron binding capacity.

The daily intra-individual variation in serum ferritin was 15% on the average. The 24 hour mean serum ferritin concentration differed less than 5% in most of the subjects.

In contrast to serum iron, there is no need to standardize the time of blood sampling for the determination of serum ferritin.

Keywords: Circadian rhythm, diurnal variation, serum ferritin, serum iron

Introduction

Serum ferritin generally reflects the size of the iron stores of the body and therefore its determination has been introduced as an important supplement to more traditional analyses in the evaluation of disturbances in iron metabolism [1–3]. Of particular interest is the possibility to replace the more cumbersome method of staining bone marrow smears for iron with the determination of serum ferritin [4]. Obviously, the usefulness of serum ferritin measurements in clinical practice requires a thorough understanding of the normal and pathophysiological mechanisms which may cause nonspecific variations in the serum ferritin concentration without simultaneous changes in total body iron. For instance, there is now good evidence that serum ferritin actually behaves like an acute phase protein [5, 6]. Increased serum ferritin is regularly found in malignant disease, tissue damage, inflammations and during heavy physical work [7–10].

On the other hand, an important aspect of normal iron metabolism is the presence of chronobiological rhythms [11]. Thus, the concentration of serum iron changes in a regular rhythm during the day [12]. Whether the same applies to serum ferritin is a matter of discussion. Some authors have reported a more or less regular biological variability in the serum ferritin concentration amounting to a 10–20% deviation from the 24 hour mean value [13, 14]. Others were not able to confirm these results [15–18]. Apparently, diverging results may in some cases be accounted for by too few serum ferritin determinations during the day. The

aim of the present study was to visualize and compare the concentration profiles of serum ferritin, serum iron and the total iron binding capacity (TIBC) in a group of healthy subjects and to learn to what extent biological rhythms may influence the concentration of serum ferritin throughout the day.

Subjects and Methods

The study was carried out in 16 healthy subjects – 7 women and 9 men – aged 21–26 years. They all had hemoglobin, blood counts, serum iron, total iron binding capacity (TIBC) and serum ferritin values within the reference interval used in the laboratory. Blood was sampled and serum iron, TIBC and serum ferritin were determined at 800, 1200, 1600, 2000 and 2200 hours on two consecutive days. The mean value of each parameter with the standard error of the mean (SEM) was calculated and related to the 24 hour mean values for the whole group.

The effect of food intake on serum ferritin was examined in 9 men who fasted overnight and then were given a meal with an iron content of about 7 mg. Serum ferritin was determined at 60 min intervals after the meal. Serum was stored at $-20\,^{\circ}C$ until assayed. Serum iron and TIBC were determined as described by Ruutu [19]. Serum ferritin was analysed by a commercial immunoradiometric assay from Ramco Laboratories Inc. (Houston, Texas) [20]. The within batch precision of the methods, i.e. the coefficient of variation, was: serum iron 3%, TIBC 3.5% and serum ferritin, 8%.

Results

The serum ferritin of the subjects was distributed over a wide concentration range with an average coefficient of variation of 64%. Figure 1 shows the deviation of serum ferritin, serum iron and TIBC from the 24 hour mean values of the group on two consecutive days. A significant circadian rhythm was found only for serum iron with high values at noon and low values late in the evening (Fig. 1b). The small fluctuations in TIBC and serum ferritin were not significant and could be explained by methodological variation (Fig. 1a, c). In male subjects serum ferritin was on the average 2 times higher than in the females (data not shown).

During the day the intraindividual variation coefficient for serum ferritin was 15% (Fig. 2a). There was no systematic pattern in the variation of serum ferritin in the individuals. From one day to the next the 24 hour mean serum ferritin concentration of most of the subjects changed only slightly (Fig. 2b). In the whole group the 24 hour mean serum ferritin value on two consecutive days differed only by about 2% (Fig. 2b). No significant change in serum ferritin concentration was found after intake of a meal (data not shown).

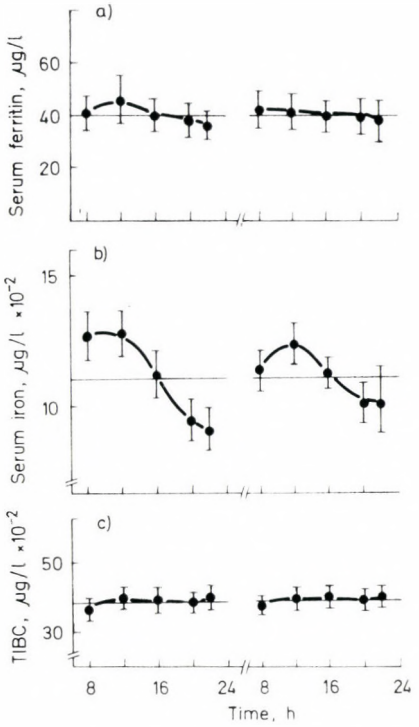


Fig. 1. Deviation of (a) serum ferritin, (b) serum iron and (c) total iron binding capacity (TIBC) from the 24 hour mean values in two consecutive days. Vertical lines represent the standard error of the mean (SEM)

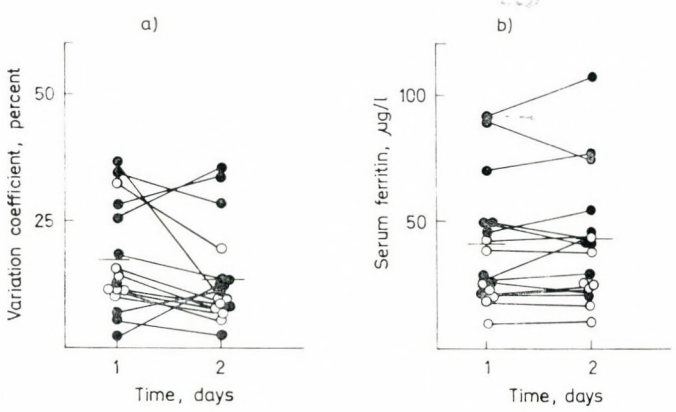


Fig. 2. (a) Daily variation coefficient for serum ferritin in each subject. (b) 24 hour mean serum ferritin concentration in each subject in two consecutive days. (○) women (●) men

Discussion

Erythropoietic and reticuloendothelial activity seems to undergo cyclic changes. This is indicated by circadian rhythms which have been described for the mitotic activity of erythropoietic cells [21], the reticulocyte count in peripheral blood [22], the release of iron from reticuloendothelial cells [23] and for the concentration of erythropoietin [22], iron [12] and bilirubin [24] in serum. Leyland et al. [13] also found a diurnal periodicity in serum ferritin in normal subjects which paralleled the rhythm for serum iron with peak concentrations before noon and low concentrations in the evening. Similar results were later reported by Pilon et al. [14]. The chronobiological rhythms for serum iron and ferritin were ascribed to a variability in the exchange of iron between plasma and the reticuloendothelial cells [23].

The physiological significance of the fluctuations in serum ferritin has been questioned by others who could not reproduce the findings of Leyland et al. [13] and Pilon et al. [14]. In some of these studies, however, the possibility to disclose a rhythmic change in serum ferritin may have been impeded by too few analyses carried out during the day [17, 18]. On the other hand, in more carefully performed studies with serum ferritin determined every second to fourth hour over a 24 hour period, a possible influence by the interrupted sleep of the subjects might be conceived. Thus, in night workers the normal serum iron rhythm is reversed with the highest value in the evening and not in the morning as usual [25]. In the present study this problem was omitted by determining serum ferritin repeatedly on two consecutive days in persons who followed their normal day and night rhythm. In keeping with previous reports [15, 16, 26] we found no significant change in the serum ferritin concentration. In normal human beings a stable concentration of serum ferritin is in agreement with the slow turnover of plasma ferritin. Worwood et al. [27] have recently shown that concanavalin A binding human plasma ferritin circulates much longer in the blood than what was thought earlier [28, 29]. They calculated the half-life to approximately 50 hours while non-concanavalin A binding ferritin, i.e. tissue ferritin, had a half-life in plasma of approximately 5 hours. The considerable sex difference in the serum ferritin levels is in agreement with the larger iron stores in men than in menstruating women [30].

Noteworthy is the finding that the 24 hour mean serum ferritin concentration was remarkably stable from day to day with a difference of less than 5% in most of the subjects (Fig. 2b). In comparison, Pilon et al. [14] who analysed morning concentrations of serum ferritin found an average intrasubject day-to-day variation of 14.5%. In a few individuals serum ferritin fluctuated more than what could be accounted for by methodological variations (Fig. 2a). The reason for this irregular change which has also been noted by others [16] is not known. In most of the subjects the variation in serum ferritin was considerably less.

In conclusion, there is no evidence for a biological variation in serum ferritin in healthy subjects. Unlike serum iron, serum ferritin can therefore be determined in blood withdrawn at any time during the day.

The author is grateful to Dr. E. H. Lehmann for helpful discussions.

References

1. Addison, G. M., Beamish, M. R., Hales, C. N., Hodgkins, M., Jacobs, A., Llewellin, P.: An immunoradiometric assay for ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. *J. Clin. Path. 25*, 326 (1972).
2. Walters, G. O., Miller, F. M., Worwood, M.: Serum ferritin concentration and iron stores in normal subjects. *J. Clin. Path 26*, 770 (1973).
3. Cavill, I.: Diagnostic methods. In: *Clinics in Haematology. Disorders of Iron Metabolism.* A. Jacobs (ed). W. B. Saunders Co., Philadelphia 1982, p. 259.
4. Roeser, H. P.: Iron metabolism in inflammation and malignant disease. In: *Iron in Biochemistry and Medicine II.* A. Jacobs, M. Worwood (eds.). Academic Press, New York 1980, p. 605.
5. Birgegård, G., Hallgren, R., Killander, A., Strømberg, A., Venge, P., Wide, L.: Serum ferritin during infection. A longitudinal study. *Scand. J. Haemat. 21*, 333 (1978).
6. Elin, R. J., Wolff, S. M., Finch, C. A.: Effect of induced fever on serum iron and ferritin concentrations in man. *Blood 49*, 147 (1977).
7. Worwood, M.: Serum ferritin. In: *Iron in Biochemistry and Medicine II.* A. Jacobs, M. Worwood (eds.). Academic Press, New York 1980, p. 203.
8. Bentley, D. P., Williams, P.: Serum ferritin concentration as an index of storage iron in rheumatoid arthritis. *J. Clin. Path. 27*, 786 (1974).
9. Birgegård, G., Hallgren, R., Venge, P., Wide, L.: Serum ferritin during inflammation. A study on myocardial infarction. *Acta Med. Scand. 206*, 361 (1979).
10. Vičnes, A., Opstad, P. K.: Serum ferritin in young men during prolonged heavy physical exercise. *Scand. J. Haematol. 27*, 165 (1981).
11. Tarquini, B.: Iron metabolism: Clinical chronobiological aspects. *Chronobiologia 5*, 315 (1978).
12. Wiltink, W. F., Kruithof, J., Mol, C., Gre Bos, M., van Eijk, H. G.: Diurnal and nocturnal variations of the serum iron in normal subjects. *Clin. Chim. Acta 49*, 99 (1973).
13. Leyland, M. J., Ganguli, P. C., Blower, D., Delamore, I. W.: Immunoradiometric assay for ferritin in human serum. *Scand. J. Haemat 14*, 385 (1975).
14. Pilon, V. A., Howanitz, P. J., Howanitz, J. H., Domres, N.: Day-to-day variation in serum ferritin concentration in healthy subjects. *Clin. Chem. 27*, 78 (1981).
15. Birgegård, G.: Serum ferritin: physiological and methodological studies. *Clin. Chim. Acta 103*, 277 (1980).
16. Dawkins, S., Cavill, I., Ricketts, C., Worwood, M.: Variability of serum ferritin concentration in normal subjects. *Clin. Lab. Haemat.* 1, 41 (1979).
17. Valberg, L. S., Sorbie, J., Ludwig, J., Pelletier, O.: Serum ferritin and the iron status of Canadians. *Can. Med. Assoc. J. 114*, 417 (1976).
18. Siimes, M. A., Addiego, J. E., Dallman, P. R.: Ferritin in serum: Diagnosis of iron deficiency and iron overload in infants and children. *Blood 43*, 581 (1974).
19. Ruutu, R.: Determination of iron and unsaturated iron-binding capacity in serum with ferrozine. *Clin. Chim. Acta 61*, 229 (1975).
20. Dempster, W. S., Knight, G. J., Jacobs, P.: An evaluation of the Ramco kit for serum ferritin assay. *S. Afr. Medical J. 56*, 1132 (1979).
21. Mauer, A. M.: Diurnal variation of proliferative activity in the human bone marrow. *Blood 26*, 1 (1965).
22. McKee, L. C., Ensign Johnson, L., Lange, R. D.: Circadian variation in reticulocyte counts and immune-detectable erythropoietin titers. *Proc. Soc. Exp. Biol. (N. Y.) 145*, 1284 (1974).
23. Lynch, S. R., Simon, M., Bothwell, T. H., Charlton, R. W.: Circadian variation in plasma iron concentration and reticuloendothelial iron release in the rat. *Clin. Sci. Mol. Med. 45*, 331 (1973).

24. Stengle, J. M., Schade, A. L.: Diurnal variations of certain blood constituents in normal human subjects: Plasma iron, siderophilin, bilirubin, copper, total serum protein and albumin, haemoglobin and haematocrit. *Brit. J. Haemat. 3*, 117 (1957).
25. Sinniah, R., Doggart, J. R., Neill, D. W.: Diurnal variations of the serum iron in the normal subjects and in patients with haemochromatosis. *Brit. J. Haemat. 17*, 351 (1969).
26. Casale, G., Migliavacca, A., Bonora, C., Zurita, I. E., de Nicola, P.: Circadian rhythm of plasma iron, total iron binding capacity and serum ferritin in arteriosclerotic aged patients. *Age and Ageing 10*, 115 (1981).
27. Worwood, M., Cragg, S. J., Williams, A. M., Wagstaff, M., Jacobs, A.: The clearance of ^{131}I-human plasma ferritin in man. *Blood 60*, 827 (1982).
28. Siimes, M. A., Koerper, M. A., Licko, V., Dallman, P. R.: Ferritin turnover in plasma: An opportunistic use of blood removed during exchange transfusion. *Pediat. Res. 9*, 127 (1975).
29. Pollock, A. S., Lipschitz, D. A., Cook, J. D.: The kinetics of serum ferritin. *Proc. Soc. Exp. Biol. (N. Y.) 157*, 481 (1978).
30. Bothwell, T. H., Charlton, R. W., Cook, J. D., Finch, C. A.: *Iron Metabolism in Man*. Blackwell Scientific Publ., Oxford 1979.

