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The Biological Role of Lactoferrin

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Lactoferrin (LF) — in various quantities — is present in human milk, secretions and polymorphonuclear neutrophils (PMN).

LF's significance lies in its bacteriostatic effect on its environment. Probably it prevents bacterial uptake of iron, leads to damage of bacteria and during phagocytosis helps the organism to combat pathogens. Most likely it regulates iron absorption, and during inflammation it takes part in the plasma iron transport. LF is believed to play an important role in the regulation of granulopoiesis in the bone-marrow. From its biological effects it appears that plasma LF determinations may be useful in the clinical diagnosis of leukaemia and other malignant diseases, as well as in the study of iron metabolism.

Keywords: Lactoferrin, human milk, iron metabolism, antimicrobial activity, regulation of granulopoiesis

Introduction

Lactoferrin (LF), an iron-binding glycoprotein, was first isolated in 1960 by Groves [24] from cow's milk, then in the same year by Johansson [26] from human milk. When iron is bound, the compound becomes red, thus Groves called it "red protein". Today's designation LF arises from its presence in milk and its similarity in structure to transferrin.

LF, which has a molecular weight of about 80 000 is present in large quantities in some mammalian secretions such as milk, tears, sperm and in neutrophil granules. Interestingly, LF in its natural form contains very little iron.

The biological significance of LF is its bacteriostatic effect, which may explain the fact that breast-fed infants do not or rarely suffer from intestinal infections.

Recently three other possible biological functions of LF have come to light. First, it probably plays a part in the regulation of iron absorption in the intestinal tract. Second, during infectious disease it participates in the transport of iron from the blood to the RES. Third, it is said to play a role in the regulation of bone-marrow granulopoiesis. The mechanism of this regulation is not clear yet.

This present study first looks at the chemical behaviour of LF because its biological role is determined by that. Following a survey of biological functions, the fields where it may be possible to utilize a quantitative assay of the LF-content of blood in clinical diagnostics will be reviewed.

I. The Chemical Behaviour of Lactoferrin

In its chemical behaviour LF is similar to transferrin [1]; i.e. both proteins can reversibly bind two ferric ions. From the structure of their protein chains, molecular weight, disulphide bridge structure and the similarity of their bacteriostatic properties, the conclusion can be drawn that they belong to the same class of protein [38]. However, their amino acid and carbohydrate components [33] and immunological properties are different. A difference can be found in the pH dependence of iron-binding capacity too: while LF retains iron even at pH = 3, transferrin loses it at pH = 4.5 [1]. It is interesting that the structure and biological role of transferrin is well known, whereas investigations of the role of LF in iron kinetics, and its biochemistry and functions, have only recently come into the focus of scientific interest.

II. The Biological Role of Lactoferrin

1. *Bacteriostatic effect of lactoferrin and its function in milk and blood*

a) *Function in milk*

a.a In vivo experiments

The fact that breast-fed infants rarely suffer from bacterial infection of the intestinal tract inspired some attempts to isolate the compound responsible for this effect from human milk. Of the compounds tested for bacteriostatic effect, LF was the most effective, but the role of other iron-chelating proteins (e.g. transferrin, conalbumin) and antibodies is also discussed [39].

Bullen and coworkers [14] conducted experiments on guinea pigs, whose milk contains high levels of LF.

Escherichia coli bacilli were administered into the intestines of the newborn animals. Then they were divided into two groups. In the group where the animals were fed artificially, coli bacilli significantly proliferated in the intestines after a few days. In the case of the suckled animals proliferation of the bacilli was much slower; in fact, after six days only 1/10 000 of the administered bacteria could be found.

That this strong bacteriostatic effect was due to LF was supported by the observation that the iron containing compound haematin given twice a day with guinea pig milk reduced the inhibitory effect. If *E. coli* bacilli are incubated with iron-saturated LF, no bacteriostatic effect can be observed such as occurs using iron-free LF. This shows that LF has a bacteriostatic effect but only in the iron-free state.

These results become somewhat less significant when it is realized that LF cannot take up the iron of haematin. Iron from haematin is taken up by the bacteria directly and it stimulates their proliferation. Consequently, this does not prove

the ineffectiveness of iron-saturated LF or, to put it another way, the effectiveness of apo-LF [9]. After these results it would be interesting to know whether the milk of any kind of mammals contains "enough" LF.

Masson and Heremans [32] determined the LF content of the milk of 10 different animals using the immundiffusion method. Their results showed that human milk contains the most LF of all of the analyzed milks. At the same time cow and sow milk are poor in LF.

The above-mentioned authors also dealt with the changes in the LF and transferrin content of milk during lactation. They studied the LF and transferrin content of the milk of 4 guinea pigs, 2 sows and 4 woman during lactation. They found that the LF level is generally at its highest in the first days after birth (with the exception of guinea pigs). This is not surprising because the colostrum is richer in protein then milk. After a few days the LF level decreases, but it remains constant during lactation in most species. In contrast to this, in guinea pigs the maximum level is reached before weaning.

Similar changes in the LF content of cow's milk have also been reported [37].

a.b. In vitro experiments

In studying the bacteriostatic effect of LF most experiments were performed *in vitro*. Some authors [37] investigated the effect of isolated iron-free LF from goat-milk on the growth of *Bacillus stearothermophilus* and *Bacillus subtilis*. It was found that LF inhibited the germination of spores and the growth of vegetative cells of *Bacillus stearothermophilus*. The bacteriostatic action of LF was suppressed by Fe^{2+} and enhanced by Zn^{2+}; Co^{2+}, Mn^{2+}, Ni^{2+} and Cu^{2+}. Regarding the LF of bovine and goat milk they contended that they possess a structure similar to human LF.

The growth controlling effect of human milk LF and transferrin on *Escherichia coli* was examined by Brock et al. [10]. Growth of *Escherichia coli* in human milk was slow during the first 10 h of incubation, but this bacteriostatic effect had disappeared by 24 h. Loss of bacteriostatic activity could be achieved by adding sufficient iron to saturate the LF in milk, or by adding enterobactin (an enterobacterial iron chelator). The lactoferrin-mediated antimicrobial activity of human milk was only very slightly affected by trypsin and chymotrypsin [8]. Neither enzyme had much effect on the iron binding capacity of purified LF.

In contrast, trypsin destroyed the antimicrobial activity of bovine colostrum and reduced the iron-binding capacity of purified bovine apo-LF.

The different resistance of the apo-LF of these species to proteolysis may reflect some structural differences.

The effect of LF on microorganisms of several types was tested by Arnold et al. [2]. The list of susceptible organisms included Gram-positive and Gram-negative microbes, rods and cocci, facultative anaerobes and aerotolerant anaerobes. Besides these microorganisms, similar other morphological and physiological types were represented among the LF-resistant bacteria.

The ability of various Neisseria species to compete with LF for iron was examined by Mickelsen et al. [34]. The ability to utilize LF as a source of iron may contribute to differences in pathogenicity among certain gonococci.

It was observed that the culture-medium and other conditions also influence LF-sensitivity. The change of culture-medium content, pH and time of LF treatment were also analyzed [3]. It was found that microorganisms in various phases of development react differently to LF, and if there was any damage, it always occurred after a certain latency time (15 minutes). While LF caused a loss of bacterial viability at pH = 5 and 6 in a glycine-containing culture-medium, this effect did not occur at any pH in a sodium phosphate culture medium.

b) *Bacteriostatic function in blood*

It is known that LF can also be found in blood, where it arises from the granules of neutrophil granulocytes (polymorphonuclear leucocytes) (PMN). The biological function of these cells is primarily participation in phagocytosis, which is an important mechanism of protection of the organism against bacteria. Besides LF other compounds are also found in the granules of neutrophils which can promote bacterial killing. One such compound is lysozyme. In the case of inflammation both lysozyme and LF are released from the granules [28], causing a bacteriostatic effect.

2. *Regulation of iron kinetics in inflammation. (Iron-transport function)*

The other function of LF released from neutrophils during phagocytosis is the regulation of iron kinetics at the site of infection or inflammation. The high level of LF resulting from damage to neutrophils in plasma or interstitial liquid changes the normal way of iron transport. As the affinity for iron of LF is much higher than that of transferrin – especially in an acidic medium – there is a flow of iron from transferrin to LF. Iron bound to LF leaves the blood circulation and enters the RES (liver, spleen, lymphatic nodes) where it is passed on to ferritin [45]. The uptake of LF-bound iron is via Fe–LF receptors on the membrane of macrophages. In this way LF facilitates the development of an iron-free medium unsuitable for the proliferation of bacteria. Considering this phenomenon from a clinical point of view, this could form the molecular biological basis of anaemia in infectious diseases. The characteristic features of this condition are low Hb concentration, serum iron level and total iron-binding capacity and high levels of serum ferritin and LF. In the normal healthy state the function of LF in iron transport is negligible [25].

Ferro-kinetic measurements were also carried out with isotope-labelled LF [7]. The investigations showed that the labelled LF immediately enters the liver, and to a lesser extent the spleen, where it accumulates for a short period. The protein moiety leaves these organs via the urine within a few hours and after 7 h it

totally disappears. The release of the iron is very much slower. First, from the liver and spleen it enters the place of erythropoiesis, i.e. the bone-marrow and finally passes into the circulation.

3. *The role of LF in the regulation of iron absorption*

In addition to the secretions mentioned, LF can be found in the fluids of the small intestine. The question arises whether or not LF takes part in iron absorption and transport in this area.

De Vet and van Gool [17] found an inverse correlation between the states of iron stores and duodenal LF concentration. They analyzed the effect of iron-saturated LF introduced into the duodenum on iron absorption. It was observed that LF inhibited iron absorption for a few hours. Others [16] confirmed the observation that LF in an iron-free state inhibits iron absorption in adults. According to a leading article in the *Nutrition Review* [27] iron bound to duodenal LF enterocytes they were able to extract it, suggesting that LF may have a role in iron transport across the mucosa. In spite of the fact that the binding constant of iron is nearly 300 times greater for LF than for transferrin, cellular uptake of iron from LF is easier than from transferrin. This would suggest that the cellular uptake of iron from iron-binding proteins is not determined by the binding affinity of the protein for iron. It was reported that the cells possess specific receptors for LF.

LF is specially important in the regulation of iron metabolism in the neonatal period. According to clinical observations [15], during their first week of life infants excrete 10 times more iron than they absorb. But others have reported [9] that in babies aged 5–8 days the daily excretion of iron is in accordance with the quantity of suckled milk and milk LF as observed by Masson et al. [32]. This observation was also supported by Saarinen et al. [41] who measured iron absorption in babies aged a few months and found it very high. Subsequently the iron absorption promoting activity of human milk is hardly questioned. Brock [9] looked for the relationship between LF found in the milk of several species and the development of offspring. According to these findings human and guinea pig milks are richer in LF than those of other species. This could be due to the slower development of offspring as during their longer suckling period they take up exogenous iron later. But one has to remember that in certain animals, such as the rat, almost 100% of the iron found in the milk is absorbed.

4. *LF in the regulation of granulopoiesis*

Broxmeyer et al. [12] first identified LF as a granulocyte derived regulator of granulocyte-macrophage colony stimulating activity (GM–CSA). The same group has also found a connection between certain myeloid diseases (for instance leukemia) and a defect in the regulatory mechanism of LF.

This mechanism is not yet clear, but an attempt will be made to summarize the concepts known at present.

It is understood that for the proliferation and differentiation of granulocyte-macrophage colony forming cells (GM–CFC, these are the progenitor cells of granulocytes) a colony stimulating factor (CSA or CSF) is needed. CSA is produced by most of the mesenchymal cells of the body, mostly monocytes and macrophages. Although some authors consider it has a physiological role in the regulation of granulopoiesis, this is not yet clearly proved. LF is an inhibitor of the production of CSA [12] but it has no effect on the growth of previously stimulated GM–CFC. Inhibition of granulopoiesis by LF was also demonstrated *in vivo* in repopulated mice [11] and in mice undergoing rebound myelopoiesis [23]. This experiment suggested that LF has some role in the physiological regulation of granulopoiesis.

LF is effective *in vitro* in very low concentrations (10^{-14}–10^{-6} M), but its iron content has a great influence on its binding to cells producing CSA and thus on the inhibition of the production of CSA. Since other metal ions (Zn, Cu) also bind to LF, it is possible that in some haematological diseases with high levels of serum copper (Hodgkin syndrome, CML) the decreased effect of LF may also play some role *in vivo* [13]. These notions, of course, still require laboratory confirmation. LF has some complicated connections to the lymphoid and other systems, too. LF does not inhibit CSA production of human lymphocytes but inhibits the production or release of so-called monocyte-"soluble factor" which has an influence on the CSA production of T-lymphocytes [4] as well as of fibroblasts [5]. These experiments suggest the possibility that the major role of mononuclear phagocytes in the regulation of granulopoiesis is played indirectly, by stimulating other cells to produce CSA. This theory might also offer supplementary ideas towards understanding the role of LF in granulopoiesis.

III. The role of LF in Clinical Diagnostics

LF is found only in small quantites in blood, so the determination of its concentration makes its diagnostic use difficult but possible. Transferrin is present at 3–5 mg/ml concentration but the LF level is between 0.4 and 2.0 μg/ml [6, 40, 44]. The concentration of the iron-binding protein, ferritin, in blood is even lower than that of LF 0.02–0.20 μg/ml. From its low concentration it follows that the occurrence and quantity of LF can be measured only with very sensitive immunometric methods.

For demonstrating its occurrence in cells and tissues, the immunofluorescent staining method seems to be most effective [30]. For determining LF level in plasma the radioimmunoassay [6, 42, 43] and enzyme-immunoassay [19, 20] techniques seem to be most suitable. The latter techniques (RIA and ELISA) make possible the diagnostic use of LF levels. The field of application can be divided into two groups:

1. *The follow-up of leukaemic disease and therapy*

As the occurrence of LF in blood is always dependent upon neutrophil granulocytes, it can be used diagnostically as a marker of neutrophil kinetics [31].

Bennett and Mohla [6] determined plasma LF levels of patients with chronic myeloid leukaemia. Corresponding to granulocyte level, a 10–20 times higher LF value (12–22 μg/ml) than normal was found, while patients on marrow suppressant therapy tended to have subnormal LF values (0.01–0.5 μg/ml). Malmquist [29] found a higher LF level in the blood of patients with chronic myeloid leukaemia and polycythaemia vera, though other observations [36] oppose these results. They followed plasma LF levels in patients with chronic myeloid leukaemia in different stages of the disease. Acute myeloid leukaemia is characterized by abnormal and deficient formation of granules. This explains the observation that 50% of the patients had subnormal LF levels [36]. Under remission they normalized. Three months prior to release LF suddenly reached a very high concentration before returning to normal. These observations must, of course, still be proven by further measurements, though the phenomenon may be useful in predicting relapse.

The same group [35] observed patients with chronic granulocytic leukaemia. The LF level of plasma became 2–8 times higher, at the same time as the LF level of each neutrophil was decreased in 70% of the cases. A comparison was made between the high values in plasma and the change of neutrophil count and a close correlation was found. The high plasma LF level can be explained by the formation of a great number of granulocytes. LF is also found in human secretions and their LF content changes considerably if the producing gland undergoes pathological changes. For the diagnosis of pancreas cancer and chronic pancreatitis the measurement of LF and trypsin content in the pancreatic juice was studied [21, 22]. In patients with pancreas cancer a low trypsin and normal LF concentration were found, whereas in the case of pancreatitis the trypsin level appeared rather changeable, while that of LF increased twofold [22, 44]. These combined measurements may be useful for the differentiation of the two diseases.

2. *LF and iron metabolism*

De Vet and ten Hoopen [18] found a relationship between the LF content of neutrophil granulocytes and iron metabolism. An examination was made of patients with granulocytosis and donors and patients with iron-deficiency anaemia receiving iron therapy. Their results showed that in the case of granulocytosis the LF content of neutrophil cells is significantly lower than in controls. Following blood-donation a definite decrease of LF concentration could be seen, while after iron-therapy the neutrophil LF level had significantly increased. This shows a direct connection between LF concentration and iron metabolism.

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Acute Myeloid Leukemia in Childhood: 12 Years Experience of Treatment in Hungary

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During the 12-year period between 1971 and 1982 leukemia was diagnosed in 759 children in Hungary. Of these, 123 (16%) had acute myeloid or myelomonocytic leukemia. This corresponds to an annual incidence rate of 6.15 cases per 10^6 children. Analysis of the presentation symptoms at diagnosis showed a higher incidence of septic signs but otherwise no difference to those in acute lymphoid leukemia. Treatment schedules were intensified successively in stepwise fashion. Remission rates rose from 34% to 66%, and remission lengths also improved. Of the prognostic factors analyzed, initial WBC count in excess of 50 G/l was found to confer bad prognosis, while the other factors had no significant effect on disease-free survival.

Keywords: childhood AML, remission rate, prognosis

Acute myeloid leukemia (AML) accounts for app. 15% of the childhood leukemias [5]. The results of its treatment have not paralleled that of acute lymphoid leukemia (ALL) which showed dramatic improvement during the past 10 years [9, 11]. We have, as yet no explanation for this considerable difference, but the "*ab origine*" existence or rapid appearance of drug-resistant cell clones is generally held to be responsible. Children who are diagnosed to have leukemia in Hungary are treated in one of the 10 centers of the Leukemia Working Party. This has enabled the collection and assessment of data from all patients since 1971. In this paper we present our findings on the morphological sub-types, prognostic factors and therapeutic results in childhood AML.

Materials and Methods

I. *Patients*

During the period 1971–1982 a total 123 children were found to have AML in Hungary. This corresponds to an annual incidence rate of 6.15 cases per 10^6 children under 15 years of age. Acute myeloid and myelo-monocytic leukemia (AMML) accounted for 16% of all the childhood leukemias (Table 1). Diagnosis

* On behalf of the Hungarian Working Party of Childhood Leukemia. Principal investigators of the W. P. are: I. Rényi, I. Zimonyi, P. Kemény, L. Velkey, I. Virág, I. Kovács, P. Kajtár, P. Cholnoky, P. Kassai.

Table 1

Distribution of leukemia sub-types in the treatment centers

Center	No. of patients			
	ALL	AML	AMML	Total
1	43	2	3	48
2	131	19	14	164
3	85	24	2	111
4	25	4	1	30
5	49	10	—	59
6	78	16	2	96
7	94	15	—	109
8	63	2	—	65
9	61	5	2	68
10	7	2	—	9
Total:	636	99	24	759
%	84	13	3	100

was established by bone marrow taps and/or biopsies. Cytochemical reactions investigated at the National Institute of Pediatrics included PAS, Sudan-black-B or myelo-peroxidase and alpha-naphthyl-acetate esterase. Once the diagnosis was made, the patient was started on the protocol used in the given period. Treatment was uniform in all the 10 centers of the Leukemia Working Party, but the number of patients, and thus experience showed considerable differences. The chance distribution of patients with AML and AMML was also uneven: for a given number of leukemic patients some centers had a higher number of AML or AMML cases than expected (Table 1).

II. *Treatment*

Treatment protocols were discussed and agreed to at regular meetings of the Working Party. Initially treatment was similar to that of ALL (Table 2.), and CNS prevention was not given or withheld until the completion of 1 yr in remission. After 1978, Ara-C and DR took the major role in remission induction, and 6-TG was introduced in maintenance. As from 1980 a considerably more intensive protocol is being used, based on the studies of the BFM group [10]. This also includes cranial irradiation for the prevention of CNS relapse.

III. *Statistical analysis*

All the most important clinical data of each patient are collected and stored in a computer. Remission durations were analyzed with the Kaplan–Meier method. Statistical tests on this distribution were made with the long-rank test [8]. The

Table 2

Outline of AML protocols 1971—1982

Treatment period	Induction	Consolidation	CNS prevention	Maintenance	"Pulse" treatment	Remission rate %
1971—1973	VCR, DR, Pred.	—	—	6-MP	VCR, DR, Pred.	34
1974—1977	Ara-C, DR, 6-TG	—	—	6-MP, MTX	—	44
1978—1979	Ara-C, DR, 6-TG	—	i. thec. MTX, after 1 yr CR: 24 Gy cranial	6-MP, MTX	Ara-C, DR, 6-TG	56
1980—1982	VCR, DR, Pred. Ara-C, 6-TG	CP, 6-MP Ara-C	24 Gy cranial + i. thec. MTX	6-TG	Ara-C, DR	66

Abbreviations: VCR: vincristine, DR: daunorubicine, Pred.: prednisolone, Ara-C: cytosine-arabinoside, 6-TG: 6-thioguanin, 6-MP: 6-mercaptopurine, MTX: methotrexate, CR: complete remission.

distribution of morphological sub-types as described by the FAB group [2] and prognostic factors was assessed by appropriate histograms, using the BMDP computer software of the UCLA [4]. Patients who failed to achieve complete remission (CR) after the induction regimen were also included in the CCR curves as failures, similarly to those lost in remission with overwhelming infection.

Results

I. *Induction of remission*

The results of remission induction are presented in Table 2. During the early 70s remission rates were below 50%, showing gradual improvement in subsequent years. Current remission rates are over 65%.

II. *Duration of remission*

Similarly to the remission rates, the duration of remission showed gradual improvement with the introduction of more intensive treatment protocols (Fig. 1). Long-term disease free survival was achieved only in a fraction of patients. Results of the latest treatment program look more promising, but so far no statistically significant improvement can be detected as yet.

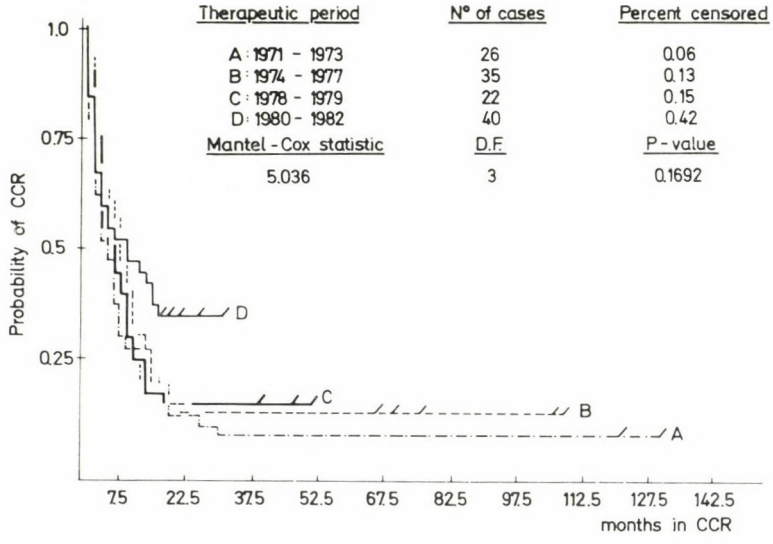

Fig. 1. Kaplan–Meier plots of probability of continuous complete remission (CCR) for 4 treatment periods

III. *Prognostic factors*

The frequency of presentation features that might influence the survival of patients is presented in Table 3. Septic symptoms were significantly more frequent in AML, whereas bone and joint lesions occurred more commonly in ALL. The occurrence of other symptoms showed no great difference.

Table 3

Some presentation features in AML and ALL

Investigated features	Frequency at diagnosis		Statistical difference (P =)
	AML %	ALL %	
Splenomegaly	6.3	10.1	0.09
Hepatomegaly	52.4	56.7	0.13
Lymphadenomegaly	39.7	43.9	0.55
Bleeding diathesis	49.2	49.5	0.99
Septic symptoms	35.7	23.8	0.01
Bone and joint lesions	16.7	28.6	0.01
Meningeal involvement	1.6	2.4	0.82

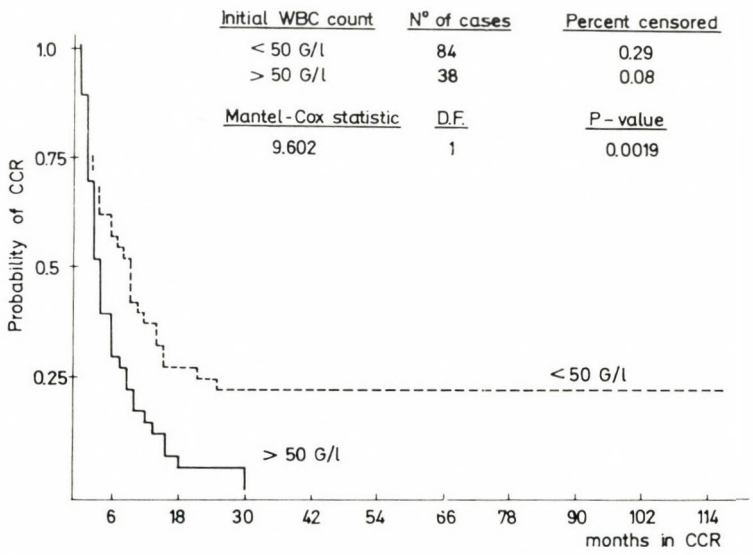

Fig. 2. Probability of CCR in AML patients with initial WBC counts less or more than 50 G/l

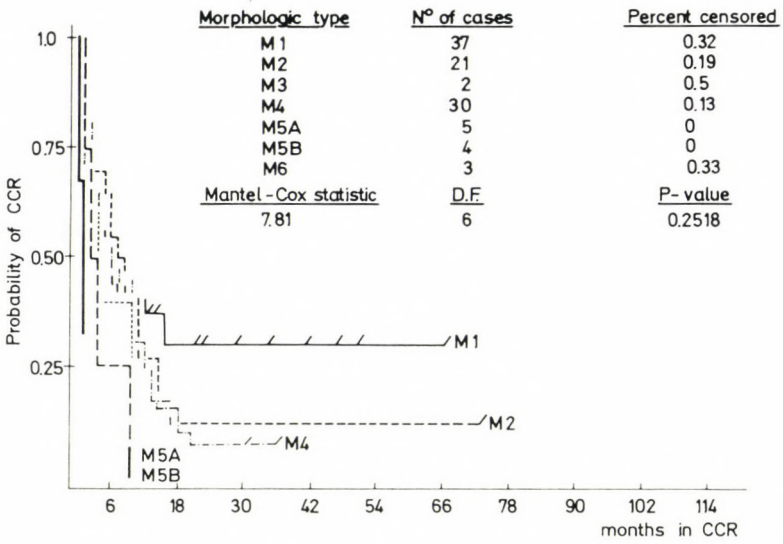

Fig. 3. Probability of CCR in AML patients with different morphological subtypes

Of the various prognostic factors investigated, age and sex seem not to play a role in the outcome of treatment. Initial WBC count was, however, found to be an important variable: children with high initial WBC counts fared significantly worse than those with lower values (Fig. 2). Organ infiltrations leading to hepatomegaly, splenomegaly or lymphadenopathy did not influence survival. Analyzing the remission lengths of the morphological sub-groups within AML, M_1 was found to have the best, M_5 the worst prognosis, but the overall statistical difference is not significant (Fig. 3).

IV. *Meningeal leukemia*

Initial meningeal involvement was only found in 1.6 % of the newly diagnosed AML cases. Meningeal relapse occurred in 4 %, which is about half of the incidence in ALL. The rate of meningeal relapse is too low to draw conclusions as to the preventive effect of cranial irradiation.

Discussion

Collection of clinical data in a national program enabled us to have reliable estimates of the incidence of childhood leukemias (Table 1). AML was found to occur in 6 out of a million children, a figure closely corresponding to other reported values [5]. It comprises some 15 % of all childhood leukemias. The distribution of morphological sub-types showed a preponderance of myeloblastic forms (M_1 and M_2) with myelo-monocytic leukemia being the other most frequent

type (Fig. 3). This is in close correspondence with other reported distribution figures [5, 7]. The effect on survival, however, seems to differ considerably, e.g.. from the results reported from the UK [5]. In their series, children with M_4 did best while M_1 had a decidedly bad prognosis. In contrast, this latter group of patients fared relatively well among our patients while the monocytic and myelomonocytic forms conferred a worse prognosis. Weinstein et al. [13] found the pure monocytic form to have had the worst outcome, but the other subtypes showed no difference in the overall survival results.

Analyzing the initial presenting symptoms some differences were found when comparing AML patients to those with ALL (Table 3). Septic signs for example were more common in AML, while bone and joint lesions were more frequently found in ALL. Organ involvement was app. the same in the two groups. In ALL initial WBC count was shown by almost everyone to be the most important prognostic variable. There is much controversy, however, as to the role of high initial WBC count in AML [1, 5, 7]. We found that patients with greater than 50 G/l WBC at diagnosis do significantly worse than those with lower values (Fig. 2). The difference could be explained by either a greater number of early deaths or by shorter remission lengths in the high WBC count group. In our material it appears to be both: 33% of these children died in the first 2 months, mostly with septic or haemorrhagic complications. The figure for the lower WBC group was 21%. Children with high WBC counts also had a shorter duration of remission: 85% relapsed before the end of the first year from diagnosis. As the results represent the cumulative AML population since 1971, it is possible that with more intensive therapy initial WBC count will no longer be a significant prognostic factor.

An important prognostic factor which has emerged during the past few years is the presence or absence of chromosomal abnormalities [3]. Our preliminary results also indicate that children with a high proportion of cytogenetically abnormal cells (aneuploidy, non-random abnormalities such as clonal translocations, extra double and/or single minute chromosomes, etc.) have a significantly shorter duration of remission. Whether the chromosomal pattern will be a clinically useful factor of predicting prognosis with more intensive treatment programs remains to be seen.

Reviewing the four main treatment periods we found a gradual increase in remission rates (Table 2) and the length of cumulative complete remission with more intensified therapy (Fig. 1). In the early 70s only a small proportion of patients entered remission and less than 10% are long-term disease-free survivors. As from 1980, a considerably more intensive therapy was introduced (Table 2) which led to a remission rate of 66% with 42% of the patients being in their first remission 6 months to 2 years from diagnosis.

Intensified chemotherapy as introduced by the Boston [12] and the BFM group [10] lead the way to better treatment results in AML. A recent update of their results indicated that over 70% of the patients enter remission and 40 to 50% can be expected to have a CCR in excess of 4 years [6, 13].

Although the two approaches show considerable differences, both represent a significantly more intensified treatment. The emphasis in the VAPA protocol is placed on the administration of a 14 month postremission induction therapy. In the BFM protocol remission induction is followed by a 2 month consolidation phase which also includes cranial irradiation for the prevention of CNS relapse.

We chose this latter approach in the treatment schedule introduced in 1980. Our treatment results clearly do not match those of the Boston or the BFM group, but *a)* show improvement in a historical perspective and *b)* compare reasonably well with other multicenter studies. Further improvement is expected from more vigorous and experienced use of supportive therapy. In a nation-wide, multicenter study some centers usually have significantly more patients and thus more experience than others. This could mean delays in diagnosing complications in the smaller centers, and subsequently delays in the administration of chemotherapy. Regular meetings and exchange of personnel are aimed at lessening the differences and improving overall results.

With more intensive chemotherapy in wider use and bone-marrow transplantation becoming available on a larger scale, AML is gradually becoming a tractable disease – at least in the majority of cases. There is much to be learnt, before treatment results approximating those in ALL can be achieved.

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Haematologia 18 (1), pp. 23–25 (1985)

To the Question of Leukaemia Transmission from Man to Man by Means of Parenteral Application of Leukaemic Blood: Report of a Case and Survey of the Literature

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During a diagnostic collection, leukaemic blood was applied intramuscularly to a nurse. She has been in full clinical health for more than two years. The reported accidents or intentional attempts at human leukaemia transmission are discussed and it is concluded that until now human leukaemia has not been transmitted by any way.

Keywords: leukaemia transmission

Some authors have assumed that human leukaemia might be of infective character, a thought greatly disturbing the health service workers who care for leukaemic patients. Recently a rare accident has occurred in our region in which leukaemic blood was injected into a muscle of a healthy person. This accident together with a brief analysis of the literature dealing with the possibility of parenteral transmission of human leukaemia will be reported.

Report of a case

M. J. a 47-year-old nurse was holding a child during withdrawal of blood for ESR on 21st July, 1980. The child suddenly jerked and the injection needle penetrated the shoulder of the nurse. The syringe was held by the physician taking blood. Less than 1 ml of the child's blood was injected in the nurse's arm. A subcutaneous haematoma measuring about 10×10 cm appeared. It healed without any side effect.

When it was ascertained that the child had leukaemia, the afflicted nurse was in a depressed state. She was examined in the haematology centre of our department. No pathological changes were found on physical examination, and she refused detailed haematological tests in the laboratory. An application of BCG vaccine was recommended for immune stimulation but after the first dose the nurse refused further treatment. Since that time she has been in full health except a respiratory tract catarrh, when her ESR was 15/32.

The data of the patient were as follows. J. B., a boy, was born on 14 February 1978. From 18 July 1980 he was treated for tonsillitis, later an acute lymphoblastic leukaemia was diagnosed on the basis of cytological and cytochemical findings in

the bone marrow. Induction tratment was done with vincristine and prednisone and intensive treatment with methotrexate and cyclophosphamide, then the skull was irradiated by 24 Gy cobalt and methotrexate was administered intrathecally. At present the child is in full remission.

Discussion

Early in this century some authors performed a few attempts at transmission of leukaemic material to volunteers suffering from lethal diseases [1, 2, 3, 4, 5]. In most cases leukaemic blood was given. All these attempted transmissions failed but in animals leukaemia was transmitted by filtered extracts in many cases [6, 7]. Then Thiersch [8, 9] performed two studies. He collected leukaemic blood or spleen or lymph node suspensions and injected this material parenterally to patients with malignant tumour or other disease where the presumed survival time was shorter than two years. He collected leukaemic material from 12 patients and injected it to 51 volunteers. He carefully followed up the recipients but observed no development of leukaemia. In another experiment he injected the bone marrow of 4 leukaemic patients to the bone marrow of 12 volunteers. Even in this case no leukaemia developed although the recipients were followed for more than two years.

Among the accidents reported there were some interesting cases which could contribute to solving the question whether or not human leukaemia could be transferred. Gramén [10] described the case of a patient with a bleeding duodenal ulcer who received blood transfusion from a seemingly healthy man. After 7 weeks the donor showed acute myeloblastic leukaemia and two weeks later he died. The recipient died two weeks after the transfusion due to bleeding from the ulcer. No examination of blood counts was, however, performed.

Mills [11] reported a case of chronic lymphatic leukaemia diagnosed in connection with an exchange transfusion given to a baby with haemolytic disease of the newborn. After transfusion a blood count typical of chronic lymphatic leukaemia was discovered. Therefore the donor was examined and it was ascertained that he suffered from chronic lymphatic leukaemia. The child's blood count had soon normalized and remained so for five months.

Sierman et al. [12] discussed experiments with 7 pairs of patients who were subjected to cross circulation. The authors wished to verify the presumption about leukaemia being caused by an inability to remove leucocytes from the blood. The lung tissue is able rapidly to remove a great number of leucocytes of foreign origin. Therefore the authors tried to form a connection between the circulation of leukaemic patients and another patient with uncurable disease. The treatment lasted 2–26 hours, 0.9–150 l of blood circulated; but improvement of the condition occurred in few leukaemic patients.

Other similar observations could also be found. One of these was leukaemia development in a man and shortly in his girl friend, with the same type of cytology [13]. Knox et al. [14] then tried to find out if leukaemic mothers were from large families. In that case there is a possibility of transmission of infective agents from

a family member and to the fetus by intrauterine way. This presumption has not been confirmed. Vianna [15] searched for social contacts among leukaemic patients but found none. Greenwald et al. [16] published a paper on the fate of subjects who received blood from donors who were later taken ill with leukaemia; they could find no increased incidence in such recipients.

The clinical observations of experienced haematologists deny the possibility of leukaemia transmission in hospital. In their 50 year experience with leukaemic patients, they did not observe a higher occurrence of leukaemia in the vicinity [17, 18]. Health service workers handling leukaemic patients are nearly "washed" with their blood, but transmission by this way was not confirmed [19].

We have thought it would be useful to draw attention to all the above similar cases. The present one was probably the first case of unintended blood transmission to a healthy adult – the experiments till now have been performed only with seriously ill volunteer subjects.

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Humoral Leukocyte Adherence Inhibition (H-LAI) with Basic Encephalitogenic Protein (BEP) in Head and Neck Cancer

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Fourty-five patients with head and neck cancer and 30 healthy subjects were evaluated by a modified humoral leukocyte adherence inhibition assay using basic encephalitogenic protein as antigen.

Ninety-three percent of cancer patients gave a positive reaction, whereas every control person remained negative. The results suggest that humoral leukocyte adherence inhibition assay performed with basic encephalitogenic protein may be useful as an early diagnostic tool in head and neck cancer.

Keywords: H-LAI, BEP cancer diagnosis

Introduction

It has been well established that cancer patients are capable of immunological recognition of their own tumour (Hellström and Hellström 1977, Baldwin 1977, Vose et al. 1977, Herbermann 1978, Bier et al. 1978, Plain and Whitehead 1981, James et al. 1981). The humoral leukocyte adherence inhibition (H-LAI) assay seems to be efficient in detecting both cellular (Halliday and Miller 1972, Gosser and Thomson 1975, Powell et al. 1978, Vetto et al. 1978, Maulish 1979) and humoral (Sanner et al. 1980, Kotlar et al. 1981), anti-tumour immune response.

Recently we (Kövesi and Fekete 1982) have introduced a new LAI assay using basic encephalitogenic protein (BEP) antigen instead of tumour tissue extracts. The BEP is a so-called common carcinoma antigen used originally in macrophage electrophoretic mobility test (MEMT), (Caspary and Field 1971, Pritchard et al. 1973, Douwes et al. 1978, Müller et al. 1977, Klausch et al. 1978, and Tautz et al. 1978).

Now the results on head and neck cancer patients obtained with H-LAI assay with BEP are presented.

Materials and Methods

Patients. Thirty-three men and 12 women with head and neck cancer aged from 24 to 80 (mean 70.3) years were selected for groups according to their TNM stages and to the histological type of cancer.

Thirty-one patients were previously treated (surgery and/or irradiation or surgery plus chemotherapy), one was under chemotherapy, one two days after surgery and 12 patients were never given any therapy.

Table 1

Distributional characteristics of the cancer patients

T. N. M. stages	I	II	III	IV	Total
Squamous cell cancer	4	4	4	8	20
Non-squamous cell cancer	6	5	9	5	25
Total	10	9	13	13	45

Control subjects. Eighteen men and 12 women aged from 20 to 80 (mean 48.1) years, suffering from benign oral diseases, without any sign of a cancer, served as controls.

Antigen preparation. Basic encephalitogenic protein (BEP) was prepared by the method of Caspary and Field (1971). The BEP powder was solubilized in RPMI–1640 (Grand Island Biol. Co., U.S.A.) and the protein concentration was adjusted to 6.5 g/l.

The BEP aliquots were stored at −20 °C until use.

Leukocytes. Venous blood was taken from healthy volunteers in the fasting state. Each ml contained 10 I.U. preservative-free heparin (Phylaxia, Hungary). Leukocytes were separated on Ficoll–Uromiro gradient according to Boyum (1976), then treated with 0.02% trypsin (DIFCO, U.S.A.) under constant stirring for 60 min at room temperature. The trypsinization was followed by repeated washings in RPMI–1640 and the cell concentration was adjusted to 1×10^7 cells/ml.

Patient sera. Blood was taken from the patients and control persons early in the morning. The blood samples were centrifuged twice and the sera were stored at −20 °C until use.

H-LAI assay; The technique of Sanner et al. (1980) was used with slight modification. Briefly, the test tubes (polyethylene, Falcon, England) contained 0.1 ml leukocytes, 0.2 ml RPMI–1640 completed with the tested serum (in 0.25% final concentration) and 650 μg BEP, or 5 T.U. purified protein derivative (PPD) (Phylaxia, Hungary) in 0.1 ml RPMI–1640. The control tubes contained 0.25 ml leukocytes and 0.75 ml RPMI–1640 containing the tested sera. The tubes were preincubated in humified atmosphere at 37 °C for 30 min gently shaking them every 10 min.

Following the preincubation period two control tubes were promptly evaluated (= base cell count). The remaining control and the test samples were trans-

ferred into glass tubes. After incubation at 37 °C in humified atmosphere for 60 minutes, cell counts were established.

The percentages of H-LAI were calculated as follows.

$$\text{H-LAI}\% = \frac{\text{test cell count} - \text{cell count in incubated control}}{\text{total cell count} - \text{cell count in incubated control}} \times 100$$

H-LAI values exceeding 29.9% were regarded as positive (Vetto et al. 1978).

Statistics. The percentage H-LAI was expressed as mean ± S.D. The differences between cancer patients and control subjects were evaluated by the two sample *t* test.

Pearson's correlation coefficient was calculated to estimate the relationship between H-LAI indices and oncological stages.

Results

Figure 1 shows the means ± S.D. of H-LAI% values obtained with BEP and PPD. While there were no statistical differences between the two groups using PPD, H-LAI indices with BEP in the cancer group were significantly higher than those of the healthy persons.

The relationship between %H-LAI and oncological stages is seen in Figure 2.

The H-LAI indices were 40.1 ± 11.3 for the stage I patients, 43.8 ± 12.5 for the stage II patients, 45.5 ± 8.6 for the stage III patients, and 47.2 ± 6.3 for the stage IV patients.