Haematologia 17 (4), pp. 439–442 (1984)

Inhibition of Granulocyte-Macrophage (GM) Colony Formation by Sera of Patients with Neoplasma of Digestive Tract in the Course of Surgical Treatment

M. SZMITKOWSKI, J. PROKOPOWICZ, A. SIWICKA

Department of Clinical Diagnostics, Institute of Biochemistry and Medical Analytics, Medical School Bialystok, M. Sklodowska-Curie 24A, 15-176 Bialystok, Poland

(Received 8 November 1982; revised 7 April 1983; accepted 19 September 1983)

The effect of sera from patients with neoplasm of the digestive tract obtained during intervention has been tested on granulocyte-macrophage (GM) colony formation. Independently of the source of blood (peripheral or tumour draining vein) and the time of sampling (during or 2–3 weeks after operation) the tested sera suppressed GM colony formation. The highest decrease was caused by blood sampled from the vein draining the tumour. It is concluded that tumour cells may be a source of the GM colony formation inhibitory activity. The possible character of the activity is discussed.

Keywords: GM colony formation, granulocytes, cancer

Introduction

The role of granulocytes in anticancer mechanism is well documented. It may be realized in two ways, by immediate phagocytosis [1] or by a cytotoxic effect [2] due mainly to the myeloperoxidase system [3].

On the other hand, *in vitro* proliferation and differentiation of granulocytes and macrophages is controlled by the glycoproteins termed colony stimulating factor (CSF) [4]. Function of CSF is intimately linked to inhibitors such as serum lipoproteins (inhibitor of CSF) [5], lactoferrin (inhibitor of CSF biosynthesis) [6] and specific chalones [7].

Concerning the regulation of granulopoiesis in patients with cancer, there are contradictory date. Tumour cells may be a source of CSF production [8, 9] or of inhibitory activity affecting GM colony formation [10].

Taking into account the role of granulocytes in cancer patients, we have focussed attention on the presence in serum of the inhibitory activity against GM colony formation.

Material and Methods

The investigations were carried out on sera obtained from 19 patients (7 men and 12 women, aged from 40 to 70) in whom cancer of the digestive tracts was diagnosed. The patients had neither radiotherapy nor chemotherapy.

The blood was collected from
A – peripheral vein of 10 controls,
B – vein draining the tumour (during surgical procedure),
C – peripheral vein (during surgical procedure)
D – peripheral vein, 2–3 weeks after the intervention.

Colony (> 50 cell/clone) formation was determined by plating 10^5 mouse ("Swiss") bone marrow nucleated cells in 1 ml of 0.3% agar medium (McCoy 5A with 15% Fetal Calf Serum), containing 0.05 ml of tested serum and 0.05 ml of stimulant (1 : 2 diluted serum of mouse treated with *Escherichia coli* endotoxin as an agent increasing the CSF level). Cultures were incubated at 37 °C in a humidified atmosphere containing 7.5% CO_2 in air for 1 week. Colonies and clusters were scored from 2–5 plates for each assay point [11, 12].

Results

The tested control sera showed a great variation of the colony count ranging from 29 to 78 (mean, 57). This mean value was arbitrarily accepted as a level of inhibition in the sera of the control group. Values above this level were considered to be under the effect of stimulation and those below this level to mirror an inhibition of GM colony formation. (Fig. 1).

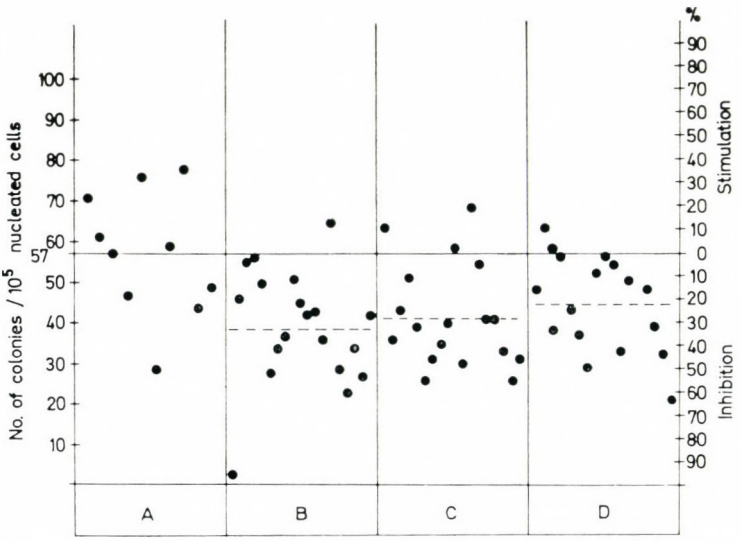


Fig. 1. Effect on granulocyte-macrophage colony formation of sera from patients with cancer of digestive tract. Symbols: ——— = mean value of control sera; – – – – = mean values of tested sera; A = control sera; B = sera from blood draining the tumour; C = sera from peripheral blood (during surgical operation); D = sera from peripheral blood (after surgical operation)

The serum samples of patients, independently from the source of blood and from the time of sampling, evidently inhibited GM colony formation. The inhibitory activity was most evident with sera obtained from a vein draining the tumour. With blood sampled 2–3 weeks after tumour excision, the inhibitory activity was somewhat weaker, but even in this case the values were lower than in the controls. It was remarkable that only a single patient's serum exerted a stimulatory effect, whereas among the control sera about 50% had such activity. Between control and patient sera there was a statistically significant difference but this was not the case with the patient sera of groups B–D.

Discussion

The blood serum of patients with digestive tract tumour exhibited an inhibitory activity on GM colony formation and the question arose concerning the source of that activity.

It has been concluded that only the tumour could be responsible for the activity, as shown by the difference between the blood obtained from a vein draining the tumour and the blood from a peripheral vein. The inhibitory substance may be present for a long time in the circulating blood, since the reticuloendothelial system is always depressed in tumour patients. Our earlier investigations done on animal Ehrlich sarcoma cells confirmed the presence of an 8 500–10 000 molecular weight peptide that had a strong inhibitory activity of GM formation [13].

At present we cannot precisely define the kind of activity responsible for the inhibition of GM colony formation in patients with cancer of the digestive tract. Our testing system gives no possibility to point to the inhibitor of CSF biosynthesis; but in our opinion the participation of the lipoprotein inhibitor of CSF should be taken under consideration. In keeping with such grounds the most attractive hypothesis would indicate the peptides like chalones [13].

Analysis of data in the literature and the present results may indicate an immediate relationship of neoplasm, regulation of granulopoiesis and function of granulocytes. It has been shown that granulocytes may destroy cancer cells [14], while cancer may affect granulopoiesis and the granulocyte count. Therefore, we may postulate that cancer brings about a diminution of the granulocyte count [10, 13] and a weakening of the granulocyte function [15].

The question of monitoring and diagnosing cancer is poorly understood. Determination of the activity inhibiting GM colony formation may be recommended as a test of the biological activity of cancer. One may assume that determination of its inhibitory activity may indicate the expansion of the tumour and also the clinical condition of the patient.

References

1. The, T. M., Eibergen, R., Lamberts, H. B., Oldhoff, J., Ploeg, E., Schrafford Koops, H., Nieweg, H. O.: Immune phagocytosis in vivo of human malignant melanoma cells. *Acta Med. Scand. 192*, 141 (1972).
2. Holtermann, O. A., Klein, E., Casals, G. P.: Selective cytotoxicity of peritoneal leucocytosis for neoplastic cells. *Cell. Immunol. 9*, 339 (1973).
3. Clark, B. A., Klebanoff, S. J.: Neutrophil-mediated tumor cell cytotoxicity: role of the peroxidase system. *J. Exp. Med. 6*, 1442 (1975).
4. Metcalf, D.: Regulation of hemopoiesis. *Nouv. Rev. Franç. Hématol. 20*, 521 (1978).
5. Chan, S. H., Metcalf, D., Stanley, E. R.: Stimulation and inhibition by normal human serum of colony formation in vitro by bone marrow cells. *Brit. J. Haemat. 20*, 329 (1971).
6. Broxmeyer, H. E., Ralph, P., Bognacki, J., Kincade, P. W., Desousa, M.: A subpopulation of human polymorphonuclear neutrophils contains an active form of lactoferrin capable of binding to human monocytes and inhibiting production of granulocyte-macrophage colony stimulatory activities. *J. Immunol. 125*, 903 (1980).
7. Paukovits, W. R., Hinterberger, W., Paukovits, J. B.: The granulocytic chalone—a specific inhibitor of granulopoiesis: molecular weight and chemical nature. *Oncology 34*, 187 (1977).
8. Sato, N., Asano, S., Ueyama, Y., Mori, M., Okabe, T., Kondo, Y., Ohsawa, N.. Kosaka, K.: Granulocytes and colony-stimulating activity (CSA) produced by a human squamous cell carcinoma. *Cancer 43*, 605 (1979).
9. Suda, T., Miura, Y., Mizoguchi, H., Kubota, K., Takaku, F.: A case of lung cancer associated with granulocytosis and production of colony-stimulating activity by the tumour. *Brit. J. Cancer 41*, 980 (1980).
10. Liu, Y. K., Stallar, S., Koo, V., Dannaher, Ch. L.: Serum inhibitor activity of granulocyte-macrophage colony formation in patients with cancer. *Cancer Res. 39*, 1640 (1979).
11. Bradley, T. R., Metcalf, D.: The growth of mouse bone marrow cells in vitro. *Aust. J. Exp. Biol. Med. Sci. 44*, 287 (1966).
12. Quesenberry, P., Morley, A., Stohlman, F., Rickard, K., Howard, D., Smith, M.: Effect of endotoxin on granulopoiesis and colony-stimulating factor. *N. Engl. J. Med. 286*, 227 (1972).
13. Szmitkowski, M., Garyel, H., Farbiszewski, R.: Inhibition of granulocyte-macrophage (GM) colony formation by basic polypeptides from mouse Ehrlich ascites tumor cells. *Experientia 37*, 1202 (1981).
14. Chee, D. O., Townsend, C. M., Galbraith, M. A., Eilber, F. R., Morton, D. L.: Selective reduction of human tumor cell population by human granulocytes in vitro. *Cancer 38*, 4534 (1976).
15. Cohen, M. C., Brozna, J. P., Ward, P. A.: In vitro and in vivo production of chemotactic inhibitors by tumor cells. *Am. J. Pathol. 94*, 603 (1979).

Haematologia 17 (4), pp. 443–448 (1984)

Possible Origin of Amniotic Fluid Constituents Influencing Fibrinolytic Activity

A. PAJOR, J. GRÓF, J. MENYHÁRT

Second Department of Obstetrics and Gynaecology, Semmelweis University Medical School Dept. of Clinical Biochemistry, Üllői út 78/a, 1082 Budapest, Hungary, and United Research Organization of the Hungarian Academy of Sciences, Budapest, Hungary

(Received 16 March 1983; accepted 20 June 1983)

An attempt was made to clarify the origin of the amniotic fluid constituents affecting fibrinolysis. For this purpose, saline extracts were prepared from various extrafetal gestational tissues (EGT) as well as from various fetal excretory products (FEP) and their effect on an euglobinolytic system was studied *in vitro*. It was found that extracts from FEPs stimulated, whereas those from EGTs inhibited fibrinolysis. It is concluded that the fibrinolysis inhibiting amniotic fluid constitutents originate from the placenta and the fetal membranes, whereas the fibrinolysis stimulating ones from various sources of fetal origin.

Keywords: fibrinolysis; pregnancy; fetal excretory products; fetal membranes; placenta

Introduction

It has repeatedly been demonstrated that among other factors, the fibrinolytic system is affected by amniotic fluid ingredients of unidentified chemical character [1, 2, 12, 14,20]. It has also been shown that the capacity of the amniotic fluid to influence fibrinolytic activity undergoes a characteristic change during pregnancy and parturition [1, 2, 13, 14]. We have recently demonstrated that such a change in the effect on fibrinolytic activity of amniotic fluid is regularly accompanied by simultaneous changes in its solute composition [14]. The origin of the substances contributing to the pregnancy related changes of the amniotic fluid remains, however, unresolved.

Methods

Extraction of extrafetal gestational tissues (EGT)

Placenta as well as amniotic and chorionic membraneous tissues were sampled immediately after the birth of mature fetuses. Chorionic villous tissue was obtained immediately after the termination of pregnancy in the 9th week of gestation. One g of amniotic and chorionic membraneous tissues, of chorionic

villous and placental tissues were homogenized in 10 ml ice cold normal saline solution by using a teflon homogenizer (1500 rpm, 1 min), and centrifuged (4500 rpm, 15 min). The pellets were discarded and the supernatants stored at $-20\,^{\circ}C$ until use.

Extraction of fetal excretory products (FEP)

Discharged urine and meconium, and mucus aspirated from the upper respiratory tract were collected from mature newborns immediately after delivery and stored at $-20\,^{\circ}C$ until processing. The meconium and mucus samples (2 g) were mixed, stirred and agitated in 10 ml normal saline solution at room temperature for 15 min. After centrifugation, the supernatants and the untreated urine samples were filtered through common filter paper. The filtrates were kept at $-20\,^{\circ}C$ until use.

Assessment of fibrinolytic activity

0.1 ml euglobin solution prepared from normal plasma mixture by the method of Kaulla and Schultz [10] was added to 0.1 ml extract or filtrate. Subsequently, 0.1 ml thrombin (Topostasin, Roche) solution was added to the mixture. The time required for the visually detectable lysis of the clot formed was measured at 37 °C. In control measurements, extracts and filtrates were substituted for normal saline solution. The effect was expressed as the ratio of control time to test time.

Results

Results are demonstrated in Table 1 and Figure 1. Normal saline extracts from various EGTs were found to inhibit fibrinolysis differentially. If the inhibitory effects were related to a unit wet weight of a particular tissue, the term placenta showed the strongest inhibition, while chorionic membraneous tissue (40th week of gestation), amniotic membraneous tissue and chorionic villous tissue (9th week of gestation) were less inhibitory in this order. On the other hand, saline extracts from all FEPs were found to stimulate fibrinolysis.

The fibrinolysis inhibitory effect of extracts of the amniotic and chorionic membraneous tissues was found to be dose-dependent. The dose–response curves of the two extracts ran parallel in concentrations ranging from 3.1 to 25.0×10^{-3} g/ml times and diverged in those ranging from 25.0 to 100.0×10^{-3} g/ml. The fibrinolysis stimulating effect of the meconium extract and of fetal urine was

Table 1

Effect on fibrinolysis *in vitro* of extracts from gestational tissues, fetal excretory products and of fetal urine

Extracts (n = 3) (0.1 g/ml)[1]	Effect on fibrinolytic activity (control time to test time: $\bar{x} \pm SD$)
Amniotic membrane[2]	0.73 ± 0.03
Chorionic membrane[2]	0.51 ± 0.06
Chorionic villus[3]	0.75 ± 0.02
Placenta[2]	0.33 ± 0.23
Mucus from respiratory tract[2]	1.04 ± 0.00
Meconium[2]	1.29 ± 0.10
Fetal urine[2,4]	1.69 ± 0.02

1: wet weight concentration 2: samples from 40th week of gestation 3: samples from 9th week of gestation 4: undiluted urine

also dose-dependent. Extract of mucus of respiratory tract origin was found to stimulate fibrinolysis in concentrations ranging from 3.1 to 12.5×10^{-3} g/ml and in those ranging from 12.5 to $100.0 \times {}^{10-3}$ g/ml its effect was diminished in a dose-dependent manner.

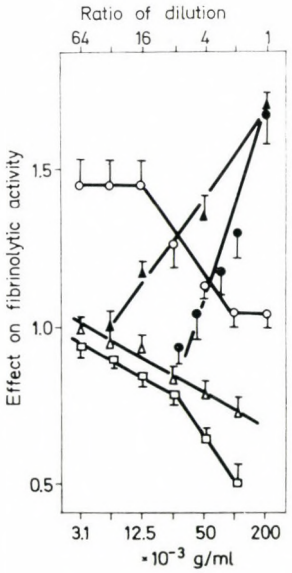


Fig. 1. Effect on the fibrinolysis *in vitro* of extracts from amniotic (△) and chorionic (□) membraneous tissues, from mucus of respiratory tract origin (○), from meconium (●), and of fetal urine (▲). Ordinate: ratio of control time to test time. Abscissa: wet weight concentration of the extracts and ratio of dilution of urine samples, respectively

Discussion

Solutes in the amniotic fluid may originate from the fetus itself (e.g. from its respiratory, urinary and alimentary tracts), from EGTs (e.g. placenta, fetal membranes) or from maternal sources (e.g. decidua, myometrium) [16]. In accordance with other literary data, our previous observations also indicated that in amniotic fluid, factors stimulating or inhibiting fibrinolysis may be present [12, 14, 17]. It has also been shown that the capacity of amniotic fluid to stimulate or inhibit fibrinolysis and thereby the relative concentration of amniotic fluid components affecting fibrinolysis in opposite direction changed gradually in the course of pregnancy, and dramatically during parturition [1, 13, 24, 25].

The data presented indicate that factors inhibiting fibrinolysis can be extracted from EGTs. This is in good agreement with previous observations that fibrinolysis inhibiting peptides with both large and small molecular mass could be isolated from the placenta [8, 19], and that both large and small molecular mass components of unidentified chemical character but with similar biological activity were found in amniotic fluid as well [14, 18, 20]. These findings suggest that fibrinolysis inhibiting factors in amniotic fluid might originate from the placenta and fetal membranes.

In the present paper it has also been demonstrated that FEPs had a fibrinolysis promoting activity. On the other hand, earlier evidence indicated that mucus from the upper respiratory tract of the fetus is easily mixed to the amniotic fluid [11]. It is also generally known that fetal urine is a natural component of amniotic fluid. In this context it is of particular interest that, immediately after delivery, blood with reduced fibrinolytic activity flows from the placenta to the fetus, while an enhanced fibrinolytic activity could be demonstrated in blood streaming in the opposite direction [5]. Consequently, the fibrinolysis promoting factors present in amniotic fluid appear to be of fetal origin.

It was also observed that the fibrinolysis promoting effect of the mucus from the respiratory tract increased paradoxically with increasing dilution. This finding may be interpreted by assuming that both fibrinolysis activating and inhibiting factors are present in the mucous extract but in different concentrations. In such an extract, the effect of the factors present in higher concentration would partially be counteracted or blocked by those with opposite effect and present in lower concentration. With increasing dilution, the latter would gradually be "outdiluted", their counteracting effect diminish and finally be abolished. At the same time, with a certain increase of dilution, the factors present in higher concentration would become less and less counteracted and their effect gradually strengthened and finally become dominant.

It was shown earlier that the fibrinolysis stimulating effect of amniotic fluid was increased at the onset and remained pronounced in the course of labour [13, 15]. It was also observed that the fibrinolysis stimulating capacity of amniotic fluid collected during labour, increased parallel with advancing pregnancy [13, 15]. All these data seem to indicate that during labour it is the fetus itself who activates

the fibrinolytic system. This capacity appears to increase parallel with fetal maturation. The fibrinolytic activity of maternal blood plasma also changes significantly during pregnancy and parturition [3, 4, 6, 7, 9]. These changes might well be due to the factors released from EGTs and the fetus itself.

References

1. Aznar, J., Hernandez, A., Parrilla, J. J., Estelles, A., Gilabert, J.: Changes in human amniotic fluid fibrinolytic inhibitor levels during pregnancy. *Thrombos. Haemostas. (Stuttg.) 35*, 382 (1976).
2. Aznar, J., Gilabert, J., Estelles, A., Parrilla, J. J.: Evaluation of plasminogen and other fibrinolytic parameters in the amniotic fluid. *Thrombos. Haemostas. (Stuttg.) 43*, 182 (1980).
3. Bonnar, J., McNicol, G. P., Douglas, A. S.: Coagulation and fibrinolytic mechanism during and after normal childbirth. *Brit. Med. J. 2*, 200 (1970).
4. Bonnar, J.: Fibrinolysis in human pregnancy. In: *Fibrinolysis: Current Fundamental and Clinical Concepts*. Gaffney, B. J. and Balkuv-Ulutin, S. (eds.) Academic Press, New York 1978 pp. 173–186.
5. Foley, M. E., Clayton, J. K., McNicol, G. P.: Haemostatic mechanism in maternal, umbilical vein and umbilical artery blood at the time of delivery. *Brit. J. Obstet. Gynaec. 84*, 81 (1977).
6. Gillabert, J., Aznar, J., Parrilla, J. J., Reganon, E., Villa, V., Estalles, A.: Alterations in the coagulation and fibrinolysis system in pregnancy, labour and puerperium, with special reference to a possible transitory state of intravascular coagulation during labour. *Thrombos. Haemostas. (Stuttg.) 46*, 387 (1978).
7. Howie, P. W.: Blood clotting and fibrinolysis in pregnancy. *Postgrad. Med. J. 55*, 362 (1979).
8. Kawano, T., Morimoto, K., Kemura, Y.: Urokinase inhibitor in human placenta. *Nature (Lond.) 217*, 253 (1968).
9. Konopka, P., Gaszynski, W., Nowak, J.: Essai d'explication du mécanisme du travail. I. Système de coagulation et de fibrinolyses au cours de l'accouchement physiologique. *Rev. franc. Gynec. Obstet. 68*, 1 (1973).
10. Kaulla, K. N., Schultz, R. L.: Methods for the evaluation of human fibrinolysis. *Amer. J. Clin. Path. 29*, 104 (1958).
11. Müller-Tyl, E., Szalay, S.: *Intrauterine measurements of tracheal pressure in the fetal lamb*. Abstracts. 7th Eur. Congr. of Perinatal Medicine Barcelona, 1980, p. 174.
12. Pajor, A., Gróf, J., Menyhárt, J., Zsolnai, B.: A magzatvíz fibrinolízist befolyásoló hatásának tanulmányozása in vitro. *Kísérl. Orvostud. 33*, 630 (1981).
13. Pajor, A., Gróf, J., Menyhárt, J., Zsolnai, B.: A vérplazma és a magzatvíz fibrinolitikus aktivitásának alakulása szülés és prosztaglandin F_2alfával indukált vetélés alatt. *Kísérl. Orvostud. 33*, 597 (1981).
14. Pajor, A., Gróf, J., Menyhárt, J.: A fibrinolízisre ellentétesen ható komponensek kimutatása terhesség és szülés alatt nyert magzatvíz mintákban. *Kísérl. Orvostud. 34*, 216 (1982).
15. Pajor, A., Gróf, J., Menyhárt, J.: Effect of amniotic fluid on haemostatic systems in vitro. In press.
16. Seeds, A. E.: Current concepts of amniotic fluid dynamics. *Amer. J. Obstet. Gynec. 138*, 575 (1980).
17. Uszynski, M., Uszynski-Folejewska, R.: Plasminogen activator and urokinase inhibitor in myometrium, placenta and amniotic fluid. *Amer. J. Obstet. Gynec. 105*, 1041 (1969).

18. Uszynski, A., Beller, F. K.: Low molecular weight peptides with anti-thrombin and anti-urokinase activity isolated from human maternal and cord blood serum and amniotic fluid. *Arch. Gynaec. 221*, 5 (1976).
19. Uszynski, M.: Isolation of peptides with anti-urokinase activity from the human placenta. *Thrombos. Haemostas. (Stuttg.) 42*, 1411 (1980).
20. Walker, J. E., Campbell, D. M., Ogston, D.: Inhibitors of fibrinolysis in amniotic fluid. *Thrombos. Haemostas. (Stuttg.) 44*, 32 (1980).

Haematologia 17 (4), pp. 449–453 (1984)

Occupational Exposure to Benzene, Toluene and Xylene and the T Lymphocyte Functions

P. MOSZCZYNSKY, J. LISIEWICZ

Immunological Laboratory, Regional Hospital, Brzesko, Poland, and Haematological Clinic of the Institute of Internal Medicine, Medical Academy, Kopernika Str. 17-501, Cracow, Poland

(Received 11 April 1983; accepted 12 August 1983)

In 72 workers having occupational contact with benzene, toluene or xylene during 31 to 122 months, the T lymphocyte functions were evaluated. Workers after a service of 55–122 months showed a decreased T lymphocyte count without, however, alterations in their functions. No clinical signs of diminished immunological reactivity were noted in the subjects studied. The E rosette test is of importance in the evaluation of early effects on the lymphocyte system of the toxic action of benzene and its homologues.

Keywords: occupational contact with toxic solvents, T lymphocytes

Deaths caused by lymphoid malignancies such as chronic lympocytic leukaemia, sarcomas and Hodgkin disease are comparatively frequent among workers having occupational contact with benzene [7, 13], in contrast with previous epidemiological studies indicating that such workers suffer mainly from acute myelogenous leukaemia [2, 3, 14]. We have reported earlier that subjects exposed to benzene, toluene or xylene show significant changes in the enzymatic composition of white blood cells and increased permeability of lymphocyte lysosomal membranes, resulting in release of those enzymes into the cytoplasm [8, 9, 10]. The purpose of the present studies was to evaluate the effect of environmental contamination with benzene, toluene and xylene on some functions of T lymphocytes, which are cells involved in antitumour immunity.

Material and Method

In the study 72 workers were involved having occupational contact with benzene, toluene and xylene during varnishing metal sheets used subsequently for packaging. All workers were incessantly in contact with the above compounds for 7–8 hours on working days 6 days every week. Out of 72 workers, 33 subjects (15 women and 18 men) aged 21 to 49 years were exposed to the above substances for 31 to 54 months ($\bar{x} = 41.4 \pm 6.14$ months), while 39 subjects (17 women and 22 men) aged 22 to 56 years have had exposures of 55–122 months ($\bar{x} = 92.9 \pm \pm 6.14$ months). The control group consisted of 38 workers (19 women and 19 men) between 21 to 55 years, of the same industrial establishment. The microclimate con-

ditions of the control group were the same as of the exposed group, only the control subjects had no contact with chemicals, especially not with benzene, toluene and xylene. Both the control group and the exposed subjects lived in the same geographical region, had similar dietary customs, comparable economical and social status.

The benzene, toluene and xylene concentrations in the air of the working places is presented in Table 1 that contains also data on the individual evaluation

Table 1

Chemico-toxicological characteristics of benzene, toluene and xylene contaminated environment and the phenol and hippuric acid concentration in the urine of exposed workers

Year of study	Chemical compound and its concentration in the air at working place in mg per m^3 (arithmetic mean and variability range)			Phenol tets mg/l (arithmetic mean and standard deviation)		Hippuric acid test mg/l (arithmetic mean and standard deviation)	
	Benzene	Toluene	Xylene	Workers not exposed	Workers exposed	Workers not exposed	Workers exposed
1968	98 (0–210)	120 (0–120)	119 (20–370)	—	—	—	—
1969	113 (0–370)	116 (0–580)	—	—	—	—	—
1970	50 (30–60)	— —	120 (110–130)	—	—	—	—
1971	—	—	102 (0–360)	—	—	—	—
1972	4 (0–20)	7 (0–30)	— —	—	—	—	—
1973	52 (15–120)	— —	— —	—	—	—	—
1974	—	—	71 (0–150)	—	—	—	—
1975	—	—	—	—	—	—	—
1976	18 (0–130)	25 (0–81)	— —	—	—	—	—
1977	17 (0.5–245)	—	17 (15–30)	7.7± 3.7	14.2± 9.6*	487± 334	812± 449*
1978	12 (5–17)	24 (0–93)	130 (10–506)	7.7± 3.7	13.8± 7.1*	487± 334	902± 596*
HPD*	100	100	100				
HPD	30	100	100				

HPD — highest permissible dose
HPD* — according to public law Dz. Ust. No 53, 1959
HPD — according to public law Dz. Ust. No 13, 1976
— — chemical estimation not performed
x — $p < 0.001$

of benzene and toluene uptake on the basis of the phenol and the hippuric acid tests described elsewhere [8].