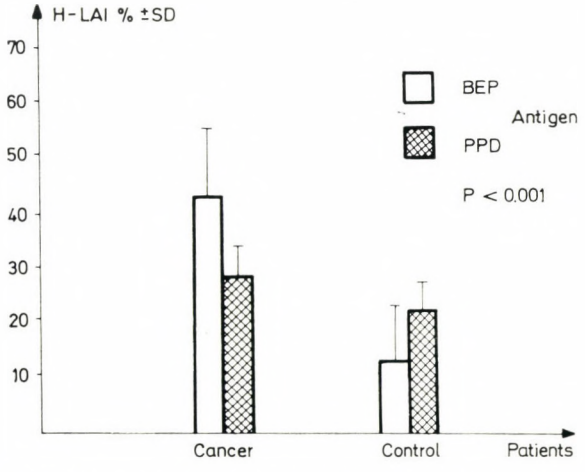

Fig. 1. Mean H-LAI indices using BEP (□) and PPD (▩) in the cancer and the control groups. H-LAI indices with BEP in the cancer group were significantly higher than those of the controls. No statistical difference was found using PPD

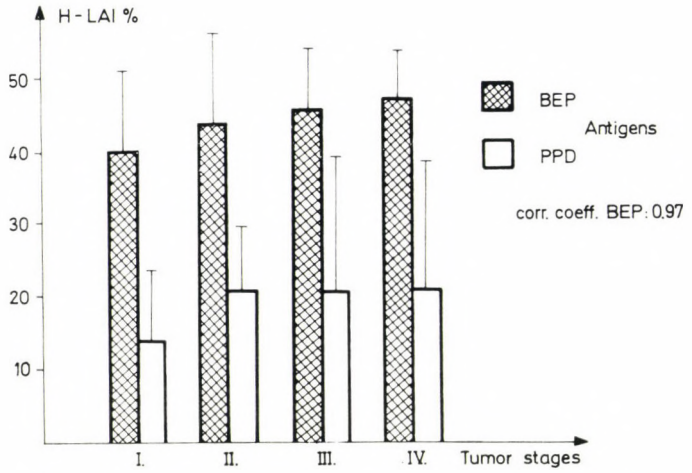

Fig. 2. Mean H-LAI indices according to tumour stages using BEP (▩) and PPD (□). The H-LAI indices did not differ in the four cancer subgroups

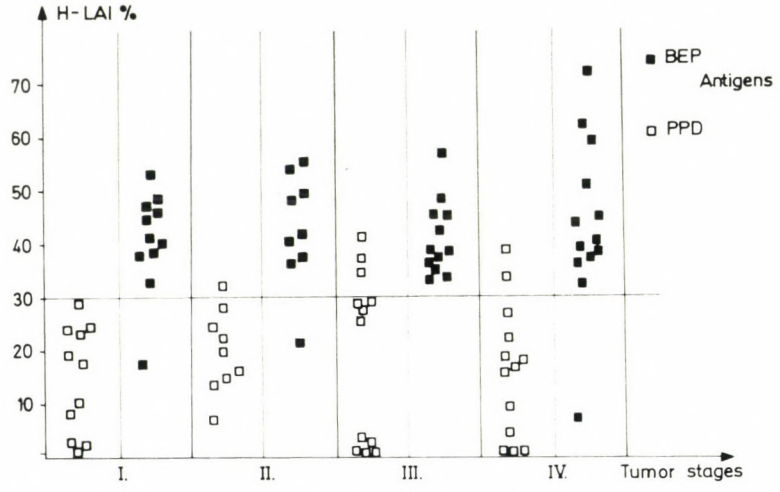

Fig. 3. H-LAI indices with BEP (■) and PPD (□) plotted against tumour stages. Using BEP only three patients were negative. The majority of the patients gave no positive reaction with PPD

The incidence for positive cases fell into the same range in all the four groups. Only three patients remained negative; one with carcinoma *in situ*, one had undergone surgery three days before sampling and one who had received cytostatic therapy (Figure 3).

A negative %H-LAI with PPD may reflect an immunological anergy due to cancer.

Discussion

Recently we have proposed the BEP as antigen in LAI assay and received good clinical correlates in oral malignancies [14]. This paper demonstrates that H-LAI by BEP can also be used for the immunodiagnosis of head and neck cancer. More than 90% of the cancer patients gave a positive reaction in the H-LAI assay. The fact that there were no differences in the levels of H-LAI indices among the four cancer subgroups means that the modified H-LAI assay is not influenced by serum blocking factors inhibiting the tube LAI in stages II–III of cancer [22]. The serum of patients with large tumour burdens contains free tumour-related antigens that coat the reactive leukocytes and block their reaction with tumour extracts in vitro, in both tube LAI and haemocytometer LAI assays. The H-LAI response is mediated through immune complexes formed in vitro. Free antigens or immune complexes already present in the serum will normally not influence the response. This may be due to the fact that LAI measurements are based on the difference in adherence found in the control samples where no antigen is added, and the adherence in the samples where the tumour extract forms immune complexes with the free anti-tumour antibodies.

Lending a great practical benefit to the new H-LAI test it can be carried out on small amount of fresh or stored sera. The introduction of BEP as antigen makes the assay very simple and reproducible. Furthermore, using PPD, the assay may assess the immunological competence of cancer patients.

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A Critical Approach to Therapeutic Apheresis in the Management of Inflammatory Dysimmune Polyneuropathies

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Data concerning 37 patients with inflammatory dysimmune polyneuropathy treated by discontinuous flow centrifugation, membrane plasma separation and cascade filtration are presented. Plasmapheresis was combined with immunosuppressants in patients with chronic or relapsing neuropathy (8 patients), cryoglobulinemic (6 patients) and paraneoplastic disease (2 patients), whereas 21 patients with acute Guillain-Barré syndrome (GBS) underwent exclusively plasmapheresis. Most patients were treated during the onset or progression of their disease and excellent or satisfactory clinical results were obtained in 18 patients with GBS, 6 with cryoglobulinemia, 2 with paraneoplastic disease and 4 with chronic relapsing polyneuropathy. Prior to therapy, 34 patients had high levels of immune complexes (CIC); this level was clearly reduced by plasmapheresis and clinical results correlated well with this removal. 3 patients with chronic dysimmune polyneuropathy, without any evidence of CIC, were completely unaffected by treatment. The possible role of CIC in demyelinating polyneuropathies is discussed on the basis of information given by cascade filtration treatment of 7 patients.

Keywords: plasmapheresis, plasma exchange, cascade filtration, immune complexes, polyneuropathy.

Plasma exchange (PE) for the clearance of undesirable circulating blood products has been used for several years, gaining more popularity and diffusion with the invention of automated separators. In neurology PE has been employed in the treatment of conditions such as myasthenia gravis in which pathophysiologic events taking place are known and in conditions such as inflammatory dysimmune polyneuropathies (IDPN) in which it is generally believed that immune mechanisms are involved, but a clear demonstration of pathophysiologic events has never been given. As a consequence, almost nothing currently known of the effectiveness of PE in IDPN satisfies modern standards for experimental proof. Pathogenetic controversies are reflected by controversies concerning the clinical outcome of PE in IDPN: and in fact, a number of anecdotal reports of benefits seemingly determined by PE in these patients [1, 2, 3] are balanced by a number of reports of dubious or negative outcomes [2, 4, 5].

In the present paper our experience with IDPN will be discussed in a manner taking into account some recent experimental informations and pheresis-derived insights into disease pathogenesis.

Inflammatory dysimmune polyneuropathies

IDPN are a heterogeneous group of acquired diseases. Although their pathogenesis remains largely uncertain, these conditions are considered dysimmune diseases occurring in immunodeficient patients, following infections, vaccination, pregnancy, connective tissue diseases, or after treatments with immunosuppressive drugs, transplantations, lymphomas and cancers. In the case of acute Guillain-Barré syndrome (GBS), this immunodeficiency was attributed to low molecular-weight inhibitors of T lymphocyte response [6]. IgM and IgG autoantibodies have been also implicated in determining neurologic damage, following demonstration of the similarity of the histological appearance of GBS and allergic experimental neuritis (EAN) in rabbits [10].

These autoantibodies were observed in a high proportion of sera of patients with GBS but also in nearly half of the samples from patients with other neurological diseases [7]. Circulating immune complexes (CIC) may also be involved [11, 12], in keeping with the presence of both Ig and complement factors in nerve biopsies and with data showing that the complement receptors may play a role in the binding of complement containing CIC in the nerves during the GBS disease process [12]. Some support to this hypothesis has been given by the good correlation between the clinical improvement and the decrease in CIC levels determined by PE in some patients [1, 13]. This CIC pathogenesis, which is not clearly demonstrated for the GBS, is clearly demonstrated for IDPN appearing in 50% cryoglobulinemic patients and in the course of tumoral diseases [14], as polyneuropathies are often combined with glomerulonephritis, vasculitis, purpura and arthralgia. Moreover, CIC vasculitis with multiple mononeuritis and typical GBS are common in systemic lupus E. S., rheumatoid arthritis, Wegener disease, Sjögren disease, temporal arteritis, polyarteritis nodosa and progressive systemic sclerosis and in general in immune complex diseases. In all these conditions, it was clearly shown that the removal of CIC by PE not only improved the vasculitis and related complications but also neurologic involvement [15, 16]. When CIC return to pretreatment levels, often but not always, a relapse occurs. In these conditions depressed T lymphocytes are common, with helper/suppressor imbalance which seems to be the basic condition that predisposes to autoimmune or immune complex diseases.

Treatment

PE was performed by discontinuous flow centrifugation or membrane plasma separation, using fresh frozen plasma as replacement solution [13]; cascade filtration (CF) was also employed [17]. In this case autologous filtered plasma was used as replacement solution. Treatments were organized into courses of 2 to 5 sessions over 3 to 10 days. The volume of each exchange ranged from 2.9 to 5.1% of body weight (BW). CIC were estimated according to Manca et al. [18]. The presence of rheumatoid factors and cryoglobulins was detected by con-

ventional methods. Patients with acute GBS were not given any cytotoxic drug or steroids, unless required for contemporary disease such as autoimmune hemolytic anemia. In other patients, prednisone (1 mg/kg BW/day) and azathioprine (2–2.5 mg/kg BW/day) were employed. Cyclophosphamide (5–6 mg/kg BW, i.v.) was added on days 2 and 9. Lymphocytapheresis was also employed as an adjunctive immunosuppressive measure in most patients with chronic relapsing GBS, when discontinuous flow procedures were performed. Two patients with paraneoplastic disease received only steroids and basic therapy for their cancer.

Patients and clinical results

Thirty-seven patients were entered into the study after we obtained their informed consent. The clinical condition was graded from 4 (function abolished) to 0 (normal function). Twenty-one patients with acute GBS, 8 with chronic relapsing GBS, 6 with cryoglobulinemic polyneuropathy and 2 with paraneoplastic disease were treated. Thirty patients underwent discontinuous flow centrifugation or membrane separation and 7 CF. In the following sections some clinical and laboratory data concerning our patients and treatments are summarized.

Acute GBS. Twenty-one patients who underwent apheretic treatment are described in Tables 1 and 2. Eleven patients were treated within 10 days after the clinical onset of their polyneuropathy (group A), 7 within 20 days (group B) and

Table 1

Results of PE therapy in patients with acute GBS treated within 10 days from disease onset (group A patients). Clinical condition is scored from 0 (normal) to 4 (function abolished). DFC: discontinuous flow centrifugation.

	Patient No.	Predisposing or combined disease	Day of PE	Type of treatment	Clinical score prior to/20 days after PE motility	ventilation
Group A	1	Enteric inf.	10	DFC	4/0	4/0
	2	Enteric inf.	9	DFC	3/0	3/0
	3	Flu-like S.	2	DFC	3/0	0/0
	4	Flu-like S.	6	DFC	4/1	1/0
	5	Enteric inf.	10	DFC	3/1	1/0
	6	Flu-like S.	8	DFC	3/1	0/1
	7	Flu-like S.	6	CF	3/0	1/1
	8	Enteric inf.	7	MPS	3/0	1/0
	9	Common cold (AIHA)	9	DFC (AZA, P)	3(4)/1	2/0
	10	Bronchitis	9	CF	4/1	1/0
	11	Common cold	5	MPS	3/0	2/0

MPS: membrane plasma separation. CF: cascade filtration. AIHA: autoimmune hemolytic anemia. AZA: azathioprine. P: steroids. Prior to PE all these patients with acute GBS had high levels of circulating immune complexes

Table 2

Results of PE therapy in patients with acute GBS and high levels of CIC, treated in the second decade (Group B) or later (Group C)

	Patient No.	Predisposing disease	Day of PE	Type of treatment	Clinical score prior to/20 days after PE motility	ventilation	Relapses or complications
Group B	12	Flu-like S.	13	DFC	4/1	4/0	
	13	Flu-like S.	14	DFC	4/1	1/0	
	14	Flu-like S.	17	DFC	4/2	0/0	(Ac. GBS 14 y. before)
	15	Bronchopneum.	18	DFC	4/2	3/1	
	16	Enteric inf.	12	DFC	3/1	0/0	
	17	Bronchitis	13	MPS	4/2	1/1	Relapse at 29 days
	18	Pulm. abscesses	11	DFC	4/3	4/3	The patient died 21 days after first treatment
Group C	19	Flu-like S.	35	MPS	4/4	0/0	
	20	Flu-like S.	23	DFC	4/4	0/0	
	21	Bronchitis	22	DFC	4/3(4)	2/2	

3 after more than 20 days (group C). Group A patients were treated either during the onset or the progression of their disease, group B when progression was halted for most symptoms and group C patients when their disease had reached its steady state. Our results appear to substantiate the value of an early treatment, as suggested by several authors [1, 2, 3] and shown in Fig. 1. Eleven patients had grade 3 to 4 improvement within 20 days from the beginning of plasmapheresis, 6 had grade 2 improvement and 3 were completely unaffected. Patients No 1 to 17 had a general pattern of response: following their first apheretic session, progression of the disease was halted and symptoms which appeared the latest, such as ven-

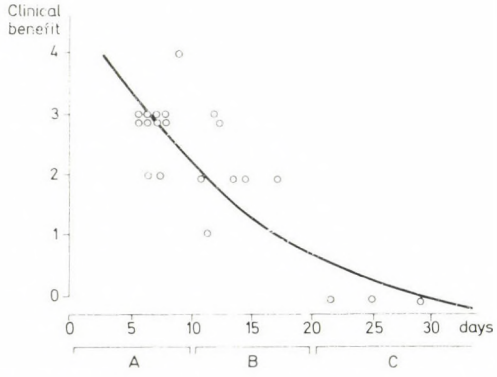

Fig. 1. Results of P. E. in 21 patients with acute GBs in respect to time of treatment. Clinical improvement at 20 days is graded from 0 (absent) to 4 (complete recovery).

tilatory insufficiency, began to improve. The following sessions brought maximum benefit and subsequently a slow and progressive improvement took place. Patient No. 18 escaped this behaviour: following each session ventilatory failure and motility showed grade 1 improvement but relapsed to pre-exchange conditions during the following 24 hours, with further progresssive deterioration. Renal insufficiency appeared with pulmonary distress syndrome which led this patient to death, 21 days after the first exchange. At necropsy multiple pulmonary and mediastinal abscesses were found, which had not been diagnosed on chest ray films obtained prior to PE treatment. Patient No. 15 too had bronchopneumonia followed by GBS: in this case adequate antibacterial therapy, by removing the cause of the polyneuropathy, seemingly, allowed PE to be effective on the neurologic damage. Group C patients had no benefit from treatment: this observation suggests that when nerve lesions have become anatomical, neither PE nor any other type of therapy can alter the course of the disease. All patients in this group had high levels of CIC prior to PE: this level was efficiently reduced by treatment, irrespective of whether we used CF or membrane plasma separation or discontinuous centrifugation. Decreased CIC level was the only laboratory evidence which correlated with patients' improvement.

Chronic relapsing dysimmune polyneuropathy (chronic GBS)

Eight patients unresponsive to steroids and azathioprine have been studied. Patient No. 1 was affected by true relapsing neuropathy and treated on onset of

Table 3

Results of PE therapy in patients affected by chronic relapsing dysimmune *GBS*. Patients 4, 6' and 7 had no evidence of circulating immune complexes. No patient but No. 2 had ventilatory insufficiency. Patients 3, 4 and 5 had reduced number of T suppressor cells. LYA: lymphocytapheresis. IS: immunosuppressants

Group	Patient No.	Predisposing disease	Days of treatment	Type of treatment	Motility: clinical score prior to/20 days after PE	Relapses or complications
A	1		4	DFC, IS	3/0	Rel. at 9 mos. regression on CF
B	2	Rheumat. arthr.	11	DFC, LYA, IS	4/3	
	3	Rheumat. arthr.	13	CF, IS	3/1 (2)	Rel. at 3 mos., poor response to DFC
	4	Scleroderma	19	DFC, LYA, IS	3/3	
	5	Rheumat. arthr.	18	CF, IS	3/1	Relapsing disease until IS started
C	6		215	DFC, LYA, IS	3/3	
	7		55	MPS, IS	3/3	
	8		87	DFC, IS	3/0	

relapses. Patients Nos 2 to 8 had the chronic type of the disease and were treated during the slowly progressive phase (group B) or during the steady phase (group C). Patient No. 1 showed a pattern of response similar to that of acute GBS patients with rapid recovery following treatment.

Patients Nos 3 and 8, of group B and C respectively showed some improvement which was much more slow than seen in acute GBS patients, reaching its maximum at 35–40 days following the completion of exchange sessions, when patients were on long-term steroids and immunosuppressants. Patient No. 2 was treated during an exacerbation of her chronic disease: some improvement was observed in ventilatory function, but the bulk of her symptoms was not clearly affected. Patient No. 5 had been treated 9 months before with some improvement, but this 7-yr-old girl rapidly relapsed to her pre-exchange condition. When we started with a second course of exchanges, she improved rapidly. Her family refused immunosuppressive drug treatment and the girl relapsed in the following 35 days, until we could start with a last course of exchanges coupled with steroids and azathioprine. Improvement was observed another time following exchanges, without relapses in the following 15 months. This patient as well as patients Nos. 2 and 3 had laboratory but not clinical signs of rheumatoid arthritis. CIC levels were very high in all patients but Nos 4, 6 and 7; none of these patients showed any improvement following exchanges. The reason for the failure of any notable improvement in some patients is not clear. It is likely that cases of chronic GBS do not constitute a homogeneous group. Since they vary in their response to steroids, it is possible that the same happens for plasma exchange [19]. However, since excellent results were obtained in 2 patients, we feel that at least PE should be tried in acute or subacute exacerbation. Clarification is needed not only concerning the most suitable patients but also concerning the role of lymphocytapheresis in these cases.

Paraneoplastic (Table 4) and cryoglobulinemic polyneuropathies (Table 5)

Patients with cryoglobulinemic disease were treated due to poor or absent response to steroids and cytotoxic drugs. Two patients with paraneoplastic disease

Table 4

Results of PE therapy in 3 patients with paraneoplastic PN and high levels of CIC

Group	Patients No.	Predisposing disease	Day of PE	Type of treatment	Motility: clinical score prior to/20 days after PE	Relapses or complications
(A)	1	Hepatoma, GN	10	DFC, CH	3/0 (1)	
	2	Renal cancer	8	MPS, CH	3/0	
	3	Lymphoma, GN	4	DFC, IS	3/0	Rel. GN.

Table 5

Results of PE therapy in patients with cryoglobulinemic PN. No patient had ventilatory insufficiency.

Group	Patients No.	Predisposing disease	Day of PE	Type of treatment	Motility: clinical score prior to/20 days after PE	Relapses or complications
(B)	1	Lymphoma	16	DFC, IS	4i2	Death 4 mos. later
	2	EMC	19	CF, IS	3/0	
(C)	3	CAH	72	CF, IS	3(4)/2	Rel. at 9 mos. (discontinued IS therapy)
	4	CAH	121	DFC, IS	3/2	
	5	EMC	63	DFC, IS	3/2	

GN: glomerulonephritis. EMC: essential mixed cryoglobulinemia. CAH: chronic active hepatitis. CH: chemotherapy. All patients had high or very high levels of CIC prior to PE

and 1 with polyneuropathy complicating the course of non-Hodgkin lymphoma (Table 4, A patients) displayed an acute onset and were treated early with excellent and rapid benefit. Patients with cryoglobulinemia (Table 5, B and C), were clinically indistinguishable from chronic GBS patients in onset and progression of the disease and the response to PE: only albumino-cytologic dissociation was lacking or not searched for. In patients with paraneoplastic polyneuropathy, this condition did not relapse even when patient No. 3 subsequently had acute renal insufficiency episodes when she suspended cytotoxic drugs against her physician's advice. Cryoglobulinemic group B patients had unequivocal but moderate improvement of those symptoms which appeared the latest. Long-lasting paralysis with muscle hypotrophy did not change or only slightly. CIC were present in all these patients and their removal seemingly determined the clinical results. Nonetheless, it is worthy of note that in 5 cryoglobulinemic patients good results were observed even in the presence of cryoglobulin and CIC levels well above the normal range.

Discussion

Our results, though uncontrolled, confirm that PE is of some utility in the management of IDPN with CIC. In view of the empirical evidence that only some patients recover when PE is instituted, instead of casting doubts on the efficacy of this therapy one should pose questions on the reasons of the failure. In our series of acute GBS the neurologic involvement appeared in the course of bacterial

pulmonary infections in patients Nos. 15 and 18. The former recovered rapidly following PE and adequate antibiotic therapy, whereas the latter was essentially unaffected by the apheretic treatment.

This might imply that the continuous production of large amounts of immune complexes in patient No. 18 was responsible for the failure. Such a hypothesis cannot be generalized; it is, nonetheless, in keeping with some previous observations such as relapsing polyneuropathies observed during successive pregnancies and recovering only after delivery [20]. On the other hand, when immune complexes are produced continuously, as in cases of cryoglobulinemia, cancer, rheumatoid arthritis and connective tissue diseases, PE can ensure only temporary relief, unless chemotherapy and lymphocytapheresis are successful in reducing the synthesis of autoantibodies involved in immune complex production or in reducing T helper/T suppressor lymphocyte imbalance. Whereas immune complex mediation of IDPN is generally accepted in cryoglobulinemia,

Table 6

Pathogenetic mechanisms of inflammatory dysimmune polyneuropathies of the GBS-type

Immune complexes	Antibodies	Lymphocytes
High levels of CIC	Analogy with EAN	T cytotoyic to Schwann cells
Receptors for CIC in nerves	IgG-Abs	Low-molecular weight inhibitors of T cells
Receptors for CIC in vessels	IgM-Abs	B/T imbalance
Analogy with IC-neuropathies	Linear IF pattern	Helper/suppressor imbalance
Granular IF pattern (C, IgG/M)	Response to steroids and immunosuppressants (CDPN)	Histologic picture
Typical GBS in LES		
Different aetiologies (viruses, bacteria, tumors etc.)	BUT	BUT
Onset after 7–14 days (as in serum sickness)	Abs in 50% of patients with different neurologic diseases	Lymphocytes necessary for Ab production
Combination with IC-GN, RA	Combination with IC-GN	
Positive results with CF	No rebound after PE (ac. GBS)	
No response to late treatment	Low association with autoimmune diseases	
Poor response to steroids and immunosuppressants	No transmission through placenta	
Poor transmission with serum	Relapses following steroids and immunosuppressants ac. GBS)	
No rebound in acute GBS		
BUT		
EAN		
CIC found in different neurological diseases		
CIC not necessarily harmful		
Lack of clear evidence		

CIC and autoantibodies are not mutually exclusive (experimental data of Koh et al. [21]

cancer and connective tissue diseases, this mechanism is under debate for polyneuropathies of the Guillain-Barré group, which probably is not a homogeneous group.

Both humoral and cellular immunological mechanisms are pathogenetically involved and there are arguments and evidence for and against both hypotheses, as summarized in Table 6.

Our data with discontinuous flow centrifugation and membrane plasma separation support a humoral mechanism: CF refines this assumption, favouring an immune complex mechanism, irrespective of the nature of antigens and antibodies involved in CIC formation. CF is capable of removing selectively IgM, CIC and high molecular weight plasma components, whereas IgG and low molecular weight components are given back to the patient. In this condition the improvement cannot be attributed to the removal of IgG autoantibodies or to the removal of low molecular weight inhibitors of T lymphocyte response [6], leaving unsolved the possible role of IgM antibodies and CIC.

It is possible, however, that autoantibodies against myelin or other nerve components, both IgG and IgM, are involved in immune complex formation and that CIC and autoantibody hypothesis are not mutually exclusive. This has recently been confirmed by the experimental study of Koh et al. [21] in experimental allergic neuritis (EAN). These authors detected CIC only in the sera of guinea pigs with clinical signs of EAN and not in animals with antibodies alone. Interestingly, immunofluorescence studies showed that immune complexes, mainly IgG and C3, were deposited in spots along the vessels of the white matter, nerves, meninges and in the choroid plexus. Vasculitis is considered to be the cause of IDPN appearing in patients with cryoglobulinemia, cancer and connective tissue diseases, which are often complicated by typical GBS [22]. On the other hand, typical GBS are often complicated by glomerulonephritis [23, 24] and temporal arteritis [25] suggesting a common pathogenetic mechanism of these conditions. On the basis of these clinical, pathological and experimental data, we feel that IDPN should be considered a group of diseases which share an immune complex pathogenesis, at least in a relevant number of cases. Since the production and deposition of immune complexes are conditioned by a number of factors in different patients, some differences in clinical expression and response to therapy are to be considered inherent with overlapping syndromes and expressions of the same basic condition which appears to be a disseminated vasculo-myelinopathy [23, 26], even though not exclusively. Further studies are needed before the role of immune complexes can be assessed, but we believe that CF as well as other selective techniques will help in solving the problem of IDPN pathogenesis.

However, until we know more about the cause (or causes) of these diseases and the effectiveness of PE in their treatment, we feel that only patients with ventilatory failure or with rapidly progressing disease should be subjected to exchange therapy before muscle wasting would develop. Neurologic abnormalities warrant vigorous treatment, since they are reversible in some instances, even if so far we do not have criteria for predicting which patients will benefit from treat-

ment. Chronic patients should only be treated when exacerbations occur or in the presence of complications related to steroids or cytotoxic drugs, since results in these cases are often limited.

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Haemoglobin Lyophilized with Sucrose: The Effect of Residual Moisture on Storage

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Sucrose is one of the most effective substances which protect haemoglobin from spontaneous methaemoglobin formation during lyophilization and subsequent storage. The dry haemoglobin–sucrose system was treated under different conditions of temperature (up to 85 °C), time of storage (up to 6 years) and residual moisture (< 0.1–7.5% H_2O), in order to reveal the main features of the hitherto unclear mechanisms of both spontaneous methaemoglobin formation and of the stabilizing effect of sucrose. In conclusion, a dry sucrose network was recognized as a significant support to the native ferrous structure of oxyhaemoglobin, while the presence of water molecules, of assumed peroxidic radicals and the action of thermal vibrations favour the oxidation and denaturation of haemoglobin.

Keywords: Haemoglobin, lyophilization, mechanism of haemoglobin stabilization, methaemoglobin, storage of haemoglobin.

Introduction

Successful lyophilization (freeze-drying) of dissolved native haemoglobin along with a fair preservation of its structural and functional properties is possible only in the presence of suitable stabilizing substances which protect haemoglobin from massive spontaneous oxidation to methaemoglobin (ferrihaemoglobin). A suitable critical concentration of a given stabilizer is necessary to achieve a standard protective effect. Among different stabilizers, sucrose seems to be most effective, followed in its effect by some amino acid salts. In spite of systematic investigation of various types of lyoprotectors, no direct correlation has yet been found between specific chemical structure and the stabilizing effect. The mechanism of stabilization is usually considered to be unclear [1–3].

According to previous investigations, the stabilizing effect (at least that of sucrose) might be elucidated in physical rather than chemical terms [2, 4, 5]. A mechanical rigidity of a dry network of associated protector molecules was assumed to be important in supporting the conformation and ferrous state of native haemoglobin during sublimation of water, as well as during storage and redissolving of the lyophilizate [4–6]. The favourable effect of low temperature upon slowing down methaemoglobin formation during long-time storage of haemoglobin is well known and frequently used [1–3, 7]. Little is known about the behaviour of lyophilized haemoglobin at temperatures above 20 °C [6].

The present experiments were aimed at investigating this problem in order to find the limitations and possible improvements for the storage of dry native human ferrohaemoglobin at room temperature. An attempt was also made to interpret in more detail some important aspects of the mechanisms of spontaneous methaemoglobin formation and of haemoglobin stabilization by sucrose.

Materials and Methods

Stroma-free haemolyzates containing 50–80 g/l haemoglobin were prepared from banked human erythrocytes by hypoosmotic haemolysis, centrifugation and filtration through membrane ultrafilters as usually [4–6]. Isotonic (0.15 mol/l) NaCl or phosphate buffers pH 6.0–8.0 represented the salt composition of the solutions. Sucrose was added to a final concentration of 0.17 to 0.2 mol/l for lyophilization experiments. Deoxygenation of haemoglobin was achieved by progressive evacuation of the haemolysate for 15 min followed by introducing nitrogen gas for 100 minutes under stirring at 0 to 5 °C. Haemoglobin solutions of 5 to 15 ml were rapidly frozen at −35 °C and freeze-dried at vacuum pressure 4 Pa and drying temperatures not exceeding 25 °C [6]. The lyophilizates were either equilibrated in humid air or dried in desiccators at 20 °C to reach different amounts of moisture estimated by final drying to constant weight over P_2O_5. Sealed freeze-dried samples were kept at defined temperatures between 5 and 85 °C. At selected time intervals they were dissolved in fresh distilled water (in non-buffered samples the final pH was usually 6.2–6.7) at 20 °C and immediately analyzed for methaemoglobin [4–6]. Isoelectric focussing [8] and biotonometric analysis of oxygenation curves [9] were performed as before.

In preliminary liquid phase experiments, the spontaneous methaemoglobin formation in crude haemolysate and in solutions of purified stroma-free haemoglobin (7) were investigated both without and with sucrose dissolved up to 2 mol/l concentration in phosphate buffer pH 7.4. The solutions were incubated at 37 °C for 48 hours. The effect of slow freezing (sample completely frozen after 4–5 hours in air at −12 °C), rapid freezing (sample completely frozen after 2–3 minutes in an ethanol bath of −40 °C) and repeated freezing and thawing (in a water bath of 20 °C) on spontaneous methaemoglobin formation was also investigated.

Results

Liquid phase experiments

At the beginning of the present work we wanted to know, whether sucrose can stabilize the native form of haemoglobin also in solution similarly as had been shown by others [10] with some nonhaemoglobin proteins. However, during incubation of haemoglobin solutions no significant stabilizing effect of sucrose

against methaemoglobin formation was observed as compared to sucrose-free samples. Moreover, spontaneous oxidation of haemoglobin was slightly (by 10–15%) higher in the presence of sucrose. This confirmed previous assumptions that the stabilizing effect of sucrose is connected with the properties of the haemoglobin–sucrose–water system in the solid phase.

Our experience with different conditions of freezing upon spontaenous oxidation of haemoglobin can be summarized briefly as follows: Slow freezing and thawing repeated twice or thrice caused a significant increase of methaemoglobin from 3% to 16% (n=5, SD=6) in stroma-free haemoglobin and to 45% (n=6, SD=3) in crude haemolysate during storage in the frozen state for 3 and 21 weeks, respectively. Five times repeated rapid freezing and thawing of both crude and purified haemolysate during 1 week did not cause any marked elevation of methaemoglobin concentration over 10%. Thus, rapid freezing is more suitable for conservation of native haemoglobin.

Experiments with lyophilizates

In orientation experiments with freeze-dried oxy- and deoxyhaemoglobin both samples were equally stabilized by sucrose against oxidation for up to 1 month storage at 20 °C (methaemoglobin remained below 10% in each group of n=5). After 5 months of storage at 20 °C oxyhaemoglobin showed 11% (SD=2) methaemoglobin while in parallel deoxyhaemoglobin samples methaemoglobin increased to 23% (SD=2). Oxyhaemoglobin was stabilized evidently better than deoxyhaemoglobin.

Oxygenation curves of lyophilized and redissolved samples were practically equal to those of non-lyophilized samples with a similar methaemoglobin content (5–15%). P50 values were usually 1.7–2 kPa (37 °C, pH 7.40), Hill's coefficients were 2.3–2.6 (cf. ref. 1).

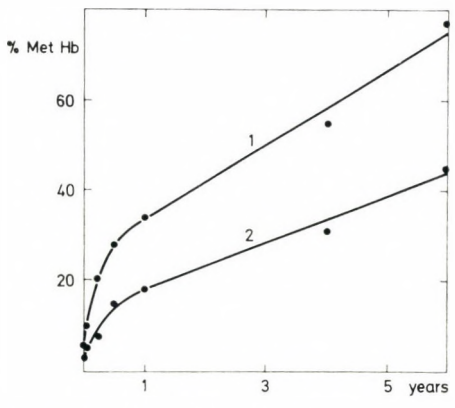

Fig. 1. Spontaneous methaemoglobin formation in oxyhaemoglobin lyophilized with sucrose. Residual moisture was 4.7% in sample 1 and 1.4% in sample 2. Stored at 20–25 °C

Only slight differences were observed between isoelectrofocussing patterns of fresh haemoglobin samples and of those lyophilized with sucrose and stored for several months at 20 °C (cf. ref. 8). After 4–6 years of storage, however, the sharpness of all haemoglobin zones decreased markedly, especially in the A_1 region.

Figure 1 shows a typical example of a time-dependent increase of methaemoglobin in lyophilized, sucrose-stabilized oxyhaemoglobin samples stored at 20 to 25 °C for several years in closed vials. The two curves were constructed from mean data of 6 randomly taken haemoglobin batches divided into two groups differing substantially in mean residual moisture ($1.4 \pm 0.6\%$ and $4.7 \pm 1.7\%$, respectively). Spontaneous oxidation to methaemoglobin proceeded during the first year exponentially in both groups and than rather linearly by an increment of $8 \pm 3\%$ of methaemoglobin per year. The oxidation was markedly higher in the more moist samples. At low temperatures (−20 °C), however the methaemoglobin increment per year was less than 5%. The marked dependence of the rate of methaemoglobin formation on residual moisture in samples kept at 20 to 25 °C for 4 years is documented in Fig. 2. Standardly lyophilized stabilized oxyhaemoglobin with less than 1% (preferably less than 0.1%) residual moisture contained after 12 months storage at 20 to 25 °C usually $18 \pm 4\%$ of methaemoglobin.

The influence of elevated temperature upon lyophilized haemoglobin is illustrated in Fig. 3. Two parallel sets of 3 samples differing in residual moisture (1.1% and less than 0.1% H_2O) were kept in closed vials at selected temperatures for 2 hours. Up to about 50 °C the slow increase of methaemoglobin concentration was similar in both samples. Above 55 °C the increase was significantly higher in the more moist lyophilizates. The time-dependence of the increase of methaemoglobin in samples heated to elevated temperatures for up to 24 hours is shown in

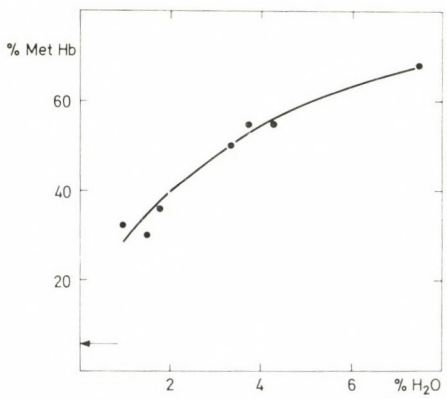

Fig. 2. Effect of residual moisture upon spontaneous oxidation of oxyhaemoglobin lyophilized with sucrose. Stored at 20–25 °C for 4 years. The original methaemoglobin content is indicated by arrow

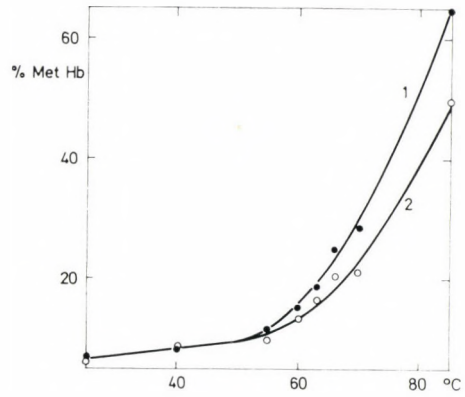

Fig. 3. Effect of temperature and moisture upon spontaneous oxidation of oxyhaemoglobin lyophilized with sucrose. Samples were treated at the given temperatures for 2 h. Residual moisture was 1.1 % in sample 1 and less than 0.1 % in sample 2

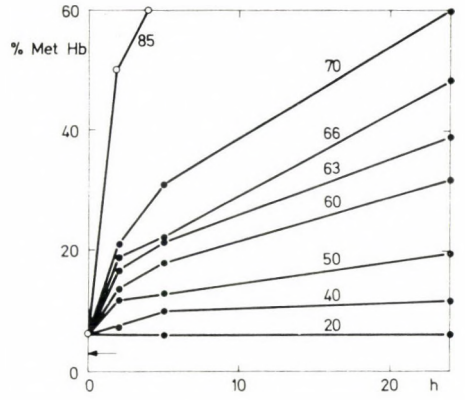

Fig. 4. Time dependent spontaneous oxidation of lyophilized and sucrose-stabilized oxyhaemoglobin. The numbering of curves corresponds to temperatures in °C. The original methaemoglobin content is indicated by arrow

Fig. 4. Since there was a formal similarity between the time-dependence demonstrated in Figs 1 and 4 it is possible to simulate and to predict roughly the methaemoglobin formation in a given sample by such accelerated aging experiments. This matter, however, needs further investigation, because there were sometimes marked and not yet satisfactorily explainable differences in absolute values of methaemoglobin between various batches. After prolonged heating to temperatures over 65 °C the differences between less and more moist samples (below 1 % H_2O) disappeared or were not significant statistically.

Discussion

The present results were in good agreement with previous experience that residual moisture, time and temperature of storage are the most important factors influencing the speed and degree of spontaneous oxidation of haemoglobin lyophilized with sucrose under standard conditions [1, 4, 5] and physiological salt concentration.

Spontaneous formation of methaemoglobin seems to be inherited to native haemoglobin since a specific methaemoglobin reductase system is always present in erythrocytes. The cause of this oxidation is unclear. Hypothetically, it could be explained either by a direct effect of oxidative contaminants upon the heme iron or by the presence of miscellaneous other substances and ions which upset the electrostatical and spatial equilibria in the heme pocket [11]. Water molecules and their dissociation products evidently belong to such disturbing factors. Induced polarization and shifts in the π orbital system of the iron–porphyrin complex might then cause, in the presence of water and proportionally to its amount, a stepwise generation of peroxidic radicals able to oxidize the heme iron.

For a better understanding of the mechanism of haemoglobin stabilization by sucrose and of the role of residual moisture in the lyophilizate it is necessary to consider the changes of the given system during the whole freeze-drying process. At the beginning, the oxy (or deoxy) haemoglobin-sucrose-water system is rapidly frozen. The randomly distributed haemoglobin molecules and their dissociated subunits are immobilized in their specific tertiary t (or r) and quaternary T (or R) conformations [12]. During evacuation and stepwise heating of the frozen system, water molecules sublimate off leaving free space while nonvolatile molecules remain at their position. In the presence of a sufficient concentration of sucrose a dry rigid network ensues of suitable shape and sufficient strength to resist various physical constraints and to conserve the native conformations of both oxy and deoxyhaemoglobin. The network is supposed to consist of sucrose molecules adhering mutually one to the other as well as to the adjacent parts of the haemoglobin molecules. H-bonds and van der Waals forces seem to be the main fixing factors here.

On the other hand, in the absence or under insufficient concentration of sucrose during lyophilization [1, 4], the native conformation of haemoglobin undergoes deep deformations caused by thermal vibrations and intramolecular interactions which are otherwise (in solution or in a wet crystal) well compensated. Due to a breakdown of the spatial and electrical features of the heme pocket the iron atom has lost its specific protection against rapid irreversible oxidation to the ferric state [4-6].

The agitating effect of water molecules upon sucrose-stabilized dry haemoglobin during storage can be explained similarly as in previous model experiments with low molecular polyethyleneglycols [5], by lowering the mechanical strength (i.e. partial dissolving) of the dry supporting sucrose network.

The lower stability of dry deoxyhaemoglobin as compared to that of oxy-

haemoglobin might be connected a.o. with the topography of a more opened heme pocket in deoxyhaemoglobin so that an intrusion and interference of residual water molecules and other contaminants is more probable.

Freeze dried oxyhaemoglobin stabilized with sucrose is relatively resistant to the denaturing effect of thermal vibrations for several hours up to a temperature of about 55 °C known to be critical for the uncoiling of helical structures of most proteins. At higher temperatures, denaturation and elevated methaemoglobin formation take place even in the presence of sucrose and at low moisture. However, short exposure (up to 5 – 15 min) to elevated temperatures (60 to 70 °C) did not cause an increase to above 10% in methaemoglobin (Fig. 4). There is a certain similarity between these results and those achieved recently by Labrude et al. [13] who desiccated haemoglobin solutions with stabilizers by atomizing them rapidly at 66 °C in a minispray-dryer.

From the present results and hypothetical deductions we anticipate that further improvement of a long-time storage of native oxyhaemoglobin can be achieved in the dry state. This would facilitate the use of haemoglobin in many branches of biochemistry, biology and medicine [2, 3, 7, 14].

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Haematological Findings in the Rat following Administration of Nephrotoxic Doses of Mercuric Chloride

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Single oral administration of a toxic dose of mercuric chloride (2.0 mg/kg) resulted in moderate reticulocytopenia and stress reaction in the white blood picture. A slight decrease in marrow cellularity followed the administration of 0.5 or 2.0 mg/kg.