The T lymphocyte count in venous blood was determined using the E rosette test. The rosette figures of lymphocytes with sheep erythrocytes were calculated after 18 h incubation. Isolation of lymphocytes and the E rosette test were performed according to the WHO instructions [1]. The lymphocyte transformation test was performed with the use of phytohaemagglutin (PHA) and the blastic index (percentage of cells undergoing blastic transformation) as well as the mitotic index (number of cells with mitotic figures calculated from 1000 lymphocytes) were determined. Lymphocyte blastogenesis was induced in cultures containing $1.5–2.0 \times 10^6$ cells to which 10 μg of PHA (Difco) was added per 1 ml of lymphocyte suspension. The culture was kept at 37 °C for 72 hours. Smears of the culture were stained with May-Grünwald-Giemsa stain. The tuberculin test was performed by intracutaneous injection of 2 units of RT_{23} tuberculin of Polish production; the diameter of infiltration was measured after 72 hours. The distreptase skin test was performed using an aqueous solution of 12 units of streptokinase and 3 units of streptodornase of Polish production; the diameter of infiltration was measured after 48 hours.

HB_sAg and anti-HB_sAg antibodies in serum were determined by electroimmunoprecipitation in 1% agar gel buffered with veronal.

Results

Examination of the workers studied revealed no disease having an effect on the immune system. Some of them showed the presence of HB_sAg or anti-HB_sAg antibodies. The frequency of respiratory and urinary tract infection was

Table 2

T lymphocyte function in workers exposed to benzene and its homologues

Group		Total lymphocyte count G/l	T lymphocyte count (E rosette test) G/l	Lymphocyte blastic transformation		Skin reactions	
				Blastic index per cent	Mitotic index per cent	Tuberculin test mm	Distreptase test mm
Healthy subjects	$\bar{X}$	2694	1849	59.7	58.0	9.8	12.5
n = 38	SD	0790	0551	5.6	3.2	0.3	0.8
Workers exposed for	$\bar{X}$	2529	1678	61.3	60.8	9.3	13.9
31 to 54 months	SD	0967	0685	7.8	5.4	0.8	1.1
n = 33							
Workers exposed for	$\bar{X}$	2208**	1501**	56.2	60.1	10.2	11.9
55 to 122 months		0623	0407	4.9	3.6	0.9	1.0
n = 39							

** — $p < 0.01$
n — number of workers studied

similar in the exposed and the control group. Total lymphocyte counts were diminished in the workers exposed to benzene and its homologues for more than 55 months (Table 2). No disturbance of T lymphocyte function was noted in the subjects studied.

Discussion

In laboratory animals poisoned with benzene a lymphocytopenia due to reduction of the T cell count was noted [4, 5, 6]. There are suggestions that the phenomenon was due to the activation of free radical processes inhibiting T lymphocyte proliferation. In mice intoxicated with benzene an increased concentration of free radicals in the hepatocyte mitochondria was shown by the paramagnetic resonance method [12]. Free radicals inhibit the in vitro lymphocyte blastic transformation in the PHA culture [11].

Our studies indicated a reduction of the T cell count in subjects having occupational contact with benzene and its homologues. The reduction might be due to the depressive effect on the lymphocyte system of benzene, especially of its free radical form – benzene epoxide. Benzene epoxide represents a transitional form resulting from the reaction of benzene and active radical oxygen which is rapidly metabolized to phenol and pyrocatechol.

It has to be emphasized that the T lymphocyte reduction was not accompanied by alterations of skin reactivity against the antigens used, and of blastic transformation of lymphocytes in the PHA culture. Moreover, the workers studied were not prone to respiratory and urinary tract infections more than the subjects of the control group.

Up to now no screening test has been elaborated to reveal early morphologic and functional alterations of lymphocytes caused by exposure to benzene and its homologues. Our studies indicate that a longer exposure to these compounds results in diminution of the T cell count. The frequent occurrence of lymphoid malignancies among workers exposed to benzene [7, 13] call for periodical control of the T cell count by the E rosette test in subjects exposed to benzene and its homologues.

References

1. Ainti, P., Cerottini, J. C., Coombs, R. R. A., Dicler, H. D., Proland, S. S. Fundenberg, H. H., Greaves, M. F., Grey, H. N., Kunkel, H. G., Natvig, J. B., Preud'homme, J. L., Rabellino, S., Ritte, R. E., Rome, D. S., Seligman, N., Siegal, F. P., Stjernswärd, J., Terry, W. D., Wybran, J.: Special Technical Report. Identification, enumeration, and isolation of B and T lymphocytes from human peripheral blood. *Scand. J. Immunol. 3*, 521 (1974).
2. Aksoy, M.: Problems with benzene in Turkey. *Reg. Toxicol. Pharmacol. 1*, 147 (1981).

3. Infante, P. F., Wagener, J. K., Kinsky, R. A., Young, R. J.: Leukaemia in benzene workers. *Lancet 2*, 76 (1977).
4. Karalov, A. V., Frash, V. N.: The mice lymphoid tissue after immunization with benzene. *Fiziol. Zh. (Kiev) 22*, 696 (1976).
5. Karaulov, A. V., Frash, V. N.: A study of the content of T- and B-lymphocytes in clinical and experimental hypoplastic conditions of hemopoiesis. *Probl. Gematol. Pereliv. Krovi 3*, 17 (1977).
6. Karaulov, A. V., Frash, V. N.: Remarks on method of evaluation of the T and B lymphocytes changes (according to experimental model of intoxication with benzene. *Zh. Microbiol. Epidemiol. 9*, 42 (1976).
7. McMicheal, A. J., Spirtas, R., Kupper, L. L., Gamble, J. F.: Solvent exposure and leukaemia among workers: an epidemiologic study. *J. Occup. Med. 19*, 234 (1965).
8. Moszczynski, P.: Cytoenzymatic studies on neutrophils in workers having contact with organic solvents containing benzene, toluene and xylene. *Fol. Haemat. 109*, 747 (1980).
9. Moszczynski, P.: The effect of work environment contaminated with organic solvents on the enzymatic composition of neutrophils. *Haematologica 67*, 482 (1982).
10. Moszczynski, P.: The organic solvents and beta-glucuronidase of leucocytes. *Med. Lav. 73*, 160 (1982).
11. Sagone, A. Jr., Cambell, R., Kamps, S.: Impaired lymphocyte function by oxygen radicals. *Clin. Res. 24*, 336A (1976).
12. Sergiejev, A. I., Najdicz, W. I., Bunto, T. W.: Alterations of paramagnetic properties of mice liver tissue after exposure to benzene vapors. *Dokl. Akad. Nauk. SSSR 234*, 234 (1977).
13. Vienna, N. J., Polan, A.: Lymphomas and occupational benzene exposure. *Lancet 1*, 1394 (1979).
14. Vigliani, E. C.: Leukemia associated with benzene exposure. *Trans. N. Y. Acad. Sci. 271*, 143 (1976).

Haematologia 17 (4), pp. 455–464 (1984)

Today's Treatment of Non-Hodgkin's Lymphomas

P. KLENER

Second Department of Medicine, Charles University Hospital,
U nemocnice 2, 128-08 Prague, Czechoslovakia

(Received 15 March 1983; accepted 3 May 1983)

Non-Hodgkin's lymphomas (NHL) are a heterogeneous group of lymphoid malignancies with different histology, clinical presentation, prognosis and response to therapy. The choice of the best therapeutic regimen is difficult and has to be settled according to the histology and the extent of the disease. All major histopathological classifications divide NHL into two groups, one with a long history (low grade malignancy, favourable histology, indolent) and those with a short history (high grade malignancy, unfavourable histology) [19]. We shall survey the modern treatment for both groups, the role of maintenance therapy and the problems arising during therapy and relapses, and mention some new drugs which appear promising in the treatment of lymphomas. Finally, a short outline of future prospects will be presented.

Keywords: Non-Hodgkin's lymphoma, treatment perspectives.

*

Therapy of indolent NHL

It has not been settled whether a deferral of initial treatment was a better approach than the immediate onset of therapy. Withholding of initial therapy might be regarded as reasonable from several points of view [21]. The disease is only slowly progressive and spontaneous regression of the disease may occur in as many as 8% of the cases [15]. Furthermore, the disease may evolve to a more aggressive, but paradoxically more "curable" histology [23]. It has been demonstrated that unlike lymphomas with unfavourable histology, indolent lymphomas are not curable, their relapse-free survival curve shows no suggestion of a plateau [20]. On the other hand, in more aggressive NHL the remission duration curve tends to form a plateau, and thus may indicate cure [26]. However, the watch and wait approach require close and careful follow-up, as the progression may be silent and thus the results of therapy once it is initiated may be compromised by the advanced state of the disease. These and some other reasons prompt us to introduce treatment immediately when the diagnosis has been confirmed. Nevertheless, the choice of initial treatment is even more controversial than the question of withholding initial therapy.

Hundreds of treatments have been given using or combining all anticancer treatment protocols (Table 1). Surgical removal of lymphoma is beneficial particularly in cases of extranodal presentation. Surgery might play a role even in advanced disease. The so-called "debulking operation" may render the lymphoma

Table 1

Different approaches in management of indolent NHL

Stage of the disease	Surgery	Radiotherapy	Chemotherapy	Immunotherapy	Supportive treatment
I	extranodal disease	involved field	—	—	—
II	—	extended field	adjuvant ??	—	—
III	—	total nodal irradiation or whole body irradiation	single agent therapy or better combination chemotherapy	BCG? Corynebacterium-parvum?	useful to manage side effects of chemotherapy and/or radiotherapy
IV	"debulking" operation	supplementary irradiation of areas of bulk disease	(COP, ABP, COPP)	Levamisole?	

sensitive to systemic treatment [4]. Radiotherapy is considered the treatment of choice in localized disease. The most frequently applied form is extended field irradiation, although some prospective trials showed no difference in disease-free interval and survival time between patients treated with extended field irradiation and involved field irradiation [18]. The former seems to be more appropriate, as in clinical practice the most patients are staged only clinically, and in such cases advanced disease cannot be ruled out (only 1 % of NHL are presenting in stage I). Stage III and IV are usually treated with chemotherapy, although adequate irradiation may ensure comparable results. Total nodal irradiation or whole body irradiation, sometimes with boost irradiation to areas of bulk disease, are suitable methods in advanced disease [5]. Whole body irradiation as a systemic treatment offers some theoretical advantages over total nodal irradiation. The techniques for NHL patients should be distinguished from techniques used for treatment of Hodgkin's disease. A widefield approach to the abdomen is used because of the high tendency to mesenteric nodal involvement even at presentation. Conversely, the infrequent involvement of the mediastinum at presentation argues for alteration of supradiaphragmatic radiation techniques, the so-called "mini-mantel", to limit it to heart and lung irradiation.

Regardless of the good results of radiotherapy, chemotherapy is the popular treatment modality in advanced disease. Table 2 summarizes some drugs commonly used in the treatment of NHL. It seems logical that simultaneous administration of two or several drugs will ensure a higher response rate. However, in recent years several authors have shown that single agent chemotherapy could be as effective as combination chemotherapy in terms of response rate, remission duration and survival [11]. In spite of this, there are some arguments which speak in favour of

combination chemotherapy. For example, with combination chemotherapy, less time is required to induce complete remission (about 6 months) than with single agents (as long as 20 months). The advantage of combination chemotherapy seems to be supported also by the recent results of NCI-group demonstrating that after combination of COPP no late relapses have been noted [1]. A longer follow-up is, however, necessary before this combination could be designated as curative. Some frequently used combinations for indolent NHL are listed in Table 3. The choice

Table 2

Response rate of NHL to single drugs
(after Frei, *Cancer Treat. Rep. 61*, 1977, 1209)

Cytotoxic drug	Response to therapy per cent
Cyclophosphamide	50–70
N-mustard	40–70
Chlorambucil	30–60
Procarbazine	20–50
Prednisone	30–80
Vinblastine	15–35
Vincristine	40–80
VM-26 (teniposide)	30–60
Adriamycin	35–65
Bleomycin	30–40
BCNU, CCNU	20–35
DTIC	10–25
Methotrexate	15–25
Cytosine arabinoside	15–20

Table 3

The most frequently used combinations in treatment of indolent NHL

Combination	Drugs in combination	Dosage (mg/m^2)	Day(s) of administration
COP (CVP)	cyclophosphamide	400	1 to 5
	vincristine	1.4	1
	prednisone	100	1 to 5
ABP	doxorubicin	75	1
	bleomycin	15	1 and 8
	prednisone	100	1 to 5
COPP (C-MOPP)	cyclophosphamide	650	1 and 8
	vincristine	1	1 and 8
	procarbazine	100	1 to 14
	prednisone	40	1 to 14

between single agent and combination chemotherapy should be probably influenced by some prognostic criteria which cannot be detailed here.

In the last decade, many papers have appeared suggesting that immunotherapy with BCG, Corynebacterium parvum or levamisole, would prolong the disease-free interval. Some other prospective trials failed, however, to confirm that immunotherapy could ensure a complete remission or its durability. Thus the question of immunotherapy for indolent NHL remains open.

Therapy of high grade malignancy NHL

High grade malignancy NHL needs aggressive treatment (see Table 4). While there is little to recommend radical surgery for localized tumours, there may be some exceptions, such as the extranodal presentation of the disease. Experience was favourable with surgical resection of the bulk of abdominal masses in Burkitt

Table 4

Different approaches in management of unfavourable NHL

Stage of the disease	Surgery	Radiotherapy	Chemotherapy	Immunotherapy	Supportive treatment
I	Extranodal disease	Extended field	Adjuvant?	—	—
II	—	Whole body irradiation?	Combination chemotherapy	—	—
III	—	Supplementary irradiation to areas of bulk disease	(CHOP, BA-COP, COMLA etc.)	???	Necessary to manage side effects of aggressive chemotherapy
IV	Debulking operation splenectomy?				

lymphoma, which rendered the patient curable with chemotherapy. Radiotherapy alone is recommended only for patients with pathological stage I, especially with supradiaphragmatic presentation. Stage II disease is better treated by chemotherapy [26]. Single agent chemotherapy is unsuitable, as it yields no more than 5% of complete responders [4]. The CVP regimen or its variations increased the complete remission rate to 15% but not the median survival time. The addition of newer agents and rational scheduling improve the potential cure which varies from 30 to 60%. The recruitment of resting cells by the cytotoxic effect of the initial agents and the rapid regrowth of residual tumour cells complicate the treatment of high grade malignancy NHL. The use of sequential antimetabolites may, however, allow for an optimal effect on recovering tumour cells. Most antitumour agents are myelotoxic and cannot be used safely where the bone marrow cells are recovering slow-

er than the tumour which is often the case. The property of leucovorin to reverse the toxicity of methotrexate allows to apply effective antitumour therapy during the periods of myelosuppression. In addition, antimetabolites cross the blood-brain barrier and therefore might possibly reduce relapses CNS. Table 5 summa-

Table 5

The most frequently used combinations in therapy of unfavourable NHL

Combination	Drugs in combination	Dosage (mg/m^2)	Day(s) of administration
CHOP	cyclophosphamide	750	1
	hydroxydaunomycin (doxorubicin)	50	1
	oncovin (vincristine)	1.4	1
	prednisone	100	1 to 5
BACOP	bleomycin	5	25 and 21
	doxorubicin	25	1 and 8
	cyclophosphamide	650	1 and 8
	oncovin (vincristine)	1.4	1 and 8
	prednisone	60	14 to 28
MEV	methotrexate	20	3
	cyclophosphamide	800	1
	vincristine	2	4
(A)-COMLA	adriamycin doxorubicin	40	1
	cyclophosphamide	1500	1
	(oncovin) vincristine	1.4	1.8 and 15
	methotrexate	120	22*
	leucovorin	25	**
	arabinosyl cytosine	300	23
M-BACOD	bleomycin	4	1
	(adriamycin) doxorubicin	45	1
	cyclophosphamide	600	1
	(oncovin) vincristine	1	1
	dexamethasone	5	1 to 5
	methotrexate	3000	14

* The same dose of methotrexate and ara-c is repeated 7 times in weekly intervals.
** Leucovorin is administered after each injection of methotrexate 6 times in 6 hour interval.

rizes the most widely used regimens for high-grade malignancy NHL. There are, of course, many variations and modifications of the presented combinations [3, 17] and it is difficult to compare the results and even those achieved with the same combination in different institutions, because of differences in patient selection, site of the disease, variable use of consolidation therapy, and particularly for lack of uniformity in restaging criteria for the definition of complete remission.

Following chemotherapy alone the majority of relapses occur at previously involved lymph node-sites. In contrast, following radiotherapy alone, recurrence in the treated area is unusual, whereas relapse in unirradiated lymph nodes or extranodal sites is the rule. That is why it has been postulated that the combination of both treatment modalities could improve the results. Unfortunately, combined modality approaches have been disappointing [12, 26], owing to additional toxicity, particularly prolonged bone marrow aplasia. Nevertheless, selected patients with bulky abdominal disease may benefit from supplementary radiation therapy or surgical debulking, or both. Immunotherapy does not seem to influence the duration of remission and survival in patients with unfavourable NHL. On the other hand, supportive treatment in an advanced disease is a salvage therapy and we cannot do without it.

Maintenance therapy

Many papers have been published demonstrating that some sort of maintenance therapy may influence the duration of remission and/or survival time [13]. It is clear that some patients with residual disease may benefit from additional induction therapy, but there is no evidence that any maintenance therapy is useful for patients in complete remission, provided the remission is really complete. Patients with unfavourable NHL who remain relapse-free after 2 years appear to be cured [4, 26]. In patients with indolent NHL, late relapse occur continuously, regardless of any maintenance therapy [20].

Therapy of relapses

The therapy of relapses remains an intriguing problem. There are several ways to overcome it.

1) Many alternative regimens were suggested, combining the drugs which have not been used for primary treatment. Such regimens are supposed to be non-cross-resistant. CVP and ABP regimens could serve an example of non-cross resistant combinations for advanced indolent NHL [2]. Similarly there are many alternative combinations at disposal for the therapy of unfavourable NHL [3, 17]. Unfortunately, the results are sometimes not as encouraging as expected, mainly because of toxicity.

2) That is why some non-toxic regimens were suggested, for example high dose methotrexate with leucovorin. These regimens sometimes bring about a dramatic response, which is often of rather short duration [14].

3) The uncertain results of therapy of relapses stimulated the search for new, modified regimens, using conventional drugs in different dosage, scheduling, and mode of administration. There have been attempts to use drugs such as bleomycin, vincristine or vinblastine in slow venous infusion [9]. This may permit a greater con-

centration of cytotoxic drug in time, so that a different spectrum or antitumour effect may emerge.
4) Combined therapy followed by bone marrow rescue deserves special attention [6]. In this regimen, patients receive high dose multiagent chemotherapy followed by total body irradiation. Then they are protected from marrow toxicity by the infusion of cryopreserved autologous bone marrow cells. Prior to marrow recovery, excellent supportive care must be given, including prophylactic antibiotic combinations to manage severe neutropenia and its complications. Most patients who had survived initial complications, displayed a beneficial response.
5) Thermochemotherapy means chemotherapy with whole body hyperthermia, by extracorporeal heating of the circulated blood or using heated blankets [8]. The damaging effect of hyperthermia may be greater in malignant cells than in their normal counterparts. There were also reports on attempts to reverse the drug resistance, for instance after pretreatment with amphotericin B [22].
6) Apart from these experimental approaches, trials with new drugs may sometimes bring surprisingly favourable results.

New drugs with promising activity in NHL

Table 6 summarizes some new drugs with promising activity in NHL. We may recognize three groups of new drugs. The first group contains derivatives of valuable compounds with known effectivity in NHL, which have been modified to attenuate their side effects without losing their cytotoxic power. AD-32 would have less cardiotoxicity than doxorubicin and a higher antitumour effect is claimed for pepleomycin, a bleomycin derivative. Vindesine has no cross-resistance to vin-

Table 6

Some newer drugs with promising activity in NHL

Derivatives of valuable compounds (original substance is in parenthesis)	Drugs originally used in the treatment of other malignancies	Drugs under investigation
(adriamycin) doxorubicin	Methyl-GAG	pyrazofurin
AD-32	hexamethylmelamin	mitoxanthrone
4'-epi-adriamycin	etoposide (VP-16-213)	spirogermanium
(bleomycin) pepleomycin	cis platinum	piperazine-dione
		peptichemio
		amsacrin (M-AMSA)
(cyclophosphamide)		
ifosfamide		
aldophosphamide		
(Vinca rosea alkaloids) vindesine		
(Chlorambucil + prednisone) prednimustin		

cristine and vinblastine. A useful drug for treatment of indolent NHL seems to be prednimustin, combining chlorambucil and prednisone.

In the second group are the agents originally designated for the treatment of other malignancies. Methyl-GAG was used for therapy of acute myeloblastic leukaemia, hexamethylmelamine for ovarian cancer, VP-16-213 for acute myelomonocytic leukaemia and cis-platinum for testicular tumours. Their activity in NHL has been demonstrated recently. Warrel [27] obtained 37% response in resistant NHL with methyl-GAG. More favourable seem to be VP-16-213 and cis-platinum [7].

The last group is composed of drugs under investigation. Although each of them has a definite effect in NHL, most remain to be of marginal significance in present day treatment. The only exception might be amsacrin (AMSA). The phase II clinical trials showed its significant activity in haematological malignances [10]. In spite of the effectivity of many of these new drugs, they still have a high toxicity which prevents to obtain better results. Nevertheless, search for new chemotherapeutic agents will remain the primary task for years to come.

Some future prospects of NHL therapy

A promising approach may be to influence the cell type from which the neoplasm is derived. If such properties are found drugs can be developed that act specifically against this cell type and do not affect normal cells. These new approaches may be directed either to metabolic or to immunological properties of the cell. Thus, future prospects may be directed by more selective and less toxic chemotherapy or a more sophisticated immunotherapy.

For example, a drug derived from this kind of research directed against the specific properties of the neoplastic target cell is 2 deoxycoformycin, an inhibitor of the enzyme adenosine deaminase, which is specifically required by the lymphocytes for their normal metabolism [25]. Another possibility might be an approach based on the immunological mechanism which undoubtedly play a part in the pathogenesis of lymphomas. Although previous attempts with passive serotherapy or active specific or non-specific boost of antitumour immunity had no definite effect the generation of monoclonal antibodies, reactive to human leukaemia and lymphoma cells, aroused interest in the passive administration of antibody in the treatment of cancer [24]. In recent years investigators have begun to utilize monoclonal antibodies in experimental models and several authors have shown that administration of monoclonal antibodies can be curative in certain situations [16]. There are, of course, some limiting factors such as the presence of circulating antigen, antigenic modulation, reactivity of monoclonal antibodies with normal cells or immune response to infused antibody. The requirement of antibody tailored to each patient puts some limitations of this approach. As monoclonal antibodies bind rapidly to target cells, they may be an ideal vehicle of cytotoxic agents delivered to tumour cells in vivo. The use of monoclonal antibodies as carrier molecules for cytotoxic drugs or plant toxin (e.g. ricin A-chain) may significantly increase the clinical effectiveness of antibody infusion.

Despite many imperfections in present day NHL therapy, it is hoped that future studies will find ways to overcome these problems and to find a cure for NHL patients.

References

1. Anderson, T., Bender, R. A., Fisher, R. I. et al.: Combination chemotherapy in non-Hodgkin's lymphom: Results of long-term follow-up. *Cancer Treat. Rep. 61*, 1057 (1977).
2. Bonadonna, G., Monfardini, S., Villa, E.: Non-cross-resistant combinations in stage IV non-Hodgkin's lymphomas. *Cancer Treat. Rep. 61*, 1117 (1977).
3. Bremer, K., Meusers, P., Brittinger, C.: Chemotherapie der Non-Hodgkin Lymphome. *Internist 21*, 512 (1980).
4. Canellos, G. P., Leonard, R. C.: Management of non-Hodgkin's lymphoma of unfavourable prognosis. *Cancer Top. 3*, 137 (1981).
5. Dobbs, H. J., Berrett, A., Rostom, Y. A. et al.: Total-body irradiation in advanced non-Hodgkin's lymphoma. *Brit. J. Radiol. 54*, 878 (1981).
6. Dauer, D., Gale, R. P.: Combined modality therapy and bone marrow autotransplantation in solid tumors and lymphomas. *Proc. AACR and ASCO* 1980, 21 Abstr. 671.
7. Hansen, M. M.: VP-16-213 in combination with cyclophosphamide doxorubicine, vincristine and prednisone in the treatment of non-Hodgkin's lymphoma. *Cancer Treat. Rep. 64*, 1135 (1981).
8. Herman, T. S., Zukoski, C. F., Anderson, R. M. et al.: Whole body hyperthermia and chemotherapy for treatment of patients with advanced refractory malignancies. *Cancer Treat. Rep. 66*, 259 (1982).
9. Hollister, D., Silver, R. T., Gordon, B. et al.: A new technique for treating resistant lymphoma. *Proc. AAR and ASCO* 1980, 21, abstr. 368.
10. Issel, B. F.: Amsacrine (AMSA). *Cancer Treat. Rep. 7*, 283 (1980).
11. Kennedy, T. J., Bloomfield, C. D., Kiang, D. T. et al.: Combination versus successive single agent chemotherapy in lymphocytic lymphoma. *Cancer 41*, 23 (1978).
12. Kinsella, T. J.: Role of combined-modality therapy in the curative management of advanced-stage "favourable" non-Hodgkin's lymphomas. *Cancer 66*, 421 (1982).
13. Klener, P., Donner, L., Ort, J.: Use of Vinblastine for maintenance therapy in malignant lymphomas. *Neoplasma 21*, 323 (1974).
14. Klener, P.: High dose methotrexate and leucovorin rescue therapy in malignant lymphomas (in Czech). *Vnitrni Lék.* 28,997 (1982)
15. Krikorian, J. G., Portlock, C. S., Cooney, D. P. et al.: Spontaneous regression of non-Hodgkin's lymphoma: a report of nine cases. *Cancer 46*, 2093 (1980).
16. Miller, R. A., Maloney, D. G., Warnke, R. et al.: Treatment of B-cell lymphoma with monoclonal antiidiotype antibody. *New Eng. J. Med. 306*, 517 (1980).
17. Muggia, F. M., Davis, H. L., Rosenzweig, M.: Current cooperative trials in the non-Hodgkin's lymphomas. *Cancer Treat. Rep. 61*, 1191 (1977).
18. Musshoff, K.: Radiotherapie der Non-Hodgkin Lymphome. *Internist 21*, 502 (1980).
19. Nathwani, B. N.: A critical analysis of the classification of non-Hodgkin's lymphomas. *Cancer 44*, 347 (1979).
20. Portlock, C. S.: Management of the indolent non-Hodgkin's lymphomas. *Semin. Oncol. 7*, 292 (1980).
21. Portlock, C. S.: Defferal of initial therapy for advanced non-Hodgkin's lymphomas. *Cancer Treat. Rep. 66*, 417 (1982).
22. Presant, C. A., Garrett, S.: Drug resistance: reversal by amphotericin B. *Proc. AACR and ASCO* 1980, 21, abstr. 552.

23. Risdall, R., Hoppe, R. T., Warnke, R.: Non-Hodgkin's lymphoma. A study of evolution of the disease based upon 92 autopsied cases. *Cancer 44*, 529 (1979).
24. Rits, J., Schlossman, S. F.: Utilization of monoclonal antibodies in the treatment of leukemia and lymphoma. *Blood 59*, 1 (1982).
25. Smyth, J. F., Chassin, M. M., Harrap, K. R. et al.: 2'-deoxycoformycin (DCF). Phase I trial and clinical pharmacology. *Proc. AACR and ASCO* 1979, 20, abstr. 47.
26. Sweet, D. L., Golomb, H. M.: The treatment of histiocytic lymphoma. *Semin. Oncol. 7*, 302 (1980).
27. Warrel, R. P., Straus, D. J., Young, C. W.: Combination chemotherapy for patients with relapsed malignant lymphoma using methyl-GAG and teniposid (VM-26). *Cancer Treat. Rep. 66*, 1125 (1982).