Repeated administration led to an elevated reticulocyte count and leukocytic stress reaction. An increase in marrow E : M ratio, proportion of erythroid nucleated cells in both femoral marrow and spleen was also found. The elevation of doses and prolongation of the time of treatment did not lead to a deterioration of marrow morphology; a diminution of the haemotoxicity for the rat marrow was revealed in the present experiments too.

Keywords: blood picture, marrow, mercuric chloride, nephrotoxicity, rat, spleen

Introduction

We could find no data on the influence of nephrotoxic doses of mercuric chloride or other compounds on the blood picture of laboratory rats except for cytochemical investigation on leukocytes [23]. Mercuric chloride administration can induce diffuse injuries with necrosis of the proximal tubular epithelium which according to the dose level is reversible in both human subjects and animals [3, 8, 10, 12, 14, 15, 17, 19, 21]. An adequate kidney function is essential for the regulation of erythropoiesis [1, 11, 13, 18]. It was of interest to see whether any haematological changes could be detected in laboratory rats after nephrotoxic doses of mercuric chloride. Experiments were therefore undertaken to study the fundamental haematological characteristics which are recommended for xenobiotic research [26]. Nephrotoxicity of doses of mercuric chloride was checked by studying the urinary sediment [10].

Material and Methods

We used 220 male Wistar rats from a conventional colony of our Institute (WIST/RIPB outbred stock). The animals were allowed to acclimatize for two weeks and included into the study at the age of 8 weeks. They were housed in the vivarium with artificial light–dark cycles of 12h/12h and under a temperature of

23–25 °C. Pelletted diet VELAZ DOS 2b and drinking water were available ad libitum.

Blood and bone marrow were investigated by methods described previously [cf. 4, 5]. Solutions of mercuric chloride (Lachema, Brno, Czechoslovakia) were administered, blood was drawn and urine collection began 0.5–1.0 h after switching on. Femoral, splenic and hepatic imprints were prepared after 1.0–1.5 h switching on and stained by the Pappenheim panoptic method. Haematological examinations preceded the procedure of urine collection. Before obtaining urine rats received 25 ml of water per kg b.w. orally and urine was collected for two hours thereafter. Microscopic examination of 0.5 ml urinary sediment [cf. 10] was used for the quantitative assessment of excreted renal tubular cells in Bürker's chamber; results were expressed as cell excretion per minute for each rat.

Mercuric chloride dissolved in water was administered (0.5 ml per 100 g b.w.) orally (LD_{50} about 37 mg/kg [22]) as follows. *Group 1* a single dose of 0.5 mg/kg; *Group 2* a single dose of 2 mg/kg (lethal effect; 78% of the animals died within 14 days); *Group 3* a dose of 0.2 mg/kg/day on five consecutive days a week for four weeks (total dose 4 mg); *Group 4* a dose of 0.5 mg/kg/day on five consecutive days a week for two weeks (total dose 5 mg of mercuric chloride); *Group 5* same as in Group 4 plus 7 days the same dose plus 1.0 mg/kg/day for further seven days (total dose 15.5 mg of mercuric chloride); *Group 6*, same schedule as in Group 5 plus 2.0 mg/kg/day on the next seven days (total dose 29.5 mg of mercuric chloride). Control animals received 0.5 ml water per 100 g b.w. orally in the same schedule as solutions of mercuric chloride.

The results are expressed as means ± s.e.m. for 6–9 animals for each point of time and each group. Data were evaluated by two-sided Mann-Whitney's U test at the significance level of 0.05.

Results

Single administration

The quantitative assessment of renal tubular cell excretion is shown in Table 1. It seems that the degree of renal injury depends on the quantity of mercury administered.

Only small changes were found in haematocrit, haemoglobin levels and erythrocyte count. Their values remained near the control ones; the greatest difference was found in the haemoglobin level 72 h after administration of 2 mg/kg: control = 168.3 ± 3.9, experimental group = 149.2 ± 3.6, $p < 0.05$. Reticulocyte counts fell within 3 days and then became normal (Fig. 1). A significant decrease of the absolute lymphocyte count and an increase of the segmented neutrophil count occurred 24 h after 2 mg/kg mercuric chloride administration (Fig. 2).

Cellularity of the femoral marrow was slightly decreased but marrow E : M ratios decreased in rats which received the highest dose of mercuric chloride (Fig. 3). The decrease in marrow cellularity (Fig. 3) 72 h after a single dose was in

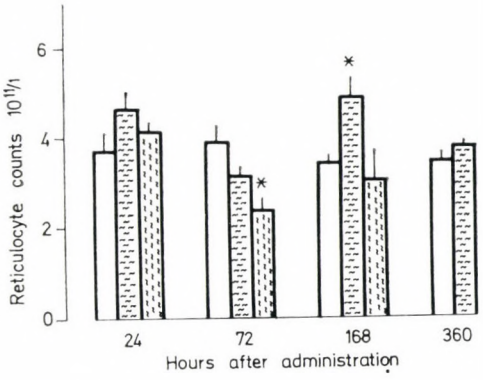

Fig. 1. Reticulocyte counts following single mercuric chloride administration. □ controls, ▨ 0.5 mg/kg, ▩ 2.0 mg/kg; ✳ statistically significant as compared with controls

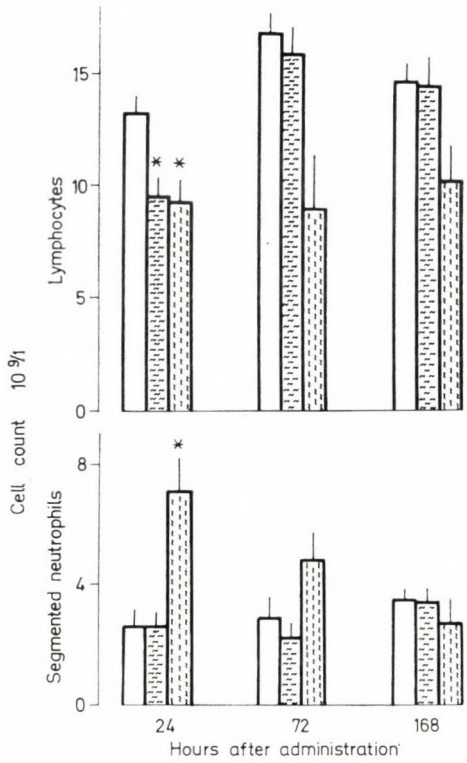

Fig. 2. Effect of single administration of mercuric chloride on circulating segmented neutrophil and lymphocyte counts. Symbols as in Fig. 1

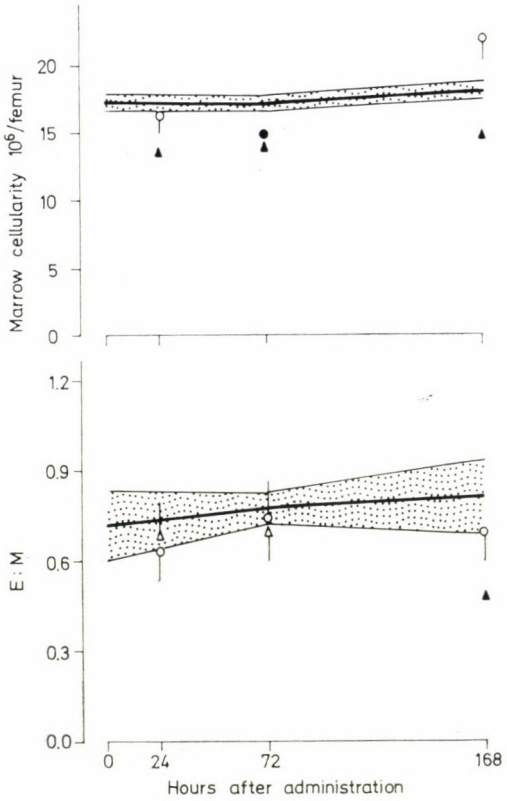

Fig. 3. Marrow cellularity and E : M ratios after single mercuric chloride administration. Dotted area — controls (mean ± s.e.m.), circles — 0.5 mg/kg, triangles — 2 mg/kg, black circles and triangles — statistically significant as compared with controls

relation to the decrease in circulating reticulocyte counts (Fig. 1). The slight increase in marrow cellularity with unchanged E : M ratios seven days after a single dose of 0.5 mg/kg was also correlated with the slight increase in the circulating reticulocyte count. The decrease in the proportion of marrow nucleated erythroid cells 7 days after 2 mg/kg of mercuric chloride was, however, not related to the restoration of the reticulocyte count. The proportion of erythroid nucleated cells in spleen and liver was not elevated after mercury administration.

Repeated administration

The quantitative assessment of renal tubular cell excretion is shown in Table 2. Excretion of epithelial and blood cells 24 h after the last dose was lower than that 24 h after a single administration (Table 1).

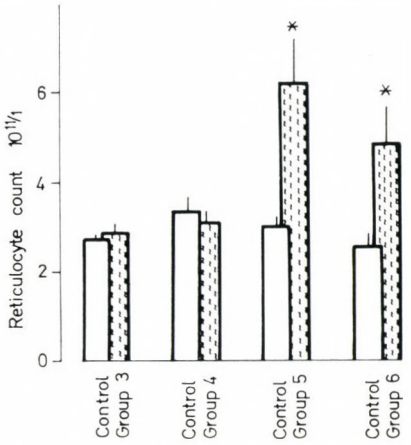

Fig. 4. Effect of repeated administration of mercuric chloride on reticulocyte counts (24 h following the last dose). Blank columns — controls, dashed columns — mercuric chloride loading, * statistically significant versus controls

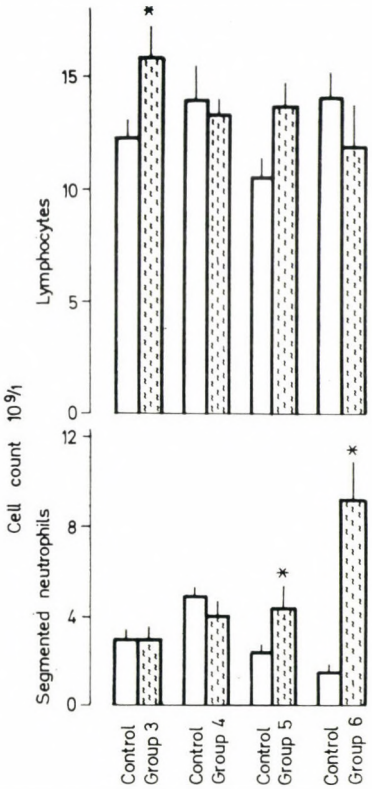

Fig. 5. Circulating segmented neutrophil and lymphocyte counts following repeated mercuric chloride administration (24 h after last dose). Symbols as in Fig. 4

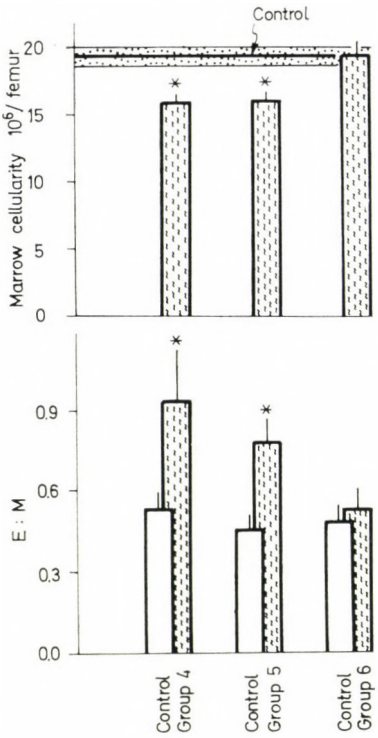

Fig. 6. Effect of repeated administration of mercuric chloride on marrow cellularity and E : M ratios (24 h after last dose). Symbols as in Fig. 3 and 4

Only small changes occurred in haematocrit, haemoglobin and erythrocyte counts. Their values remained near the control ones. The greatest difference was shown in the haemoglobin levels: i.e. controls = 169.7 ± 4.0, and Group 6 =

Table 1

Urinary sediments following single dose of mercuric chloride

Dose (mg/kg)	Time after administration	Epithelial cells/min/rat		Leucocytes/min/rat	
		Mean	Range	Mean	Range
Control	24 hours	35.7	28.5–50.9	0.0	0.0–0.0
	7 days	66.8	27.8–194.4	5.9	4.6–37.0
0.5	24 hours	4270.8[a]	1666.7–8854.2	1101.2[a]	416.7–2187.5
	7 days	35.7	27.8–55.6	8.6	4.6–13.9
2.0	24 hours	417.0[a]	23.1–1562.5	47.9[a]	4.6–208.3

[a] statistically significant as compared with control values

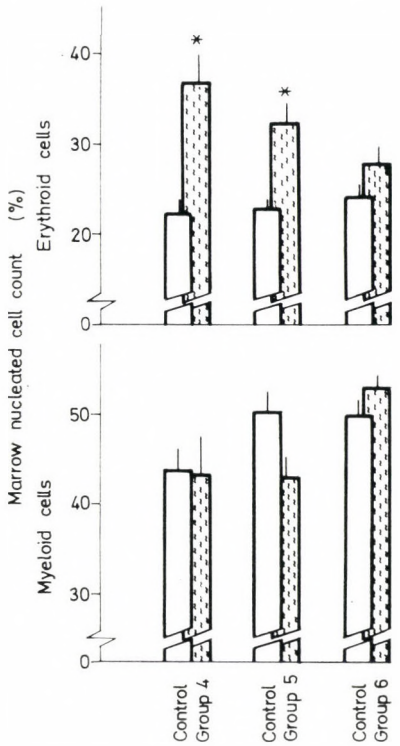

Fig. 7. Marrow erythroid nucleated and myeloid cell counts following repeated mercuric chloride administration (24 h after last dose). Symbols as in Fig. 4

Table 2

Urinary sediments following repeated administration of mercuric chloride (24 h after the last administration)

Dose	Epithelial cells/min/rat		Leucocytes/min/rat		Erythrocytes/min/rat	
	Mean	Range	Mean	Range	Mean	Range
Control	90	56–167	0	0–0	0	0–0
Group 3 (0.2 mg/kg/d)	2622[a]	1354–3958	0	0–0	17	0–104
Control	65	51–74	0	0–0	0	0–0
Group 4 (0.5 mg/kg/d)	505[a]	69–2292	37[a]	5–104	99[a]	5–521
Control	52	37–83	1	0–5	2	0–14
Group 5 (0.5+1.0 mg/kg/d)	382[a]	222–917	6	0–18	6	0–9
Control	66	28–116	0	0–0	0	0–0
Group 6 (0.5+1.0+2.0 mg/kg/d)	2135[a]	625–5312	104	0–312	17	0–104

[a]statistically significant as compared with control values

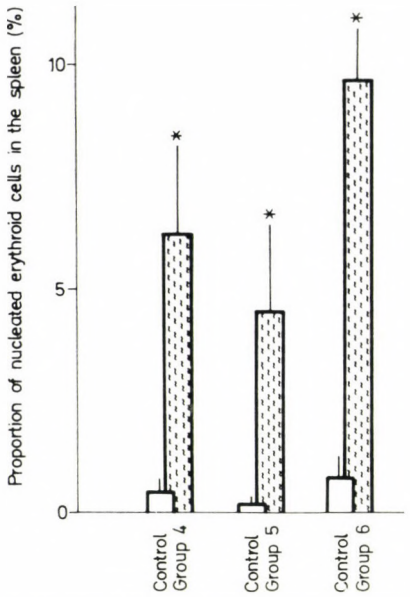

Fig. 8. Proportion of nucleated erythroid cells in the spleen after repeated mercuric chloride administration (24 h after last dose). Symbols as in Fig. 4

$= 143.5 \pm 4.5$, $p < 0.05$. The reticulocyte count was increased in Groups 5 and 6 (Fig. 4). The absolute lymphocyte count showed only small changes while the absolute segmented neutrophil count increased distinctly in Group 6 (Fig. 5).

Bone marrow cellularity decreased slightly in the group of the dose of 0.5 mg/kg/day for two weeks or after 0.5 plus 1.0 mg/kg/day (Group 5) while it remained within normal limits in Group 6. In Group 4 the marrow E : M ratio increased after two weeks but decreased after administration of higher doses in Groups 5 and 6 (Fig. 6). Variations in bone marrow morphology were associated especially with an elevated erythroid count (Fig. 7). In contrast to the findings that marrow E : M ratios and proportions of marrow erythroid nucleated cells returned to normal values on prolonged treatment and with increases in the dose, the proportion of erythroid nucleated cells in the spleen remained markedly increased (Fig. 8).

The reticulocyte count was not correlated with the proportion of marrow nucleated erythroid cells after mercury loading. It seems that part of the circulating reticulocytes may have originated in the spleen at the later part of chronic administration.

Discussion

The cause of the haematological changes is certainly a complex one. The slight fall in reticulocyte counts after a single dose of mercuric chloride may have been due to a decrease of renal erythropoietin production [1, 13, 20] and/or the

cytotoxic effect of waste products [18, 24, 25]. The elevated reticulocyte count found in multiple dose trials could be a consequence of a decrease in the erythrocyte mass after mercury treatment similarly as that induced by a blood loss and/or some haemolytic effect [2, 7, 16]. The observed changes in bone marrow morphology may be induced by factors similar as those affecting the circulating reticulocyte count.

Single administration of mercuric chloride suppressed erythropoiesis, while repeated doses did not inhibit but stimulated it. It is suggested that the effect is based upon metabolic changes caused by the mercury; whereas mercuric chloride inhibited haemolysis at a concentration of 10^{-3} M it became a potent lytic agent at lower concentrations [2].

The erythrocyte count, haematocrit and haemoglobin level were not affected by mercury treatment. As they all decrease with a blood loss exceeding 20% of the total blood volume [5, 6], it is assumed that after mercury treatment necessary for the activation of erythropoiesis blood loss in the rats did not exceed 20%. The changes observed in the segmented neutrophil count can be explained as a stress reaction [cf. 9].

The findings indicate that the character of haematological changes in laboratory rats following administration of nephrotoxic doses of mercuric chloride depends not only on the dose level but also on the duration of loading.

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Obituary

Dr. Robert R. Race died on April 15th 1984 after a long illness. During the period beginning 1942, when he took over the directorship of the Galton Laboratory Serum Unit until 1973, when he retired, Dr. Race exercised a tremendous influence on the development of his chosen field: the genetics of human red cell alloantigens. Undoubtedly, the greatest single influence on his development as a scientist was the personal teaching which he received from R. A. Fisher. From Fisher, Race learned the essential role played by statistics in unravelling the dependence or independence of one alloantigen from another and from the first his papers were characterised by the use of tests of probability.

In the early work on the Rh system Race's role was difficult to separate from that of Fisher, whose concept of three pairs of closely linked genes provided a framework into which almost all the "new" antigens of the Rh system could be fitted. However, in 1946 he moved with his unit (now the Blood Group Research Unit) to the Lister Institute in London and soon showed that he had his own powerful contribution to make. From then until 1973, when he retired, his unit made one important discovery after another; amongst the most memorable of these were: the findings of -D-, ce and ce^s (V); the discovery of the O_h ("Bombay") phenotype and of the "null" phenotypes S-s- and Fy (a-b-); the recognition of an acquired form of the B antigen and of the first human chimera and, finally, the discovery of the antigen Xg^a, determined by an X-borne gene. This astonishing catalogue, which is far from complete, establishes Race's central role in the development of his subject.

In 1946 Ruth Sanger arrived from Australia to work in Race's unit on a Fellowship and her collaboration with Race led almost immediately to an important discovery. A serum sent from Australia was identified as containing an antibody, anti-S, reacting with an antigen shown to be linked to M and N. The discovery indicated that Rh was not the only blood group system with a complex locus; indeed, it later became evident that those blood group systems that appeared to be simple were simply those about which least was known.

After the termination of her Fellowship, Ruth Sanger became a member of the Blood Group Research Unit and soon played such an important part in its work that, in the field of human blood groups, her name came to be linked inseparably with that of Race. They were married in 1956. During the last few years of his life she virtually gave up work in order to care for him.

The book "Blood Groups in Man" by Race and Sanger which was published in 1950 brought them world-wide fame in medical and scientific circles. Numerous workers in fields related only distantly to blood groups who turned to the book for information discovered they had found something unique: a textbook which was at the same time of the highest scientific standard and yet full of humour. For a period of 25 years, which separated the first and sixth editions, this book was in a class of its own as a source of reference, of insight and, one might well say, of inspiration.

Race was rather a retiring man and was happiest with very small groups of people. Throughout his career he carried out much of his serious business in "pubs" (Inns). There he met his scientific friends, speculated about the interpretation of recent findings and even wrote lectures. At large meetings he could be nervous and did not like to take part in discussions unless to communicate something which he had prepared with great care. When he did give a paper he was masterly because he had the power to make complex subjects simple and he had a command of the English language which reflected his strong interest in literature.