Haematologia 17 (4), pp. 465–471 (1984)

Improved Survival in Multiple Myeloma with Combination Chemotherapy and Plasmapheresis

A. WAHLIN, J. HOLM, L. NYSTRÖM

Department of Internal Medicine, University Hospital,
S-901 85 Umeå, Sweden

(Received 31 May 1983; accepted 23 July 1983)

The relation of different cytostatic regimens and plasmapheresis to survival time was studied in patients with stage III myeloma. The median survival time for 23 melphalan-prednisolone treated patients was 9 months. Nine melphalan-prednisolone treated patients, who were in addition treated with plasmapheresis survived longer (median 23 months), but the difference was not significant. Twenty-five patients, treated with plasmapheresis and cytostatic combination (melphalan, cyclophosphamide, BCNU, vincristine, prednisolone) survived significantly longer, for 61 months (median). Cytostatic combination therapy seems to prolong survival in stage III myeloma. Plasmapheresis may also have a favourable effect.

Keywords: cytostatics, multiple myeloma, plasma cell myeloma, plasmapheresis.

Introduction

A minority of patients with advanced multiple myeloma survive for more than one year without treatment [1]. In 1964, Waldenström [2] reported encouraging results with melphalan treatment. Alexanian and co-workers later reported that treatment with melphalan and prednisone in combination prolonged survival times in patients with multiple myeloma [3]. The melphalan-prednisone combination then became standard treatment for multiple myeloma. No single drug has been demonstrated to induce remission more effectively than melphalan [4]. During the last few years multiple drug combinations have also been used [5]. Drug combinations containing vincristine have been demonstrated to produce higher remission rates than the same combinations without vincristine [6]. In 1977, Case and co-workers reported higher remission rates and longer survival times with the M-2 protocol than other investigators using conventional therapy [7].

We have retrospectively studied survival times for patients with advanced myeloma during the period 1971–1980. During the first five years melphalan was the only cytotoxic drug which was used. During the period 1976–1978 most patients were treated with melphalan, cyclophosphamide and vincristine. Since 1979 all patients have been treated according to a modification of the M-2 protocol. During the ten-year period plasmapheresis was used on many patients. The aim of the study was to analyze the relationship of different cytotoxic therapies and plasmapheresis to survival times.

Material and Methods

Patients

During the ten-year period 1971–1980 105 patients with myeloma diagnosis were registered at the Department of Internal Medicine, University Hospital of Umeå. All patient data were reviewed. Two patients had solitary plasmocytoma. In 11 cases patient data were inconclusive or missing. The remaining 92 patients fulfilled the criteria for myeloma diagnosis proposed by the Chronic Leukemia and Myeloma Task Force [8]. Staging and subgrouping with respect to absence (A) or presence (B) of impairment of renal function (serum creatinine > 2 mg/dl) was performed according to Durie and Salmon [9]. Seventy-five patients had stage III disease, indicating high myeloma cell mass and poor prognosis (Table 1). The present study was confined to these 75 patients, 22 of whom were treated for stage III B disease. The median age of the stage III patients was 67 (range 41–88) years. Thirty-four were males, 41 females.

Table 1

Criteria for stage III myeloma [9]

One or more of the following findings:
B-hemoglobin < 85 g/l
S-calcium > 3 mmol/l
Multiple lytic bone lesions
IgG > 70 g/l or IgA > 50 g/l or urine light chains > 12 g/24 h.

Cytotoxic treatment

During the period 1971–1975 melphalan was the only cytotoxic drug which was used. A total dose of 50–100 mg of melphalan orally and 200–400 mg of prednisolone was given during each treatment cycle which was restarted every five weeks. A treatment-free interval of 10–14 days preceded the start of the next cycle. Thirty-two patients received melphalan-prednisolone treatment (Group M, Table 2).

During the period 1976–1980 most patients were treated with combinations of cytotoxic drugs. Eleven patients were treated with melphalan and prednisolone in the same doses and time intervals as Group M, but in addition these patients also received 1–2 mg of vincristine and 1000–2000 mg of cyclophosphamide during each five week cycle. Since 1979 all new stage III patients were treated according to the following protocol: vincristine 0.03 mg/kg b.w. intravenously day 1, BCNU 0.5 mg/kg b.w. intravenously day 2, cyclophosphamide 10 mg/kg b.w. intravenously day 2, melphalan 0.1 mg/kg b.w. daily orally days 2–5, melphalan 0.03 mg/kg b.w. daily orally days 6–26, prednisolone 1 mg/kg b.w. daily orally days 2–5. A new

Table 2

Clinical data of prognostic significance

	Combination drug therapy	Combination drug therapy and plasmapheresis	Melphalan therapy	Melphalan therapy and plasmapheresis
Age (years)	65±8	61±14	68±9	66±7
B-hemoglobin (g/l)	100±26	99±25	101±25	101±23
S-calcium (mmol/l)	2.5±0.3	2.5±0.3	2.5±0.4	2.5±0.3
Plasma cells (%)	39±24	40±25	39±27	24±13
Size of M-component:				
S-IgG (g/l)	55±31	50±30	49±18	51±18
S-IgA (g/l)	31±17	37±15	32±18	49±64
U-light chains (g/l)	8.1±7.2	9.8±8.0	6.4±6.3	
Number of patients	32	25	32	9

Mean values ± SD.

cycle was started on day 35. A total of 32 patients received one or another type of combination treatment (Group C, Table 2). Eleven patients with stage III myeloma received no cytotoxic treatment at all. Seven were diagnosed in the period 1971–1975, four during the period 1976–1978.

Plasmapheresis

Plasmapheresis was used on some patients during and after 1973. During the period 1973–1978 the access to plasmapheresis was limited, but increasing. Since 1979, all new stage III patients, a total of 34 were treated with plasmapheresis as a complement to cytotoxic treatment. A total of 34 patients were treated with plasmapheresis (Table 2). Patients were not randomized to plasmapheresis treatment, but they were treated so if our limited resources permitted it. There was no conscious selection of patients for plasmapheresis treatment, especially no regard was paid to the size of the M-component. Plasma exchange was performed with Haemonetics 30 on three consecutive days immediately before starting a new treatment cycle. The plasma volume exchange was 2000–3000 ml daily. Albumin was used as a plasma substitute.

Statistical methods

Life table analyses were performed and actuarial survival calculated according to the method of Kaplan and Meier [10]. Evaluation of the observed differences between the patient groups was done using Gehan's test [11]. Patients had been observed till death or June 1981 when the calculations were performed.

Results

The median survival time was longer for the multiple-drug combination group (C) (42 months) than for the melphalan-prednisolone group (M) (19 months). The difference was significant ($p < 0.001$, Fig. 1).

The median survival time for melphalan-prednisolone treated patients who were not subjected to plasmapheresis was 9 months (Fig. 2). Group M patients, who were in addition treated with plasmapheresis, survived longer (median 23

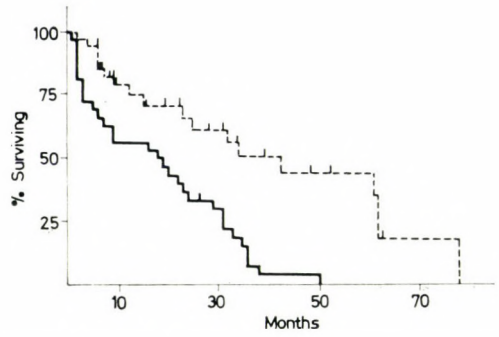


Fig. 1. Stage III myeloma. Relationship of drug regimen to survival. Patients treated with combination drug therapy had longer survival times than patients treated with melphalan-prednisolone ($p < 0.001$). - - - Combination therapy, 32 patients. —— Melphalan-prednisolone, 32 patients. | Patients still under observation

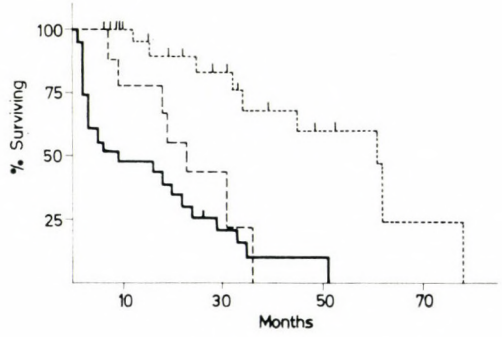


Fig. 2. Stage III myeloma. Relationship of plasmapheresis treatment to survival. Patients treated with combination drug therapy and plasmapheresis survived longer than patients treated with melphalan-prednisolone and plasmapheresis ($p = 0.006$). Patients treated with plasmapheresis in addition to melphalan-prednisolone survived longer than patients treated with melphalan-prednisolone alone, but the difference was not significant ($p = 0.17$). - - - Combination drug therapy and plasmapheresis, 25 patients. — — — Melphalan-prednisolone and plasmapheresis, 9 patients. —— Melphalan-prednisolone alone, 23 patients. | Patients still under observation

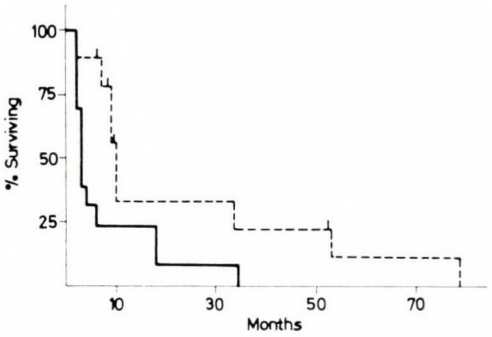


Fig. 3. Stage III B myeloma. Relationship of drug therapy to survival. Patients treated with combination drug therapy survived longer than patients treated with melphalan-prednisolone ($p = 0.03$). - - - Combination drug therapy, 9 patients. ——— Melphalan-prednisolone, 13 patients. | Patients still under observation

months), but the difference was not significant ($p = 0.17$, Fig. 2). Patients treated with cytostatic combination, Group C, and plasmapheresis survived for 61 months (median). The difference between plasmapheresis-treated Group M and Group C patients was significant ($p = 0.006$, Fig. 2).

The survival times for patients with impaired renal function, stage III B, were also analyzed according to treatment (Fig. 3). The median survival time for 13 patients treated with melphalan-prednisolone was 3 months. The median survival time for 9 patients treated with combinations of cytostatic drugs was 10 months. The difference was significant ($p = 0.03$). Two melphalan-prednisolone treated patients with stage III B disease were treated in addition with plasmapheresis. The combination-treated group of stage III B patients contained seven plasmapheresis-treated patients.

The median survival time for 11 patients treated neither with cytostatics nor with plasmapheresis was 1.5 months.

Patients with stage III myeloma had longer survival times when diagnosed during the period 1976–1980 (median 22 months) than during the period 1971–1975 (median 17 months), but the difference was not significant ($p > 0.05$).

Discussion

During the last five years the majority of the patients were treated with combination chemotherapy and plasmapheresis. Patients treated with combination therapy survived longer than patients treated with melphalan-prednisolone. The difference in survival time between the cytostatic treatment regimens was also evident in patients with very poor prognosis; stage III B patients treated with combination chemotherapy survived longer than patients treated with melphalan-prednisolone.

Patients receiving no cytotoxic treatment at all survived for 1.5 months (median). The difference in survival times between treated and untreated patients is not attributable only to the presence or absence of treatment. In some cases no treatment was started because the patients were regarded to be too ill to benefit from the treatment. Since 1979, however, all patients received combination treatment regardless of their condition. Consequently, a larger number of patients in critical condition were included in the combination group than in the melphalan-prednisolone group.

Plasmapheresis seems to be important for the survival time. Melphalan-treated patients who were treated with plasmapheresis survived longer than those who were not treated with the same. Also, patients treated with combination chemotherapy and plasmapheresis survived longer than those who were only treated with cytostatics. However, the difference in favor of plasmapheresis was not significant statistically. Yet, as far as we know, the median survival time of 61 months for stage III myeloma patients treated with combination chemotherapy and plasmapheresis was the longest median survival time reported for this category of patients.

The median survival time was found to increase insignificantly during the studied period of time. Woodruff and co-workers also found increasing survival times during the observation period 1956–1976 [1]. The median survival time for treated stage III patients studied by Woodruff and coworkers was only 6 months [1], a figure close to the median survival time of 9 months for those of our patients who were treated with melphalan-prednisolone alone. Other investigators have also reported survival times of one year or less for stage III patients treated with melphalan.

The slight improvement of survival times recorded during the last five years may be attributed to factors other than the intensified cytostatic treatment, for example better antibiotic treatment and improved treatment of hypercalcemia. In our clinical experience, however, such factors alone do not explain the substantial improvement of survival times in patients treated with combination chemotherapy and plasmapheresis. Our conclusion is that patients with stage III myeloma, especially stage III B disease, benefit from multiple drug therapy and probably also from plasmapheresis. As can be seen from Table 2, the parameters known to be of the greatest prognostic importance in myeloma, were essentially equal in the different treatment groups, so the different survival times are probably attributable to the different treatment schedules.

We cannot explain why patients treated with plasmapheresis had longer survival times than patients not receiving this treatment. One hypothesis is that some factor in plasma has a suppressive effect on DNA synthesis in myeloma cells. Lowering the concentration of this factor in plasma through plasmapheresis might increase the DNA synthesis in the malignant cell clone, thus rendering the cells more sensitive to cytostatic drugs. We have no experimental proof of this hypothesis, but it has recently been demonstrated that mouse myeloma cells produce factors capable of depressing antibody production [12].

In summary, it is reasonable to assume that the present finding of a median survival of 61 months for patients with stage III myeloma is due to the favorable effect of the combination of multiple drug chemotherapy and plasmapheresis. The possible effect of plasmapheresis on survival time will be studied in a prospective randomized trials.

*

This study was supported by grants from Norrlands Njursjukas forskningsfond, the Medical Faculty of Umeå and the Swedish Cancer Union.

References

1. Woodruff, R. K., Wadsworth, J., Malpas, J. S., Tobias, J. S.: Clinical staging in multiple myeloma. *Brit. J. Haemat. 42*, 199–205 (1979).
2. Waldenström, J.: Melphalan, therapy in myelomatosis. *Brit. Med. J. 1*, 859 (1964).
3. Alexanian, R., Haut, A., Khan, A. U., Lane, M., McKelvey, E. M., Migliore, P. J., Stuckey, W. J., Wilson, H. E.: Treatment for multiple myeloma: combination chemotherapy with different melphalan dose regimens. *J. Am. Med. Assoc. 208*, 1680–1685 (1969).
4. Vrana, M., Bunn, P. W. Jr.: A review of therapeutic trials in multiple myeloma and perspectives for future trials. In: *Progress in Myeloma. Biology of Myeloma.* Ed. Potter, M. Elsevier/North Holland, New York, Oxford, Amsterdam 1980. pp. 239–262 (1980).
5. Bergsagel, D. E., Bailey, A. J., Langley, G. R., MacDonald, R. N., White, D. F., Miller, A. B.: The chemotherapy of plasma-cell myeloma and the incidence of acute leukemia. *New Engl. J. Med. 301*, 743–748 (1979).
6. Alexanian, R., Salmon, S., Bonnet, J., Gehan, E., Haut, A., Weick, J.: Combination therapy for multiple myeloma. *Cancer 40*, 2765–2771 (1977).
7. Case, D. C., Lee, B. J., Clarkson, B. D.: Improved survival times in multiple myeloma treated with melphalan, prednisone, cyclophosphamide, vincristine and BCNU: M-2 protocol. *Am. J. Med. 63*, 897–903 (1977).
8. Committee of the Chronic Leukemia-Myeloma Task Force National Cancer Institute: Proposed guidelines for protocol studies. II. Plasma cell myeloma. *Cancer Chemotherapy Reports 4*, 145–158 (1973).
9. Durie, B. M. G., Salmon, S. E.: A clinical staging system for multiple myeloma. *Cancer 36*, 842–854 (1975).
10. Kaplan, E., Meier, P.: Nonparametric estimation from incomplete observations. *J. Am. Statist. Ass. 53*, 457–582 (1958).
11. Gehan, E. A.: A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. *Biometrika 52*, 203–223 (1965).
12. Kennard, J., Zolla-Pazner, S.: Origin and function of suppressor macrophages in myeloma. *J. Immunol. 124*, 268–273 (1980).

Haematologia 17 (4), pp. 473—481 (1984)

Heterogeneity of β-Thalassemia in Azerbaidzhan

L. V. DERGUNOVA,[1] A. P. RYSKOV, P. A. SLOMINSKY,[2]
R. SH. RUSTAMOV,[3] S. A. LIMBORSKA[2]

Institute of Molecular Biology, the USSR Academy of Sciences, Moscow[1]; Institute of Medical Genetics, the USSR Academy of Medical Sciences, Moscow[2]; Azerbaidzhan Institute of Hematology and Blood Transfusion, Baku[3], USSR

(Received January 23, 1983 accepted 12 July 1983)

Globin chain synthesis, RNA and D NA of β-thalassemic patients from Azerbaidzhan were analyzed. A β-mRNA deficiency was found in β^+-thalassemic reticulocytes. In one case of β^+-thalassemia, it was possible to study nuclear and cytoplasmic RNA from spleen erythroid cells. The α/β mRNAs sequence ratio was 5 in the nuclear and cytoplasmic RNAs. In patients with β°-thalassemia a different level of β-globin mRNA was demonstrated. Gene mapping analysis indicated that the general organization of the β-globin gene was normal in the investigated cases of β-thalassemia.

Keywords: gene mapping analysis, globin synthesis, mRNA ratio, β-thalassemia

Introduction

β-thalassemias are inherited disorders that are caused by defects in the expression of β-globin genes. A decreased synthesis or absence of the β-globin chains determines the typical aspect of homozygous β-thalassemia which can be of β^+ or β° type. β-thalassemia has a high incidence in Greece, Italy, Turkey, Algeria, Israel, in the Far East, in American Blacks and African Blacks [1, 2]. In some southern republics of the USSR, the frequency of β-thalassemia gene has been estimated at 4 per cent in Central Asia [3], and at 10 per cent in Azerbaidzhan [4]. As in this condition the structure and function of individual globin genes are not quite clear [5], we have analyzed the RNA and DNA isolated from β-thalassemic patients from Azerbaidzhan using the hybridization technique. In order to study structural alterations in the DNA of some patients, we analyzed the restriction fragments of the DNA containing the globin gene and compared them with those from normal DNA.

Materials and Methods

Globin chain synthesis was studied using previously described methods [6].

RNA isolation. Total reticulocyte RNA was prepared from red cell hemolysates by phenol-chloroform and detergent extraction [7]. Globin mRNA for reverse transcription was further purified by sucrose gradient centrifugation and poly(U)-

Correspondence: S. A. Limborska, Institute of Medical Genetics, Kashirskoe sh., 6a. 115478, Moscow, USSR.

Sepharose column chromatography [8]. Nuclear and cytoplasmic RNA from β-thalassemic spleen was obtained by the slightly modified hot phenol fractionation procedure [9]. A suspension of spleen cells in 0.14 mol/l NaCl (about 10 vol to 1 vol of packed cells) was treated with an equal amount of water-saturated phenol pH 6 in the cold. The mixture was shaken for 15 min, centrifuged for 30 min at 3000 r.p.m. and the interphase layer was collected. It was washed four times in the cold with a mixture (v/v) of 0.14 mol NaCl and phenol pH 6 (15 min shaking and 10 min centrifugation at 5000 r.p.m.). Then the interphase layer was suspended in 0.14 mol/l NaCl containing 1 per cent sodium dodecyl sulphate (SDS) (about 10–20 vol of the mixture per 1 vol of the interphase), mixed with an equal volume of phenol, and shaken at 65 °C for 15 min. Practically all the nuclear RNA was isolated in this step. Aqueous layers obtained after extraction at 0 °C (cytoplasmic RNA) and 65 °C (nuclear RNA) were treated according to the standard procedure.

DNA preparation. DNA was prepared from normal and thalassemic blood cells by the method of Gross-Bellard et al. [10].

Human globin cDNA. Human globin cDNA (labeled with ^{3}H dCTP, 24 cI/mmol) was synthesized using RNA-dependent DNA polymerase of avian myeloblastosis virus under conditions which yielded predominantly full-length cDNA [8].

β-Globin cDNA was prepared by hybridizing (α + β)-globin cDNA with JW-101 plasmid DNA containing human α-globin cDNA. Denatured plasmid DNA (5–10 mg) was immobilized on nitrocellulose filters according to Dergunova et al. [11]. The filters were preincubated overnight at 65 °C in threefold Denhardt's medium containing 2 × SSC (1 × SSC is 0.15 mol/l NaCl in 1.5 mol/l sodium citrate). (α + β)-globin cDNA was applied to a filter in 50 µl of a solution containing 0.3 mol/l NaCl, 0.005 mol/l tris-HCl pH 7.4, 0.1 per cent SDS and threefold Denhardt's medium. Hybridization was done under mineral oil at 65 °C for 4 h. The nonhybridized (β-globin-enriched) component was eluted with 0.3 mol/l NaCl, 0.005 mol/l tris-HCl pH 4.7, 0.1 per cent SDS at 65 °C for 2 h and extracted with chloroform. The β-enriched cDNA was subjected to a second purification cycle of hybridization to JW-101 plasmid DNA. Its purity, as estimated by hybridization to plasmid DNA containing α- and β-globin genes, was approximately 90 per cent. The β-globin cDNA had a 2 per cent S_1-nuclease resistant component. α-globin cDNA was prepared by hybridizing (α + β)-globin cDNA to JW-102 plasmid DNA containing human β-globin cDNA. Its purity, as estimated by hybridization to JW-101 and JW-102 plasmid DNA, was approximately 85–90 per cent. The α-globin cDNA had a 10 per cent S_1-nuclease resistant component.

RNA-cDNA hybridization assay was performed as described previously using a constant amount of cDNA and a variable amount of reticulocyte RNA [7]. The extent of hybridization was determined by measuring the percentage of cDNA which had become resistant to digestion by single-stranded specific S_1 nuclease.

Detection of globin gene fragments by blotting filter hybridization. Southern transfer and filter hybridization were done as described by Bernards et al. [12].

The filter was hybridized to ^{32}P-labeled JW-102 plasmid DNA at 65°C for 48 h. Phage lambda DNA was digested with Eco RI restriction enzyme to generate molecular weight standards.

Plasmid DNA was labeled with ^{32}P by nick translation [13]. The recombinant DNA level of P3-EK2 was used.

Results and Discussion

Table 1 lists the results of globin chain synthesis in intact peripheral blood cells of patients with homozygous β-thalassemia. In some cases there was no β-chain synthesis in peripheral blood cells (β°-thalassemia). In others, β-globin

Table 1

Globin chain synthesis and globin mRNA content in homozygous β-thalassemia

Patient	α/β-globin chain synthetic ratio	α/β-globin mRNA content (by hybridization)
β⁺-thalassemia		
Ah. Sh.	3.1	2.8
M. T.	3.8	
U. Z.	5.2	4.6
Sh. S.	6.0	5.0
M. M.	12.2	
A. S.	13.4	20
β°-thalassemia		
S. S.	—	40
A. A.	—	
Ag. Sh.	—	?*
T. Sh.	—	
A. M.	—	11.2

* In this case, there was either no, or there were only traces of β-globin mRNA (Fig. 3b).

chain synthesis was decreased (β⁺-thalassemia). The data indicated that β⁺-thalassemia in Azerbaidzhan was heterogeneous with respect to the level of reduction in β-chain synthesis; the α/β synthetic ratio varied from 3.1 to 13.4.

Saturation-hybridization assays were performed using a constant amount of α or β-globin cDNA and variable amounts of reticulocyte RNA for a time sufficient to allow total hybridization of RNA to cDNA.

Figure 1 shows the results of reticulocyte RNA hybridization in patient S. S. with β°-thalassemia. The hybridization of α- and β-globin cDNA occurred in a single transition. When (α + β)-globin cDNA was hybridized to thalassemic

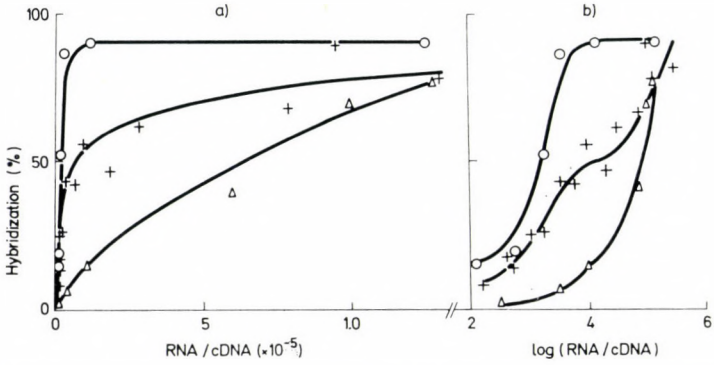


Fig. 1. Hybridization of α-, β- and (α + β)-globin cDNA to reticulocyte RNA of patient S. S. with β°-thalassemia. a = saturation curves; b = hybridization as a function of the logarithm of RNA/DNA ratio. — ○ — α-cDNA; — △ — β-cDNA; — + — (α + β)-cDNA

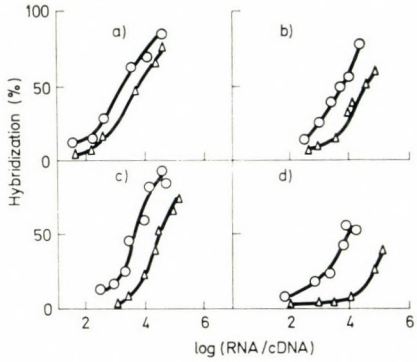


Fig. 2. Hybridization of α- and β-globin cDNA with reticulocyte RNA of patients with β^{+}-thalassemia. a = control RNA; b = patient Ah.Sh.; c = patient U.Z.; d = patient A.S.

RNA, hybridization became clearly biphasic showing a fast hybridization (α-mRNA) followed by a slow one that was obviously due to β-globin mRNA. These data indicate that the amount of reticulocyte β-globin mRNA was decreased.

The results obtained from one control subject and three patients with β^{+}-thalassemia are illustrated in Fig. 2. Normal (nonthalassemic) human reticulocyte RNA hybridized with α- and β-globin cDNA at 85–90 per cent, and the ratio of the RNA inputs achieving half-saturation with α- and β-cDNA was 1.12 : 1. When reticulocyte RNA from β^{+}-thalassemic patients was used, β-globin cDNA annealed at an RNA concentration several times higher than that of α-globin cDNA. If the RNA input gave half-saturation of hybridization to β-globin cDNA, a ratio of the relative amounts of α- to β-globin mRNA sample

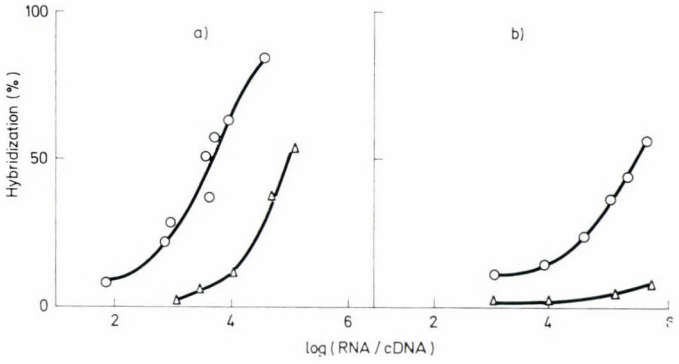


Fig. 3. Hybridization of α and β-globin cDNA to reticulocyte RNA of patients with β°-thalassemia. a = patient A.M.; b = patient Ag.Sh.

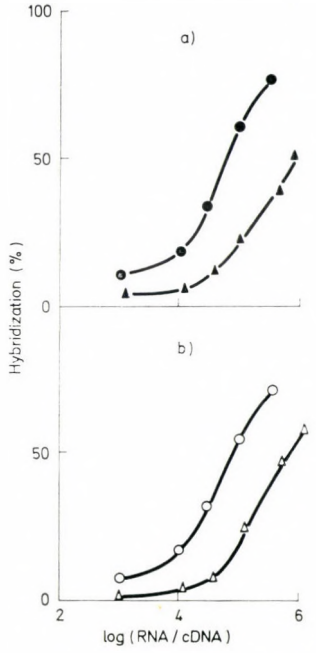


Fig. 4. Analysis of nuclear (a) and cytoplasmic (b) RNA from the spleen of Patient A.S. with β^{+}-thalassemia. —○— α-cDNA; —△— β-cDNA

is obtained. The α/β ratios of reticulocyte RNA of β-thalassemic patients are summarized in Table 1. In β^{+}-thalassemic patients, a close correlation was found between α/β globin chain synthesis and the α/β mRNA content determined by molecular hybridization.