When, in the very early 1940s, Race started to play an important part in the field of human blood groups, only the ABO, MN, P and Rh systems had been described and then only at a rather rudimentary level. In determining the pace

at which the other systems were revealed, in exploring the complexing of blood group *loci* in general and in establishing the importance of blood groups in human genetics, he was pre-eminent.

P. L. Mollison

Abstracts

Characterization of a (Ca^{2+} + Mg^{2+})-ATPase activator bound to human erythrocyte membranes. B. D. Roufogalis, C. T. Elliott and G. B. Ralston (Department of Biochemistry, University of Sydney, Sydney, N.S.W., Australia). *Cell Calcium 5*, 77 (1984).

Incubation of human erythrocyte ghosts with an equal volume of 0.2 mM EDTA in isotonic KCl decreased both the activity and Ca^{2+} sensitivity of the (Ca^{2+} + Mg^{2+})-ATPase remaining associated with the membrane. Readdition of the EDTA-extract activated the (Ca^{2+} + Mg^{2+})-ATPase activity. The activator activity was trypsin sensitive, heat stable and retained by a phenothiazine affinity column, consistent with properties expected of calmodulin. However, unlike calmodulin, the activity was not retained by DEAE Sephadex A-50 and it eluted from Sephacryl S-200 as heterogeneous peaks of activator activity of apparent molecular weight between 107,000 and 178,000. Nevertheless, the activator in the EDTA extract both before and after gel filtration contained calmodulin, as determined by radioimmunoassay and by its activation of calmodulin-deficient phosphodiesterase. SDS-gel electrophoresis of the activator isolated by gel filtration showed a protein of M_r 56,000 in addition to a low molecular weight protein corresponding to calmodulin. It is suggested that the red cell membrane contains a calmodulin binding protein that tightly binds calmodulin as a polymeric complex in a Ca^{2+}-independent manner.

G. Gárdos

Distribution of insulin receptors in human erythrocyte membranes. Insulin binding to sealed right-side-out and inside-out human erythrocyte vesicles. J. H. Im, J. Cuppoletti, E. Meezan, C. E. Rackley and H. D. Kim (Department of Pharmacology and Medicine, University of Alabama in Birmingham, Birmingham, Alabama, USA). *Biochim. Biophys. Acta 775*, 260 (1984).

Analyses of insulin binding to human erythrocytes and to resealed right-side-out and inside-out erythrocyte membrane vesicles have revealed that high affinity insulin binding receptors are present on both sides of the erythrocyte membranes. Insulin binding to human erythrocytes was examined with the use of a binding assay designed to minimize the potential errors arising from the low binding capacity of this cell type and from non-specific binding in the assay. Scatchard analysis of equilibrium binding to the cells revealed a class of high affinity sites with a dissociation constant (K_d) of $(1.5 \pm 0.5) \cdot 10^{-8}$ M and a maximum binding capacity of 50 ± 5 sites per cell. Interestingly, both resealed right-side-out and inside-out membrane vesicles exhibited nearly identical specific sites for insulin binding. At the high affinity binding sites, for both right-side-out and inside-out vesicles, the dissociation constant (K_d) was $(1.5 \pm 0.5) \cdot 10^{-8}$ M, while maximum binding capacity was 17 ± 3 per cell equivalent. These findings suggest that insulin receptors are present on both sides of the plasma membrane and are consistent with the participation of the erythrocyte insulin receptors in an endo-

cytic/recycling pathway that mediates receptor-ligand internalization/externalization.

G. Gárdos

Stabilizing factors of phospholipid asymmetry in the erythrocyte membrane. V. Dressler, C. W. M. Haest, G. Plasa, B. Deuticke and J. D. Erusalimsky (Department of Physiology, Medical Faculty, RWTH Aachen, Aachen, FRG and Department of Biological Chemistry, Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel). *Biochim. Biophys. Acta 775*, 189 (1984).

Transbilayer reorientation (flip) of exogenous lysophospholipids and changes of the transbilayer distribution of endogenous phospholipids were studied in human erythrocytes and membrane vesicles. (1) Exogenous lysophosphatidylserine irreversibly accumulates in the inner membrane layer of resealed ghosts of human erythrocytes. (2) This accumulation even occurs after complete loss of asymmetric distribution of endogenous phosphatidylethanolamine and partial loss of phosphatidylserine asymmetry in diamide-treated cells. (3) Formation of inside-out and right-side-out vesicles from erythrocyte membranes results in a loss of endogenous phospholipid asymmetry as well as of the ability to establish asymmetry of exogenous lysophosphatidylserine. Rates of transbilayer reorientation of lysophospholipids for the vesicles, however, are comparable to those for intact cells. (4) Loss of endogenous asymmetry of phosphatidylserine is also observed in vesicles isolated from erythrocytes after heat denaturation of spectrin. The asymmetry in the residual cells is maintained. (5) In contrast to the loss of asymmetry of phosphatidylethanolamine and of phosphatidylserine, the asymmetry of sphingomyelin is completely maintained in the vesicles. (6) The stability of phospholipid asymmetry in the native cell is discussed in terms of a limitation of access of phospholipids to hypothetical reorientation sites. Such a limitation may either be the result of interaction of phospholipids with the membrane skeleton as in case of phosphatidylserine and phosphatidylethanolamine, or the result of lipid-lipid interactions as in the case of sphingomyelin.

G. Gárdos

Cholinergic agonists stimulate calcium uptake and cGMP formation in human erythrocytes. L. C. Tang, E. Schoomaker and W. P. Wiesmann (Division of Medicine, Walter Reed Army Institute of Research, Washington, DC, USA). *Biochim. Biophys. Acta 772*, 235 (1984).

Human erythrocytes possess a muscarinic cholinergic receptor sensitive to cholinergic agonists which stimulate transient increases in calcium uptake and subsequent cyclic GMP formation. These phenomena can be blocked by atropine and EGTA. The cholinergic stimulation of cyclic GMP formation depends on Ca^{2+} uptake from external media. The effects of cholinergic agonists on the erythrocyte resemble their effect on calcium channels in nervous tissue. The cholinergic stimulation of Ca^{2+} uptake in erythrocytes may affect the calcium-sensitive mechanism involved in the shape, permeability and rigidity of these cells.

Ilma Szász

The Ca^{2+} uptake and the hydrolysis of various nucleotide triphosphates by human platelet membranes. M. de Metz, J. Enouf, M. Lebret and S. Lévy-Toledano (U 150 of Institut National de la Santé et de la Recherche Médicale, 75 010 Paris, France). *Biochim. Biophys. Acta 773*, 325 (1984).

Several nucleotide triphosphates (NTPs) were tested as energy source for the Ca^{2+} uptake by human platelet membrane vesicles. The Ca^{2+} uptake by these membranes was driven by ATP, GTP, ITP, UTP and CTP. The steady-state level of accumulated Ca^{2+} was equal to the different NTPs. The highest uptake velocity was found with ATP, but about 40–80% of the velocity with ATP could be accomplished with the other nucleotides. The highest affinity was also found with ATP (K_m apparent = 15 μM). The liberation of Pi from the various NTPs was measured simultaneously with the Ca^{2+} uptake. The coupling ratio (moles of Ca^{2+} taken up/moles of P_i liberated) varied from 0.4 for ATP to 2.3 for UTP and was almost independent of the NTP concentration. The enzyme activity with ATP as substrate is strongly dependent on the Ca^{2+} concentration in contrast to the activity with GTP, ITP, UTP or CTP.

Ilma Szász

Book Reviews

The Reticuloendothelial System. A Comprehensive Treatise. By H. Friedman, M. Escobar and S. M. Reichard. Vol. 4. *Immunopathology.* By N. R. Rose and B. V. Siegel. Plenum Press, New York and London 1983, 440 p., 23 figs

Reading the foreword by the general editors, these series of books want to focus attention of the readers on the interactions between various cell types belonging to RES from the viewpoint of different disciplines. The previous three volumes published between 1980 and 1982 have dealt with the morphology, biochemistry and metabolism, as well as with the phylogeny and ontogeny of the RES.

This Vol. 4 reviews 14 different topics of immunopathology of the RES. The first four chapters summarize data concerning mediation of tissue injury by the RES. B. V. Siegel et al. in Chapter 1 discuss some of the general aspects of the RES-mediated tissue lesions, while in the next chapter we can read about T cell mediated tissue injury.

M. D. P. Boyle and T. Borsos report on the mechanism of the complement mediated tissue damage, while D. E. Tracey writes interestingly on macrophage mediated tissue injury.

The second part of the book has chapters on the immunopathology of diseases caused by facultative intracellular parasites (M. J. Lefford, on mycotic (S. B. Salvin and R. Neta) and on viral infections (M. R. Escobar and P. D. Swenson). A chapter written by L. D. Berman on virus-induced tumours of experimental animals leads us right on to the third part of the book that deals with the immunopathology of malignant disease in mice (A. E. Reif) and in humans (R. D. Moore).

The last four chapters sum up different mechanisms preceding autoimmune diseases. N. R. Rose surveys some of the general aspects of the topic, while immunopathology of the immune complex disease is reconsidered by U. E. Nydegger and associates. G. O. Solley deliberates upon IgE-mediated inflammatory reactions and, in the last chapter, T. Yoshida and M. Suko summarize the immunopathology of delayed hypersensitivity.

In spite of its somewhat staved off publication (the last data in literature are from 1980), the book — as says in its title — is really comprehensive, and can be recommended with good reason to immunologists and to all others who are involved in theoretical and clinical immunology.

G. Füst

Leukaemia Reviews International: Volume 2. Edited by Marvin A. Rich. 208 p., bound, illustrated. 1984.

Through laboratory studies of leukemias in human and in model experimental systems, we are gaining a greater understanding about all aspects of the disease. *Leukaemia Reviews International* stands at the forefront of this growing knowledge, providing state-of-the art coverage of every branch of basic and clinical leukemia research.

Packed with timely, authoritative insight from leading international laboratories, *Volume 2* deals with leukemia cell biology and virology, including recent research on adult T cell leukemias and the oncornavirus associated with them. Additionally, *Volume*

2 covers research in such diverse areas as virus induced leukemias in mice, the suppression of hematopoiesis in leukemias lymphokine production by lymphoid cell lines, and much more.

For clinicians requiring the latest developments in their efforts to improve leukemia detection, prevention, and patient management, and researchers seeking enhanced awareness of worldwide advances, *Leukaemia Reviews International Volume 2* is a good source for their continuing work in leukemia research. Also recommended for hematologists, oncologists, cell and molecular biologists, virologists, geneticists, immunologists, biochemists, pathologists, and epidemiologists.

A. Mód

Atlas d'hematologie et de cytologie du chien et du chat (Atlas of Haematology and Cytology of Dogs and Cats) by P. Groulade and I. F. Guelfi. 249 p., 450 colour pictures, legends in French, English and Spanish. Conf. Nat. Vétérinaires Spec. de Petits Animaus, Paris 1983.

The neatly designed handbook describes 1) notions and data dealing with blood physiology and cytology, blood physiopathology, as well as data about main haemopathias and diseases with blood changes; 2) staining techniques, techniques for punctioning, bone marrow, lymph nodes, spleen, liquid or compact masses. Examples of myelogrammes in pathological cases as well as clinical data are also presented.

Further, the atlas gives a summary of the results of clinical observations where cytology is confronted with the histologic examination after biopsy or necropsy in some cases, which confirms the accurate diagnosis.

The book intends to show the definite interest of the investigational techniques given to the practitioner by haematologic, lymphatic, splenic, bone marrow cytology as well as the cytology of tumoral growth and cavitary effusions; to define sampling methods in agreement with clinical signs and the rules to be followed; to show pictures where specific characteristics of the observed cells are useful for diagnosis and prognosis. Such informations will allow to differentiate an inflammatory from a tumoral process and to appreciate the being or malignant character of this process. On the other hand, the presence of some elements is indicative of other aetiologies (e.g. epithelioid cells in tuberculosis).

The authors also try to convince the practioner that smears (or imprints) of the sample in appropriate conditions provide an information as important as X-ray records which can be submitted to a specialist in difficult cases.

One objection to this book is the poor translation of the legends into English and the surprising number of emendable setting errors, particularly in the English text.

In spite of these shortcomings, however, individuals interested in veterinary pathology will find valuable informations in this atlas.

Gy. Lelkes

Bone Marrow Biopsies. Updated New Prospects for Clinical Diagnostics. Satellite Symposium of the 7th Congress of the International Society of Haematology, European and African Division, Barcelona, September 4–9, 1983. Volume Editors: B. Frisch, Tel Aviv, and R. Bartl, München; Basel, New York, Kargel, 1984. (Bibliotheca Haematologica No. 50), VIII + 132 p., 48 figs, 40 tab., hard cover.

Based on a symposium, the volume presents some convincing data on the increasing diagnostic and prognostic value of bone marrow biopsies.

The total of eight reports open with an overview of the diagnostic possibilities of bone marrov biopsy through studies based on cryostat sections, imprints and plastic embedding. A number of techniques (histochemical and immunological, electron microscopical) are explored in terms of their application in clinical situations.

The second and third chapters focus on bone marrow biopsies in myelodysplastic and myeloproliferative disorders. Both chapters supply ample opinions and counter-opinions. Still, the book is by no means academic only; its findings are footed on the investigation of more than 3,000 patients.

The references following each work are up-to-date, the black and white pictures are outstanding. Recommended for haematologists and for pathologists.

J. Tamáska

VIIIth MEETING OF THE INTERNATIONAL SOCIETY OF HAEMATOLOGY EUROPEAN AND AFRICAN DIVISION

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Vitamin K-Dependent Carboxylase

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Vitamin K-dependent carboxylase is found in the liver, where it is involved in the synthesis of four blood coagulation factors and protein C. The hepatic enzyme has partly been purified and several mechanisms have been postulated for the vitamin K-dependent carboxylation reaction. Recently the enzyme has also been detected in other tissues including the lung, kidney, spleen, testis, bone and arterial vessel wall. The proteins produced by these non-hepatic carboxylases are now being characterized, but in most cases their function is still unknown. This paper is meant to review our present knowledge in this field.

Keywords: Blood coagulation, γ-carboxyglutamic acid, carboxylase, coumarin, vitamin K, warfarin.

Introduction

Vitamin K is involved in a post-translational modification of proteins and recently several authors have reviewed its role in the formation of the blood clotting factors II (prothrombin), VII, IX and X [1–4]. The vitamin K-dependent step in protein biosynthesis is the carboxylation of a number of discrete glutamic acid (Glu) residues into γ-carboxyglutamic acid (Gla) [5, 6]. This reaction can be inhibited in vivo by the administration of vitamin K-antagonists such as warfarin, phenprocoumon and dicoumarol [7, 8], which leads to the production of non-carboxylated and hence inactive coagulation factors [9]. The three drugs mentioned above are therefore currently used as anticoagulants in rodenticides (rat poison) and in medicine for the control of thrombogenic episodes [10, 11].

Soon after the identification of Gla in blood coagulation factors, other proteins were reported to contain this abnormal amino acid [12–15], and every year a number of proteins is added to those which are called the "Gla-containing" or "vitamin K-dependent" proteins. In this paper we intend to summarize our present knowledge in this field and hope to make it clear that vitamin K-dependent carboxylation is a process which is not restricted to four blood coagulation factors, but that it is a normal modification undergone by numerous other proteins.

Hepatic carboxylase

All vitamin K-dependent proteins discovered up to now belong to what are called the "secretory proteins". These proteins are excreted into the extracellular fluid or to the outer cell membrane after their maturation has been completed. The mechanism by which they are synthesized has been clarified by the work of Blobel and Dobberstein [16, 17] and Meyer et al. [18], whereas the groups of MacGillivray and Davie [19, 20] have studied in particular the biosynthesis of the coagulation factors. The basic concept is the presence of a strongly hydrophobic "signal-" or "leader-sequence" at the N-terminal side of the growing peptide chain. Once it has been synthesized and emerges from the ribosome the signal sequence mediates in a process which leads to the binding of the ribosome to the endoplasmic reticulum. Here the polypeptide chain continues to elongate and the hydrophobic signal sequence penetrates into the endoplasmic membrane, thus facilitating the transport of the growing peptide chain. Signal sequences have been found in all secretory proteins examined until now, except albumin, which seems to have an internal signal sequence. It should be emphasized that only a small fraction of these proteins contain Gla-residues and that the mechanism responsible for the selection of carboxylatable Glu-residues is still unclear.

It has been shown, that non-carboxylated coagulation factors are excreted in the blood stream during in vivo treatment with vitamin K-antagonists [9, 21, 22] and that warfarin also induces an accumulation in the liver of clotting factor precursors [23, 24]. A few years later it was found that these precursors could be carboxylated in vitro in a cell-free system obtained from the postmitochondrial supernatants of these livers [25]. Both the precursor proteins and a vitamin K-dependent enzyme (carboxylase) were present in the microsomal fraction and the addition of vitamin K and $H^{14}CO_3^-$ promoted the carboxylation reaction. Since then the vitamin K-dependent carboxylation reaction has been studied most extensively in liver microsomal preparations from vitamin K-deficient or warfarin-treated rats and from warfarin-treated cows [26, 27]. Its main characteristics are summarized in Table 1.

Exogenous substrates; development of synthetic peptides

In the early studies of carboxylase, the enzyme system was invariably obtained from the liver of vitamin K-deficient or warfarin-treated animals. The hepatic microsomal fraction contained both, carboxylase and an endogenous substrate, which had accumulated during the in vivo warfarin treatment [24–26]. This endogenous substrate is readily carboxylated upon adding vitamin K to the in vitro system. Although the use of such systems has several advantages (e.g. the correct substrate is complexed to carboxylase in such a way that it is readily carboxylated upon adding vitamin K), their drawbacks are also obvious. During attempts to purify carboxylase, for instance, the substrate may be removed from its enzyme, which inevitably will lead to the complete loss of detectable enzyme

Table 1

Properties of vitamin K-dependent carboxylase

Absolute requirements:
- vitamin K quinone + reducing agent (NADH, DTT) or vitamin K hydroquinone
- O_2
- CO_2
- carboxylatable substrate

Stimulating agents:
- DTT
- pyridoxalphosphate or Mn^{2+}
- detergents
- antichaotropic agents
- ketones or DMSO

Inhibiting agents
- chloro-K
- 2,3,5,6-tetrachloro-4-pyridinol
- coumarin-like anticoagulants
- sulphydryl reagents
- chaotropic agents
- CN^-

Abbreviations: NADH, nicotinamide adenine dinucleotide (reduced form); DTT, dithiothreitol; DMSO, dimethylsulphoxide; chloro-K, 2-chloro-3-phytyl-1,4-naphthoquinone.

activity. A second drawback of using the endogenous substrate as a $^{14}CO_2$-receptor is that kinetic studies may require that the substrate is present in varying and well-defined amounts.

Since it was known that the coagulation factors II, VII, IX and X as well as protein C are synthesized in the liver, and that the factors II and X are the most abundant Gla-containing proteins in blood plasma, it was to be expected that precursors of prothrombin and factor X were identified to constitute the main part of the hepatic endogenous substrate for carboxylase [28, 29]. On comparing the N-terminal aminoacid sequences of the bovine plasma-proteins [13, 30] it can readily be seen that the first two Gla-residues occur in the homologous sequences Phe-Leu-Gla-Gla-Val (prothrombin, factor X) or Phe-Leu-Gla-Gla-Leu (factor VII, protein C), whereas the sequence for rat prothrombin is Phe-Leu-Gla-Gla-Ile [31]. Two research groups have independently started to synthesize similar pentapeptides in which Gla was replaced by Glu [31, 32]. When offered to the carboxylating system these synthetic pentapeptides were all readily carboxylated. A drawback was, however, the relatively high K_M (4 – 10 mM) of carboxylase for these substrates [31, 33, 34]. Since the natural substrate is present in the endoplasmic reticulum in a concentration of 0.001 mM or less [31], it is obvious that additional structural elements are required for an efficient recognition of the substrate by carboxylase. The fact that only the first Glu-residue is carboxylated and not the second one [35, 36] also indicates that the synthetic substrates are still

inferior to the natural ones. Later studies, in which the synthesis of numerous analogues of these pentapeptides was reported [37] did not reveal a virtually better substrate. It cannot be excluded, therefore, that the presence of the earlier mentioned hydrophobic signal sequence is essential for the binding and hence for the efficient carboxylation of these peptides. Nevertheless it is needless to say that the availability of synthetic substrates has greatly facilitated further investigations concerning the purification of carboxylase and the mechanism of the carboxylation reaction.

Partial purification of carboxylase

When we try to solubilize carboxylase from the microsomal filaments, the enzyme acts as a typical integral membrane protein. Relatively high concentrations (1–2%) of detergent (CHAPS, Triton X-100, Lubrol) are required and even after solubilization the enzyme remains associated with phospholipids and contaminating proteins. Further desintegration of these structures inevitably leads to an almost complete loss of the carboxylase activity. Recent reports by Girardot [38, 39] about the purification of carboxylase after solubilization with CHAPS have not been confirmed by other laboratories.