We have measured the α- and β-mRNA contents in three β°-thalassemic patients (Figs 1 and 3). In one patient (S. S., Fig. 1), the content of β-mRNA was 2.5 per cent of that of α-mRNA, the α/β ratio was 40; in another case (A.M., Fig. 3a) the level of nonfunctional β-mRNA was 9 per cent of that of α-mRNA, the α/β ratio was 11. In a third patient (Ag.Sh.), there was either none or there were only traces of β-globin mRNA (Fig. 3b, Table 1).

The detected variability in the β-mRNA level in β°-thalassemia suggests that β°-thalassemia in Azerbaidzhan is truly heterogeneous with respect to the presence and the steady-state level of nonfunctional β-globin mRNA. Chang and Kan [14] and Moschonas et al. [15] characterized β-thalassemia in which biologically inactive β-mRNA was identified. They demonstrated that a single nucleotide change in the codon for the amino acid number of either 17 (AAG–UAG) or 39 (CAG–UAG) of the β-chain created a terminator for translation, a nonsense mutation. How common this form of β°-thalassemia is remains uncertain but several positions within the β-globin sequence could in theory be mutated to yield a chain terminator [5].

Losson and Lacroute have investigated the effect of amber nonsense mutations on eukariotic mRNA metabolism in yeast. Their result shows clearly that nonsense mutations interfere with the stability of the corresponding mRNA according to their position in the gene [16]. One may suppose that, in the case of β°-thalassemia, the level of nonfunctional β-globin mRNA depends on the position of a nonsense codon within the locus.

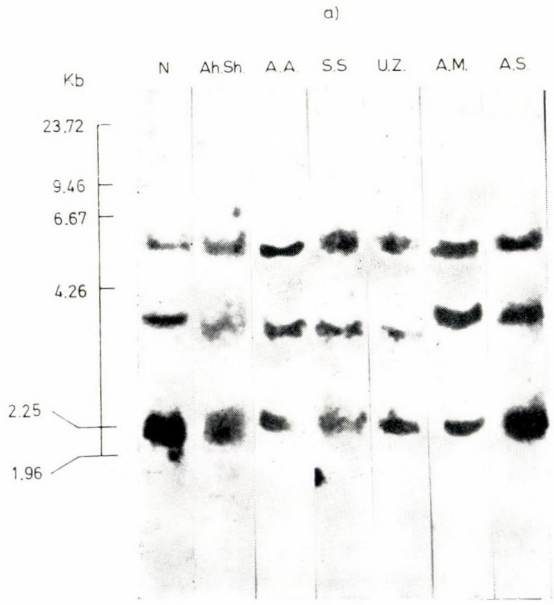
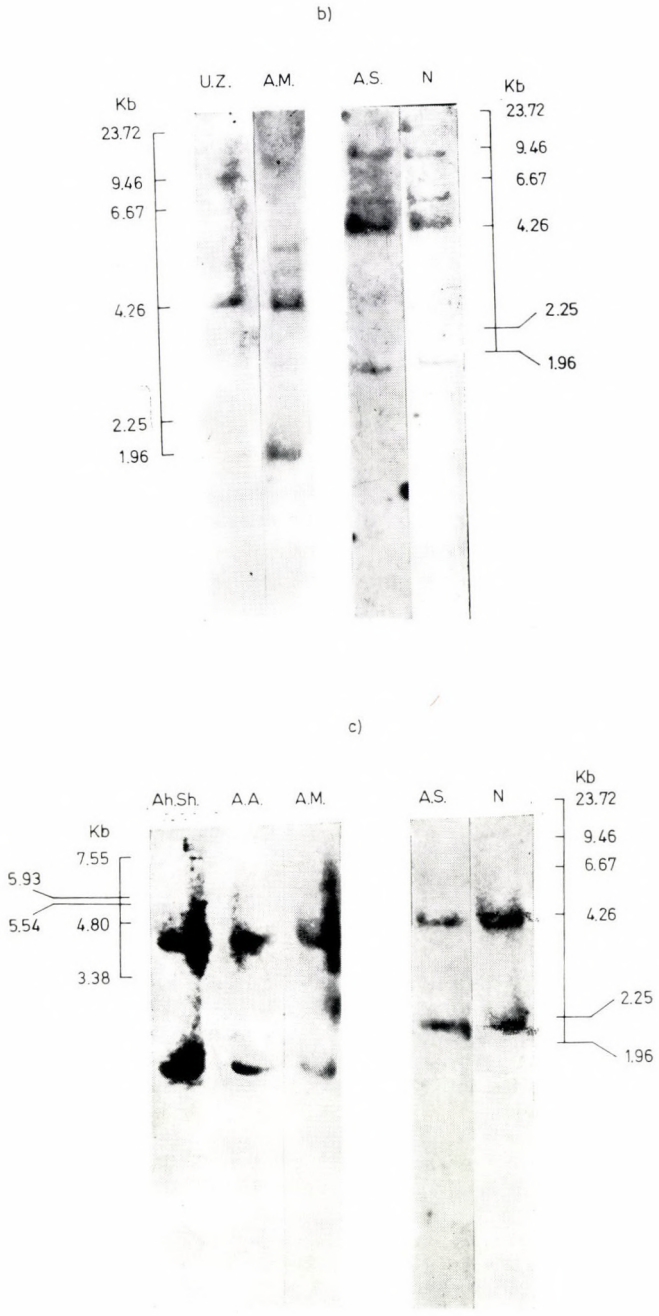


Fig. 5. Autoradiograph of β-globin gene fragments from the DNA of normal (N) and thalassemic patients Ah.Sh., A.A., S.S., U.Z., A.M., A.S. DNA was digested with Eco RI (a),



Bam HI (b), Pst I (c). The size of the hybridizing fragments was determined by co-electrophoresis of Hind III and Eco RI digested lambda DNA

In one case (A.S.) of β^+-thalassemia, it was possible to study nuclear and cytoplasmic RNA from spleen erythroid cells. The results are illustrated in Fig. 4.

The concentration of globin mRNA sequences in the spleen nuclear sample was the same as in the cytoplasmic one. Similar results were obtained for nuclear and cytoplasmic RNA fractions from spleen erythroid cells by Comi et al. [17]. There was an increase in the total concentration of globin mRNA sequences when nuclear spleen and cytoplasmic spleen RNAs were compared to reticulocyte RNA (Fig. 2).

In this patient, the α/β mRNA ratio in the nucleus was the same as in the cytoplasm ($\alpha/\beta = 5$). The results suggested that the deficiency of β-globin mRNA in this case of β^+-thalassemia was probably due to a mutation affecting either the transcription or the early stages of mRNA processing. At the same time, the α/β mRNA ratio in reticulocytes was 20 (Fig. 2d) which could have been due to an impaired stability of β-globin mRNA of this patient in comparison with the α-globin mRNA.

The DNA from the blood cells of individuals homozygous for β-thalassemia was cleaved with a variety of restriction endonuclease and analyzed by the Southern blotting technique and the filter hybridization to ^{32}P-labeled JW-102 DNA containing β-globin cDNA. In the cases analyzed here, no difference could be seen between the DNA of β-thalassemia patients and the control DNA from hematologically normal subjects (Fig. 5). The data suggest that deletions greater that 100–200 base pairs in fragments containing β-globin genes have not been present in these cases of β-thalassemia.

The authors wish to thank Prof. B. G. Forget for the gift of JW-101 and JW-102 plasmids, Dr. A. A. Ianulaitis for the gift of restriction endonucleases, and Prof. G. P. Georgiev for the helpful discussions.

References

1. Belhani, M., Morle, F., Colonna, P., Godet, J.: Heterogeneity in β^0-thalassemia from Algeria: genetic, clinical and molecular studies. *Hum. Genet. 54*, 251 (1980).
2. Cividalli, G., Kerem, H., Rachnulewitz, E. A.: Globin synthesis in severe and intermediate homozygous β-thalassemia in Israel. *Ann. N. Y. Acad. Sci. 344*, 132 (1980).
3. Garkavtseva, R. F., Ginter, E. K., Sotnikova, E. N., Asanov, A. Yu., Bahramov, S. M.: *Probl. Gemat.* (in press).
4. Rustamov, R. Sh., Dadasheva, T. S., Limborska, S. A.: Varying forms of β-thalassemia among children of Azerbaidzhan. *Probl. Gemat. 9*, 3 (1981) (In Russian).
5. Orkin, S. M., Nathan, D. G.: The molecular genetics of thalassemia. *Advanc. Hum. Genet. 11*, 233 (1981).
6. Limborska, S. A.: A method of investigation of globin protein synthesis in patients with thalassemia. *Lab. Delo 8*, 464 (1979). (In Russian.)
7. Limborska, S. A., Frolova, L. Yu., Maleeva, N. E., Shipitsina, G. I., Skobeleva, N. A., Belkin, V. M., Lunc, M. G., Evdokimova, A. I.: Molecular investigation of thalassemia. 1. Determination of globin mRNA content in homozygous β-thalassemia by human globin complementary DNA hybridization. *Molec. Biol. 12*, 787 (1978). (In Russian).

8. Limborska, S. A., Frolova, L. Yu.: Isolation of human globin mRNA and synthesis of complementary DNA. *Acta Biol. Med. Germ. 36*, 335 (1978).
9. Georgiev, G. P., Ryskov, A. P., Coutelle, Ch., Mantieva, V. L., Avakyan, E. R.: On the structure of transcriptional unit in mammalian cells. *Biochem. Biophys. Acta 259*, 259 (1972).
10. Gross-Bellard, M., Odet, P., Chambon, P.: Isolation of high-molecular weight DNA from mammalian cells. *Eur. J. Biochem. 36*, 32 (1973).
11. Dergunova, L. V., Slominsky, P. A., Rustamov, R. Sh., Dadasheva, T. S., Amzashvili, M. G., Limborska, S. A.: Molecular investigation of thalassemia. *Genetika 7*, 1045 (1982) (In Russian).
12. Bernards, R., Kooter, J. M., Flavell, R. A.: Physical mapping of the globin gene deletion in β-thalassemia. *Gene 6*, 265 (1979).
13. Wilson, J. T., Wilson, L. B., De Riel, G. K., Villa-Komaroff, L., Efstratiadis, A., Forget, B. G., Weissman, S. M.: Insertion of synthetic copies of human globin genes into bacterial plasmids. *Nucl. Acid Res. 5*, 563 (1978).
14. Chang, J. C., Kan, Y. W.: β°-thalassemia, a nonsense mutation in man. *Proc. Nat. Acad. Sci. USA 76*, 2886 (1979).
15. Moschonas, N., De Boer, E., Grosveld, F. G., Dahl, H. H. M., Wright, S., Shewmaker, C. R., Flavell, R. A.: Structure and expression of a cloned β-thalassemic globin gene. *Nucl. Acid Res. 9*, 4391 (1981).
16. Losson, R., Lacroute, F.: Interference of nonsense mutations with eukaryotic messenger RNA stability. *Proc. Natl. Acad. Sci. USA 76*, 5132 (1979).
17. Comi, P., Giglioni, B., Barbarano, L., Ottolenghi, S., Williamson, R., Novakova, M., Mazera, G.: Transcriptional and post-transcriptional defects in β°-thalassemia. *Eur. J. Biochem. 79*, 617 (1977).

INDEX TO VOLUME 17

CONTENTS

Author Index

Subject Index

INSTRUCTIONS TO CONTRIBUTORS

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

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24
H-1113 Budapest, Hungary

Three copies of the manuscript should be submitted. They should be typed double-spaced on one side of a good quality paper with proper margins. The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which the work has been carried out; (4) name and full postal address of the author to whom communications regarding the manuscript should be directed; (5) a short title not to exceed 40 characters (including space) to be used as a running head. The second page should contain an **abstract** of 50—100 words, summarizing the reasons for the study, the methods used, the results, and the major conclusions. This page should also contain 4—8 **keywords** placed in alphabetical order. Original papers should not exceed 15 printed pages including tables, figures, and references. Case reports should not be more than four, technical notes and Letters to the Editor not more than two printed pages in length. In the manuscripts the approximate location of tables and figures should be indicated in the margin. The manuscript of original papers should be divided into summary, introduction, materials and methods, results, discussion, acknowledgements and references. Review articles should also be appropriately divided. SI units should be used in the manuscript, except that, for the time being, litre (l) may be used as a unit of volume.

References: Only papers closely related to the author's work should be referred to. In the text citations should include the name of the authors and/or the reference number in parentheses. A list of references, numbered according to the order of their occurrence in the text, should be compiled at the end of the paper. It should be typewritten, double-spaced, on a separate sheet.

References to periodicals should be typed double-spaced and mention: (1) name(s) and initials of all the authors; (2) title of the paper; (3) international abbreviation of the periodical; (4) volume; (5) first page number; (6) year of publication in parentheses. Thus: 4. Liebermann, J. E., Gordon-Smith, E. C.: Red cell pyrimidine 5'-nucleotidase and glutathione in myeloproliferative and lymphoproliferative disorders. *Brit. J. Haemat. 44*, 425 (1980).

References to books should include: (1) author(s)' name(s); (2) title; (3) editor(s); (4) publisher; (5) place and year of publication. Thus: 5. Guyton A. C., Taylor, A. E., Granger, H. J.: *Circulatory Physiology. II. Dynamics and Control of Body Fluids.* Saunders, Philadelphia 1975. Or: 6. Rappaport, J. M.: Granulocyte transfusion. In: *Haematology of Infancy and Childhood.* D. G. Nathan, F. A. Oski (eds). Saunders, Philadelphia 1974, p. 875.

Illustrations must be cited and numbered in the order they appear in the text. All line drawings should be submitted as clear, glossy, black and white originals or good quality photographs. The author's name and the title of the paper together with the serial number of the figure should be written on the back of each print. Legends for illustrations should be typewritten, double-spaced, on a separate sheet, and included at the end of the manuscript. Tables, each bearing a title, should be self-explanatory and numbered consecutively.

Authors will receive one set of proofs which must be corrected and returned to the Editor-in-Chief *within three days of receipt*. Major alterations of the text cannot be accepted.

Authors are entitled to 50 reprints free of charge.

HAEMATOLOGIA
VOLUME 17 · NUMBER 4 **1984**

Contents

Index: 26861