Attempts to partly purify rat carboxylase prior to solubilizing the enzyme were reported by Canfield et al. [40] and Wallin and Suttie [41], who extracted sequentially the microsomal membranes with a number of detergents and chaotropic agents. By this procedure the specific enzyme activity could be increased about 100-fold, but this increase was due to the elimination of an inhibitor of carboxylase rather than to the removal of a substantial amount of contaminating proteins. Hence this procedure, although useful, can hardly be qualified as a purification of the enzyme.

A partial purification of detergent-solubilized bovine carboxylase has been reported by De Metz et al. [29] who had observed that after warfarin-treatment of the animals, 60–70% of the samples consisted of a precursor of blood coagulation factor X. Purified antibodies against normal factor X were covalently bound to Sepharose and used for extraction of the solubilized microsomes. Because carboxylase is firmly bound to the hepatic substrates, the binding of factor X-precursor to the insolubilized antibodies resulted in the specific extraction of enzyme/substrate complexes. Probably because of conformational differences between normal factor X (against which the antibodies had been raised) and the factor X-precursor the extraction process is slow (Fig. 1), but after 24–48 h at 4°C about 60% of the total amount of carboxylase can be bound. It should be pointed out that in most cases it is desirable to use an excess of solubilized microsomes so that a maximal amount of enzyme is bound per mg of Sepharose in a short time. Non-bound proteins may be washed off from the Sepharose, resulting in a 50–100 fold purification of carboxylase.

The "Solid-phase carboxylase" thus obtained did not contain any reductase (see below) but, as analyzed on polyacrylamide gels, only 4–5 proteins, two of

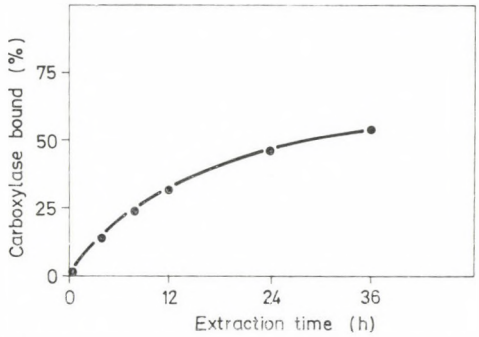

Fig. 1. Adsorption of carboxylase to Sepharose-bound antifactor X. Antibodies against bovine factor X were raised in goats and purified by immunoadsorption onto insolubilized purified factor X. The antibodies were eluted with 2 M KCNS, dialyzed and coupled to CNBr-activated Sepharose. Bovine liver microsomes were prepared from warfarin-treated cows (27), solubilized with 0.5% CHAPS and centrifuged for 1.5 h at 150 000 g. Aliquots (5 ml) of the supernatant were extracted with Sepharose-bound antifactor X (0.5 ml of slurry) and at the indicated times the Sepharose beads were extensively washed with a buffer containing 1 M KCl and 0.05 M Tris-HCl, pH 7.5. The insolubilized carboxylase (solid-phase carboxylase) was assayed by supplementing 0.05 ml of Sepharose with 10 mM DTT, 10 mM Phe-Leu-Glu-Glu-Leu, 0.01 mCi $NaH^{14}CO_3$ and 0.1 mM vitamin K hydroquinone (29) in 0.25 ml reaction mixtures at 25°C. After 1 h the reaction was terminated with 1 ml trichloroacetic acid (5%, w/v) and the samples were shortly boiled before counting

which strongly bound [^{3}H]vitamin K [42]. Apart from Sepharose beads, the antibodies and the factor X-precursors, solid-phase carboxylase consists of only 60% of proteins. Phospholipids (mainly phosphatidylcholine) constituted the other 40% of the enzyme [43]. When the phospholipids are removed from the carboxylase complex, the enzyme activity is completely destroyed, but it can be restored by adding mixed micelles of purified phosphatidylcholine and cholate [43].

Various mechanisms have been proposed for the way in which vitamin K is involved in the carboxylation of Glu-residues [44–47]. Since these mechanisms have been postulated on the basis of results obtained in partly purified systems, it is impossible to decide whether one of them is correct. Therefore, a considerable amount of fundamental research on vitamin K-dependent carboxylase awaits purification of the enzyme.

Carboxylase and reductase

In the liver three metabolites of vitamin K have been identified: vitamin K quinone (K), vitamin K hydroquinone (KH_2) and vitamin K epoxide (KO). In crude microsomal systems all these metabolites are able to serve as a coenzyme for carboxylase, provided that the reaction mixtures contain DTT as a reducing agent. In the absence of DTT, carboxylase activity is only found in the hydro-

Table 2

Stimulation of carboxylase by DTT

Coenzyme added	DTT concentration (mM)	$^{14}CO_2$ incorporated (dpm)
None	0	270
None	10	9 783
Vitamin K hydroquinone	0	216 495
Vitamin K hydroquinone	10	374 23
Vitamin K quinone	0	287
Vitamin K quinone	10	338 342
Vitamin K epoxide	0	256
Vitamin K epoxide	10	331 278

Reaction mixtures (0.125 ml) contained: 2 mg of solubilized bovine liver microsomal proteins, 0.15 M KCl, 1 M $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 7.5, 10 mM Phe-Leu-Glu-Glu-Leu, 0.4% (w/v) 3-([3-cholamidopropyl]-dimethylammoniol)-1-propane sulphonate (CHAPS), 12% (v/v) ethylene glycol, 0.01 mCi $NaH^{14}CO_3$ and 0.4 mM vitamin K (if added). After 1 h at 25°C the mixtures were supplemented with 1 ml of 5% (w/v) trichloroacetic acid, boiled for 1 min and counted. Vitamin K hydroquinone and vitamin K epoxide were prepared as described elsewhere [27]

quinone-containing mixtures (Table 2). It has also been demonstrated that the partly purified solid-phase carboxylase is active only in the presence of vitamin K hydroquinone, whether DTT is present or not [29]. These observations gave rise to the idea that in the liver vitamin K quinone, which is the most common form of the vitamin, is first converted into the hydroquinone before it can be used by carboxylase. The reducing enzyme (K-reductase) has been purified by Wallin et al. [48]. In vitro the enzyme makes use of dithiols such as DTT, which serve as a reductant. Obviously DTT is not used in vivo, but its physiological counterpart has not yet been identified. An explanation for all these observations is, that in crude microsomal systems both carboxylase and K-reductase are present, whereas the latter enzyme has been removed from solid-phase carboxylase. It is not known whether the reduction of vitamin K quinone and that of vitamin K epoxide is accomplished by the same enzyme system or by two different enzymes.

Using the partly purified solid-phase carboxylase system, De Metz et al. demonstrated, that during the carboxylation reaction vitamin K epoxide accumulates in the reaction mixture [46]. This observation strongly supported the hypothesis that vitamin K is interconverted in a cyclic way as represented in Fig. 2. From the experiments of Bell and Stark [49] it became clear, that the vitamin K-antagonists which are generally used for oral anticoagulant therapy (phenprocoumon, dicoumarol and warfarin) interfere with the 'vitamin K-cycle" by inhibiting the two reductases rather than carboxylase: during in vivo anticoagulant treatment of rats the relative abundance of hepatic vitamin K epoxide

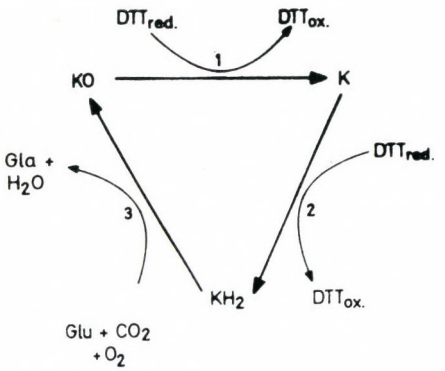

Fig. 2. The vitamin K cycle, simplified form. Steps 1 and 2 are inhibited by oral anticoagulants. Gla-residues are formed in step 3. Abbreviations used are: KO, vitamin K epoxide; K, vitamin K quinone; KM_2, vitamin K hydroquinone; $DDT_{red,}$, dithiothreitol (reduced form); $DDT_{ox,}$, dithiothreitol (oxidyzed form)

was strongly increased. Hence the anti-vitamin K drugs do not affect carboxylase in a direct way, but they induce a scarcity of vitamin K hydroquinone and the rate of carboxylation is slowed down by a lack of coenzyme. In in vitro carboxylating systems it could be shown, that low concentrations of anticoagulant inhibited the K- and KO-driven reaction but not the KH_2-driven one. Only at 100-fold higher anticoagulant concentrations the KH_2-driven reaction was inhibited (Fig. 3).

Although these observations all supported the idea of the vitamin K-cycle one question remained to be solved. It is well known that in vivo oral antico-

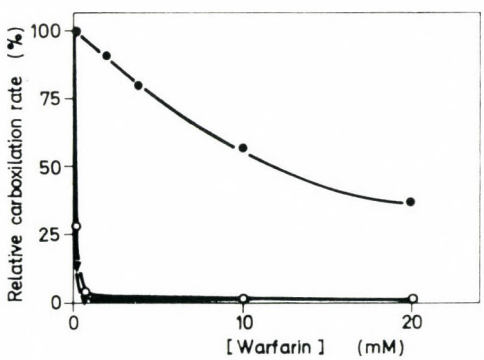

Fig. 3. Inhibition of carboxylase/reductase by warfarin. The relative carboxylation rate was determined in reaction mixtures containing 4 mg detergent (CHAPS)-solubilized microsomes from normal cows. Carboxylation was performed in the presence of either 0.1 mM vitamin K hydroquinone (●—● or 0.1 mM vitamin K quinone (○—○) or 0.1 mM vitamin K epoxide (▼—▼). Further reaction conditions were as described in the legend to Figure 1. In the non-inhibited reaction 420 dpm were incorporated per minute and per mg of microsomal protein

agulant treatment can efficiently be counteracted by giving the patient extra vitamin K. When we tried to repeat that in vitro in a crude microsomal system, we observed that the effect of warfarin on carboxylase/reductase could not be counteracted by extra vitamin K quinone or epoxide. It soon turned out that there is a second K-reductase, which uses NADH as a reductant [48]. As shown in Table 3, the NADH-dependent K-reductase has a rather high K_M for vitamin K, which means that a high concentration of vitamin K quinone is required before the hydroquinone is produced in sufficient amounts. A second difference with the DTT-dependent reductases is that the NADH-dependent system is relatively insensitive for coumarin-like vitamin K-antagonists (Table 3). Since during oral anticoagulant therapy these drugs efficiently interact with synthesis of the clotting factors II, VII, IX and X, we have to assume that under normal conditions the

Table 3

Kinetic constants of carboxylase/reductase

Reductant	Apparent K_M for vitamin K (mM)	IC-50 of warfarin (mM)
DTT	0.020	0.015
NADH	0.140	22

The apparent K_M of the carboxylating enzyme complex was determined from the initial carboxylation rates at various vitamin K concentrations and in the presence of either DTT (10 mM) or NADH (2 mM). Carboxylase was obtained from the livers of normal, non-treated cows. The IC-50 of warfarin was measured in carboxylating reaction mixtures containing vitamin K quinone, which was present in a concentration of 5 times the K_M.

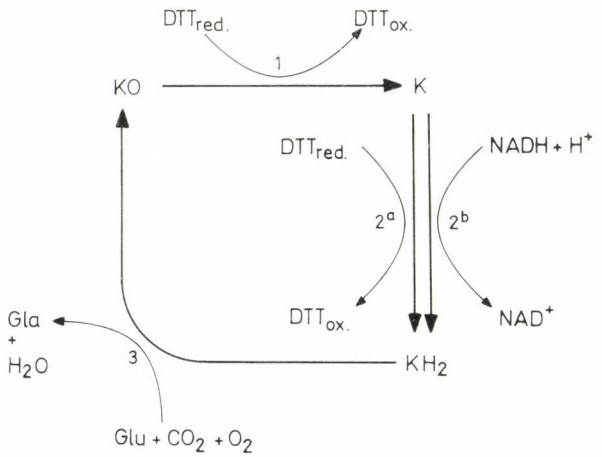

Fig. 4. The vitamin K cycle, complete form. Steps 1 and 2^a are inhibited by warfarin at low concentration. Steps 2^b and 3 are relatively insensitive to warfarin

coumarin-sensitive DTT-dependent reductases play a dominant role in the generation of vitamin K hydroquinone. Only if high amounts of vitamin K are administered to the patient, also the NADH-dependent reductase comes into operation. The latter is insensitive to vitamin K-antagonists, so that in vivo the effect of coumarin-like drugs can efficiently be counteracted by high doses of vitamin K. These observations lead to some modifications of the vitamin K-cycle (Fig. 4). This form is now generally accepted.

Inhibition of carboxylase by aspirin

On several occasions it has been reported, that salicylate and acetylsalicylate (aspirin) have, besides their effect on platelet aggregation, a direct effect on the plasma level of the vitamin K-dependent clotting factors [50, 51]. This was demonstrated once more in an experiment in which the effects of warfarin and salicylate were compared in 9 groups of 5 rats each (Table 4). Groups 1–4 were treated with various amounts of salicylate and groups 5–8 with warfarin. Group 9 served as a control. After 18 h blood was taken by heart puncture and the plasma concentration of the various factors was measured. It is obvious that both

Table 4

Effect of salicylate and warfarin on the synthesis of blood coagulation factors

Drug administered (mg/kg body weight)	Plasma level of clotting factor (%)				Endogenous substrate in liver (dpm)
	FII	FVII	FX	FV	
None	100	100	100	100	1 285
Salicylate, 4× 50	98	67	83	100	7 852
Salicylate, 4× 100	79	37	51	104	8 542
Salicylate, 4× 200	52	29	39	94	10 960
Salicylate, 4× 200 + vitamin K, 1× 20	109	98	96	97	857
Warfarin, 1× 0.2	78	40	78	94	5 430
Warfarin, 1× 0.5	30	12	32	98	7 083
Warfarin, 1× 1.0	22	5	5	96	10 280
Warfarin, 1× 1.0 + vitamin K 1× 20	85	97	85	100	725

Salicylate and warfarin were dissolved in 0.15 M NaCl, pH 7.5 and injected in a volume of 1 ml. Vitamin K (if added) was mixed with the drugs shortly before injection. Because of the short half-life time of salicylate in blood, administration of this drug was repeated every 6 hours. After 20 h blood (taken by heart puncture) was collected in citrate and used for determination of the coagulation factors, whereas the livers were removed for detection of the accumulated substrates for carboxylase. The amount of hepatic carboxylatable proteins was determined by preparing the microsomal fractions and measuring the incorporation of $^{14}CO_2$ under standard conditions in the absence of exogenous substrate. All data are the means of 5 different animals.

drugs induced a decrease of the plasma level of factors II, VII and X, whereas the level of factor V remained unchanged (factor IX was not determined). The effects of both drugs could be completely reversed by a simultaneous injection of vitamin K. It is also clear from Table 4 that warfarin is a much more potent inhibitor than is salicylate.

In man the effect of salicylate on clotting factor synthesis is only measurable at very high dosages (4 – 6 g daily). Since the effect of salicylate is additive to that of warfarin, it may be expected that much lower doses will interfere with oral anticoagulant therapy. With this therapy it is intended to reduce the plasma concentration of the vitamin K-dependent clotting factors to 5 – 10% of normal [10]. Low amounts of aspirin may further reduce this level, for instance to 0 – 5%, which would give rise to a high risk of bleeding. In non-anticoagulated subjects, on the other hand, a similar aspirin-induced decrease of clotting factors (from 100 to 95% of normal) would not be detectable.

Whereas in man and in many animal species coumarin-derivatives induce the appearence of non-carboxylated descarboxy factors in blood, the same could not be demonstrated with aspirin. Neither in human volunteers nor in experimental animals, descarboxy factors have ever been detected after the aspirin or salicylate-induced decrease of the plasma clotting factor concentration. This is in agreement with data reported by Owens and Cimino [52, 53], who showed that the perfused rat liver is able to produce normal clotting factors and that in this system too, warfarin and salicylate inhibit the synthesis of the vitamin K-dependent factors. Whereas in the presence of warfarin the clotting factors were excreted

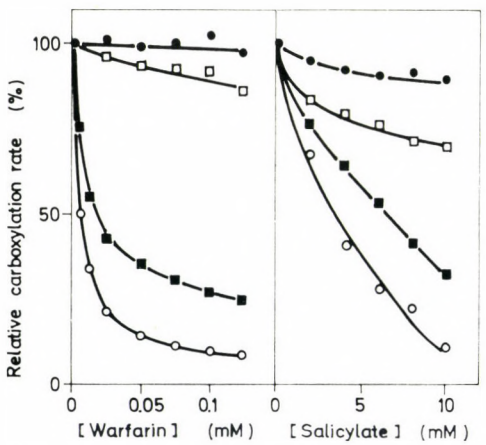

Fig. 5. Activity of carboxylase/reductase in the presence of increasing concentrations o inhibitor. The inhibition of warfarin (left panel) and salicylate (right panel) was measured at 5 times the K_M value of either vitamin K hydroquinone (●—●), vitamin K epoxide + DTT (10 mM, ○—○), vitamin K quinone + DTT (10 mM, ■—■) or vitamin K quinone + NADH (2 mM, □—□). The experiments were performed with microsomes from normal, non-treated animals under standard conditions (see Fig. 1)

as biologically inactive antigens (presumably descarboxy factors), salicylate induced a parallel decrease of the production of functional clotting factors and of clotting factor antigens. At present we have no data available to explain on a molecular basis the discrepancy between the effect of these two drugs.

Therefore, Hildebrandt and Suttie [54] and our group independently started to investigate the possible inhibition by salicylate of the in vitro microsomal carboxylase/reductase complex. In a typical experiment we have compared the influence of warfarin and salicylate on the carboxylation reaction, using as a coenzyme either vitamin K quinone, vitamin K hydroquinone or vitamin K epoxide. The vitamin K concentrations used in this experiment were 4 times the K_M and the results are shown in Fig. 5. It was confirmed that warfarin strongly inhibited the DTT dependent reactions, whereas the NADH-dependent reaction was only slightly affected. Similar results were obtained with salicylate: the DTT-dependent systems are inhibited more than the NADH-dependent ones, whereas hardly any effect was measured on the vitamin K hydroquinone-driven reaction. As was the case with warfarin, inhibition by salicylate of the DTT-dependent systems could not be counteracted by increasing the vitamin K concentration.

Non-hepatic carboxylase

It has been assumed for many years that vitamin K-dependent carboxylase was found exclusively in the liver, where it was thought to synthesize the "vitamin K-dependent coagulation factors". Consequently it was thought that the coumarin-like vitamin K-antagonists specifically inhibited the synthesis of these coagulation factors and not that of other proteins. In 1976, however, Hauschka et al. demonstrated the presence of vitamin K-dependent carboxylase in the kidney [55] and later on similar enzyme systems were detected in bone [56], placenta [57, 58] and the lung [59]. The importance of these non-hepatic carboxylases can only be estimated when we know a) the relative amounts of carboxylase in the various tissues, b) what are the products of these carboxylating systems and c) where these Gla-containing proteins exert their function. In this and the following three sections we will discuss our current knowledge of these subjects.

In an attempt to obtain some insight in the relative abundance of the various carboxylating systems we have anticoagulated a young steer by the oral administration of 1.5 g warfarin daily for 1 week. It was assumed that during this period all vitamin K-dependent enzymes were blocked. Consequently precursors of Gla-containing proteins will have accumulated in the microsomal fractions of carboxylase-containing tissues. After one week the animal was slaughtered and the various organs were excised and used for the preparation of crude microsomes. The amounts of carboxylase and accumulated endogenous substrate are shown in Table 5. Whereas the hepatic substrates could readily be bound to Sepharose-bound antibodies against prothrombin and clotting factor X [29], the non-hepatic substrates had only very little affinity to the antibodies against one of the clotting factors. Therefore, this experiment showed that in the

Table 5

Microsomal carboxylase in various tissues

Tissue	Amount of carboxylase (%)	Amount of endogenous substrate (%)
Liver	100	100
Testis	210	130
Kidney	73	12
Spleen	70	41
Lung	63	6
Thyroid	31	7
Pancreas	11	6
Thymus	11	3
Arteries	62	10
Cartilage	40	16
Epiphysis	25	8

Soft tissues were homogenized and used for the preparation of crude microsomes as described for liver. Cartilage and epiphysis were frozen in liquid nitrogen, crushed to powder and extracted with a buffer containing 0.1 M EDTA, 0.8 M KCl and 0.02 M Tris-HCl, pH 7.8. The extracts were homogenized as described for the soft tissues. The amounts of carboxylase and endogenous substrate were assessed as described in ref. 27. In both cases the amounts in liver were arbitrarily considered to be 100%. No carboxylase was found in heart muscle, skeletal muscle, diaphragm, veins, lymphocytes, bone marrow, bone membrane and brains.

non-hepatic tissues proteins are synthesized that do not belong to the coagulation factors. This became even more apparent from results reported by Buchtal and Bell [15], who found that most of them are rather small (< 10 000 D). On the other hand also molecular weights of more than 80 000 D have been reported [60], so we may conclude that vitamin K-dependent carboxylation is a very common modification for a wide variety of proteins.

In order to ensure that all carboxylating systems are inhibited and endogenous substrates for carboxylase accumulate in the endoplasmic reticulum, high amounts of warfarin are generally given to the experimental animal in this kind of experiments. The dose used for therapeutical purposes in man is about 100 times less [10], which led to the question whether the non-hepatic carboxylases are inhibited under the conditions of oral anticoagulant therapy. The effect of coumarin-related drugs on non-hepatic tissues was studied in vitro by comparing the IC-50 (inhibitor concentration required for 50% inhibition) of the various vitamin K-antagonists in hepatic carboxylase with that in a number of non-hepatic carboxylases [14]. No significant differences could be detected in this way.

Using the rat as an experimental model system we have tried to verify these results in vivo. Warfarin was given in the drinking water and the effect was measured by the decrease of the plasma procoagulant activity. At a daily intake

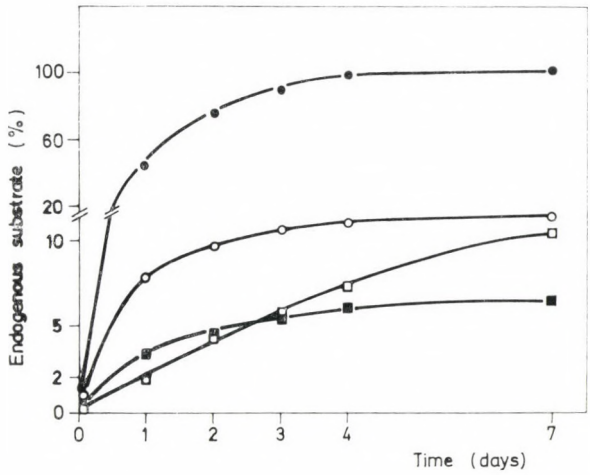

Fig. 6. Effect of low doses of warfarin on various tissues. Accumulation of endogenous substrates was measured under standard conditions and the results are expressed as a percentage of the amount of hepatic substrate that had accumulated after 7 days of treatment with 0.25 mg of warfarin per kg body weight. The following tissues were examined: liver (●—●), lung (○—○), spleen (■—■) and testis (□—□)

of 0.7 mg of warfarin/kg body weight the plasma clotting activity (as measured with Thrombotest) had completely disappeared after 2 days. Higher concentrations of warfarin did not shorten this period. A daily intake of 0.25 mg of warfarin/kg body weight induced a 4 times slower decrease of the plasma clotting activity with a residual activity of 10% after 7 days. So we decided that under these conditions vitamin K-dependent carboxylase was only partly inhibited, a situation which is comparable with that during oral anticoagulant treatment of patients. In a second experiment the same low dose of warfarin was administered to 30 rats and on each day 6 of them were killed and the liver, spleen, lungs and testes were excised for the production of the respective microsomal fractions. The presence of carboxylatable proteins was determined in these preparations using an in vitro assay and the results are shown in Fig. 6. In all the tissues investigated, the accumulation of endogenous substrates for carboxylase started without delay, as soon as the warfarin-treatment was started. After 7 days the level of the substrates had increased about 10-fold in all tissues. At that time the amounts of carboxylatable proteins had reached plateau levels in the liver, spleen and lung microsomes, whereas in the testis the accumulation of substrate still continued in a linear way. This experiment therefore demonstrates that also at low plasma concentrations, warfarin inhibits the vitamin K-dependent reactions in non-hepatic tissues in a similar way as it does in the liver. Since it is plausible that this effect is not restricted to rats, it may be expected that the same phenomenon occurs in patients on oral anticoagulant therapy. Whether this side-effect

of anti-vitamin K drugs is harmful, neutral or beneficial for the patient, can only be deduced when the functions of the various non-hepatic vitamin K-dependent proteins will be known.

Identification of Gla-containing proteins

Except for the coagulation factors II, VII, IX, and X, blood plasma contains three more Gla-containing proteins, the proteins C, S and Z. Whereas protein C seems to be synthesized in the liver, the place of origin of the other two proteins is less clear. Bovine protein C is a glycoprotein composed of a heavy and a light chain, the molecular weight of which is 41 000 and 21 000, respectively [61]. The 11 NH_2-terminal Glu-residues in the light chain are carboxylated to Gla [30]. Like the coagulation factors, protein C occurs in plasma in a zymogen form and can be activated into a serine protease by proteolysis with either the factor X activator from Russell's viper venom [62], trypsin [62] or thrombin [63]. The function of activated protein C seems to be the inactivation by proteolytic degradation of activated factor V and hence it is involved in one of the control mechanisms which limit the coagulation process whenever it has started. Another similarity with the coagulation factors mentioned above is that the activity of activated protein C is greatly enhanced by the presence of Ca^{2+}-ions and phospholipids.

Protein S is a glycoprotein consisting of a single polypeptide chain with a molecular weight of about 65 000 D, and containing 9 Gla-residues [13]. Purified protein S does not affect the activity of factor Va either in the presence or in the absence of Ca^{2+}-ions and phospholipids, but it enhances the rate of inactivation of factor Va by activated protein C in the presence of Ca^{2+}-ions and phospholipids. Therefore, protein S is now generally regarded as a cofactor of protein C.

Protein Z is also a single-chain glycoprotein with a molecular weight of approximately 50 000 D [30], containing 13 Gla-residues per molecule but its function is not known.

Atherocalcin is a protein found in hardened atherosclerotic plaques [60]. With its molecular weight of 80 000 D it is the heaviest Gla-containing protein known at this moment. It contains approximately 12 Gla-residues per molecule and several authors consider it to be a unique, plaque-specific protein, not related with any of the plasma proteins [1]. Low levels of atherocalcin were found in fatty streak and fibrous plaque lesions, whereas increased amounts were observed in calcified plaques. Although it has not been demonstrated unequivocally, it seems probable that atherocalcin is synthesized by the carboxylating enzyme system present in the arterial vessel wall. In this respect it is suggestive that both vitamin K-dependent carboxylase and atherosclerotic plaques are found in arteries but not in veins. It is not certain whether atherocalcin is required for the binding of Ca^{2+} in the atherosclerotic plaque or, contrarily, that it mediates (at least in non-pathological situations) the transport of Ca^{2+} through the vessel wall. In

principle none of these possibilities can be excluded, so that the function of atherocalcin remains obscure.

Osteocalcin is a Gla-rich protein found in bone [64, 65] and it is among the six most abundant proteins in man. It comprises over 25% of the non-collagenous proteins in bone. Osteocalcin is synthesized by the osteoblasts [66] and it is detectable in the growing fetus at the moment when the initial mineral deposition can be observed histologically. Human osteocalcin contains 49 aminoacid residues, the sequence of which is known. The sequence homology with calf osteocalcin is over 90% and that with swordfish osteocalcin is 50%. Three Gla-residues are found among the first 24 aminoacid residues, whereas further in the molecule two normal Glu-residues are present [67]. We do not know the precise function of osteocalcin, but that it plays a crucial role in bone development is clear from a number of observations. In the first place it is well known that in the human fetus serious bone deformations are seen in the so-called "fetal warfarin syndrome" (chondrodysplasia punctata), which occurs when women take vitamin K-antagonists during the first trimester of pregnancy [65]. The bone abnormalities arise from the excessive precipitation of calcium phosphate in the rapidly growing parts of bone. Growth stops in the calcified areas, whereas in the surroundings it continues. As demonstrated by Price et al. [68], after birth vitamin K-antagonists may still have a serious impact on the development of bones. When young rats were treated with vitamin K-antagonists for 9 months and their bones were compared with those from normal animals it became clear that the growth plates of the experimental animals had completely fused by an excessive calcium precipitation, whereas those of the control animals had remained open. The fusion of the growth plates in the treated animals had caused a complete absence of longitudinal growth. It has also been shown that in vitro purified osteocalcin strongly binds to hydroxylapatite and that it is able to stop the growth of calcium phosphate crystals from supersaturated solutions of calcium and phosphate [64]. Therefore it is generally thought that osteocalcin has a regulatory function in the deposition of calcium in bone. A similar function has been ascribed to a number of Gla-containing proteins found in dentin [69].

As early as in 1976 Hauscha et al. [55] reported the presence of a membrane-bound Gla-containing protein in the kidney. The protein was not further characterized, but presumably it is similar to or related with the Gla-containing protein found in calcified renal stones [70]. This protein, which is extremely acidic, has a molecular weight of 17 000 D and contains about 7 Gla-residues per molecule. Patients with a history of recurrent renal stone disease appear to excrete increased levels of Gla-proteins [65] so that these proteins might serve as a nucleating or stabilizing factor in renal stone formation.

Finally, in our laboratory a Gla-containing protein has been found in human sperm cells. The molecular weight is about 30 000 D but the number of Gla-residues per molecule has not yet been determined. The protein is probably related to or identical with the protein that accumulates in the testicles during in vivo warfarin treatment. Initially it was thought that the sperm Gla-protein

(S.G.P.) might be a precursor of acrosin, a serine protease with a 50% sequence homology with thrombin. Later experiments, however, in which we used purified antibodies against acrosin clearly demonstrated that this was not the case [71]. The fact, that in the testes endogenous substrates accumulate during in vivo treatment with low doses of warfarin indicates that under these conditions the production of S.G.P. is affected. Whether this influenced the fertility of the sperm cells has not yet been demonstrated. At this moment we have been informed about three patients who were capable of begetting offspring after more than one year of continuous oral anticoagulant therapy. Nevertheless we would like to plead for statistical analysis of the fertility of patients on long-term oral anticoagulant therapy.

Exogenous substrates; preparation of descarboxy proteins

After the development of a number of synthetic substrates for carboxylase it became clear that the K_M of carboxylase for all these substrates was high (4–10 mM). Because the synthetic peptides are small they can be added to the reaction mixtures in high concentrations, so that the carboxylation reaction can proceed. On the other hand it is clear that the natural substrates for carboxylase cannot be present in such amounts in the microsomes. So it seems that the synthetic substrates lack a "recognition site", which is recognized by carboxylase, thus creating a much higher affinity between the enzyme and its substrate. This recognition site may be a certain aminoacid sequence directly before or after the carboxylatable Glu-residue, or a secondary or tertiary folding of the Glu-containing polypeptide chain. Alternatively the recognition site might also be hidden in the hydrophobic aminoacids which are present at the N-terminal end of growing peptide chain and which are cleaved off during the maturation of secretory proteins.

In order to test the first possibility we have prepared and purified descarboxy prothrombin and descarboxy factor X from warfarin-treated cows. These substrates were added to a substrate-free carboxylating system from normal bovine liver and tested for their ability to serve as a substrate. It was found that the descarboxy factors (especially descarboxy factor X) are very bad substrates for carboxylase, with K_M values in the millimolar range [34]. When however, they are fragmented by limited proteolysis, peptides can be obtained with a much lower K_M. The best results were obtained when we digested descarboxyprothrombin with subtilisin [34]. This treatment generated a substrate composed of the aminoacids 13–29, which contained 6 carboxylatable Glu-residues and which had a K_M of 0.002 mM. The substrate was designated as fragment-Su and it was the first exogenous substrate for which carboxylase was reported to have a low K_M. When treated in a similar way, descarboxy factor X could also be fragmented into an active peptide (unpublished data). Since the aminoacid sequence in this part of the molecule is almost identical to that of descarboxy

prothrombin, it is to be expected that the subtilisin-derived fragments are also closely similar.

Chymotrypsin is a second protease, the descarboxy prothrombin digest of which rendered an active substrate for carboxylase [34]. Analysis of the digest showed that it contained a peptide composed of the first 44 N-terminal amino-acids of descarboxy prothrombin [72] and this is the largest polypeptide chain derived from any clotting factor, which is still a good substrate for carboxylase. Larger peptides such as descarboxy fragment-1 have comparable affinities for carboxylase as has the descarboxy prothrombin itself. So it seems that from descarboxy factors peptides can be derived which are efficiently recognized by carboxylase, but that the recognition site is destroyed or hidden when the amino-acid chain is longer than about 50–60 residues. It remains intriguing that the structure of fragment-Su contains the information which is required for being a good substrate, whereas this information is apparently absent in a number of smaller synthetic peptides [32, 37]. It may therefore be expected that further proteolytic degradation of fragment-Su (for instance by the subsequent cleavage of the C-terminal aminoacids with carboxypeptidase-C) will worsen this substrate, thus giving us more insight in the essential structural requirements for the recognition of a substrate by carboxylase. It is clear that for this type of investigation huge amounts of plasma have to be obtained from warfarin-treated cows. Since warfarinized cows cannot be used for consumption, the method is expensive; moreover, the purification procedure for the descarboxy factors is laborious.

Fortunately a technique has recently been developed by which normal clotting factors can artificially be converted into their descarboxy form [73]. So normal prothrombin and normal factor X may be purified from normal plasma. The purified proteins are lyophylized and heated at 110°C for 6 h under vacuum. During this treatment they are decarboxylated and it has been established in our laboratory that these "artificial" descarboxy factors may serve for the preparation of fragment-Su.

The method of decarboxylation of Gla-containing proteins may be applied for the detection of these proteins in a number of tissues or biological fluids. The place of synthesis of these proteins can easily be found by using warfarin-treated animals for the production of carboxylase. During anti-vitamin K treatment precursors of the Gla-containing proteins accumulate in the cellular endoplasmic reticulum and they are readily carboxylated in vitro upon the addition of vitamin K. In this way it could be established that Gla-containing proteins are synthesized in the liver and in many non-hepatic tissues. We know that the hepatic proteins are precursors of a number of plasma proteins (4 clotting factors, protein C) which are excreted into the blood shortly after their maturation. It is believed that the non-hepatic Gla-containing proteins are secretory proteins, but in most cases we do not know their function and neither where they exert their function. In a purified protein preparation Gla-residues can easily be determined by HPLC analysis [74], but in crude samples such as plasma this is impossible because of the large amount of contaminating proteins. Therefore we

have subjected a number of crude protein preparations to conditions of thermal decarboxylation. Afterwards the proteins were dissolved and used as substrates for bovine liver carboxylase. In this way we have been able to localize two non-hepatic Gla-containing proteins: Bone Gla-Protein (B.G.P.), obtained from bovine bone and Sperm Gla-Protein (S.G.P.), obtained from human sperm cells. The preparation and characterization of these two proteins will be discussed below.

B.G.P. is obtained by extracting bone powder with 1 M EDTA and it is readily converted into descarboxy-B.G.P. (d-B.G.P.) by thermal decarboxylation [75]. It was demonstrated that purified B.G.P. is identical with osteocalcin. The K_M of carboxylase for d-B.G.P. was determined to be 0.025 mM, which is 10 times higher than that for fragment-Su but about 200 times lower than that of FLEEL (4.8 mM). Therefore d-B.G.P. was the first complete exogenous protein substrate for which carboxylase had a low K_M. The fact that the K_M for d-B.G.P. was 10-fold higher than that for fragment-Su might be due to the fact that d-B.G.P. is three times larger than fragment-Su, so that its diffusion rate will be lower, or to the fact that fragment-Su contains six carboxylatable Glu-residues and d-B.G.P. only three. Finally it is also possible that hepatic carboxylase (which is used in all these assays) has a higher affinity to fragment-Su (which is derived from a hepatic protein) than to d-B.G.P., which in vivo is synthesized in the bone osteoblasts.

S.G.P. was prepared by washing human sperm cells with a buffer containing 0.25 M sucrose, 0.14 M NaCl, 0.004 M $CaCl_2$ and 0.02 M tris-HCl, pH 8.0.

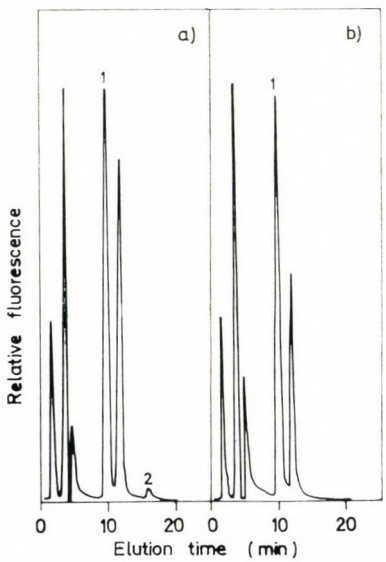

Fig. 7. Demonstration of Gla in S.G.P. HPLC chromatogram of an alkaline hydrolysate of S.G.P. (panel A) and decarboxylated S.G.P. (panel B). Peaks 1 are Glu and peak 2 is Gla. Technical details of the Gla-determination are described in ref. 74

The washed cells were extracted with a buffer containing 20 mM benzamidin-HCl and 50 mM EDTA, pH 8.0, and centrifuged at 150 000 *g* for 1 h and the extract was desalted by size exclusion chromatography. After alkaline hydrolysis Gla could be detected in the supernatant (Fig. 7). The Gla-containing protein(s) were decarboxylated by lyophilizing the preparation and heating at 110°C under vacuum.

When decarboxy-S.G.P. (d-S.G.P.) was added to a carboxylating enzyme system from bovine liver, it was readily carboxylated and in this way we were able to recover the substrate after fractionation by high performance liquid chromatography (HPLC) using ion exchange and size exclusion columns. From its elution time on the latter ones the molecular weight of d-S.G.P. was estimated at about 30 000 D and its K_M in the hepatic carboxylating system was 0.08 mM.

When considering the various peptide and protein substrates for carboxylase, we see that the small synthetic peptides (5–8 aminoacid residues) have a high K_M. They are carboxylated only because they can be added to carboxylase in a high concentration (> 10 mM) and in this way they are extremely useful in laboratory practice. Nevertheless they should be considered as bad substrates. Longer peptides such as fragment-Su (17 residues), the N-terminal chymotryptic fragment of descarboxy prothrombin (41 residues) and d-B.G.P. (49 residues) have low K_M values (0.002–0.08 mM) and hence they may be regarded as good substrates. They probably contain special structural features which are recognized by carboxylase and which are absent in the small peptides. At still higher molecular weights we see that d-S.G.P. (MW 30 000) is a good substrate but that descarboxy fragment-1 (MW 21 000), descarboxy prothrombin (MW 72 000) and descarboxy-factor X (MW 54 000) are extremely bad substrates.

The discrepancy between the substrates of high molecular weight might be caused by the presence of oligosaccharides which in the case of coagulation factors are known to be linked to the polypeptide chain, quite near to the Gla-residues (in the case of prothrombin for instance at Asn-77 and Asn-101). When glycosylation occurs in vivo after carboxylation, these sugars might inhibit the binding between carboxylase and the artificially decarboxylated coagulation factors. The validity of this hypothesis cannot, however, be checked, since at this moment nothing is known about the eventual presence of carbohydrates in d-S.G.P.

Substrate specificity of carboxylase

When it had been shown that prothrombin contains a number of carboxylated Glu-residues, the question arose why only the first 10 Glu-residues were carboxylated and the others were not. Similarly, it remained intriguing why prothrombin contained Gla-residues and other proteins, such as albumin, did not. Both proteins are secretory proteins of comparable size and both are synthesized in the liver. These questions will only be answered after we have detected which substrates are recognized by carboxylase and which ones are not. We have therefore studied the affinity of carboxylase to a number of substrates.

In a first attempt to obtain some insight into the problem we have prepared carboxylase from four different tissues: liver, lung, kidney and testis. In these carboxylating systems we have measured the K_M values for four different substrates: the pentapeptide Phe-Leu-Glu-Glu-Leu, fragment-Su (both derived from descarboxy prothrombin), decarboxylated Bone Gla-Protein and decarboxylated Sperm Gla-Protein. When the in vitro substrate for an enzyme system is also its natural substrate (for instance: fragment-Su in hepatic carboxylase or d-S.G.P. in testicular carboxylase), the system is defined as homologous.

Table 6

Substrate specificity of carboxylase

Origin of carboxylase	Carboxylation of substrate:			
	FLEEL	Fragment-Su	d-B.G.P.	d-S.G.P.
Liver	● ●	■ ■	▲ ▲	◆ ◆
Testis	● ●	■	▲ ▲	◆ ◆ ◆
Lung	● ●	■	▲ ▲	◆ ◆
Kidney	● ●	■	▲ ▲	◆

K_M values were determined from the initial carboxylation rates at various substrate concentrations under standard conditions. The K_M of hepatic carboxylase for each of the various substrates is arbitrarily represented by a double symbol. A single symbol represents a 10-fold higher K_M and a triple symbol a 10-fold lower one.

Unfortunately we have not yet determined all K_M values to an accuracy that warrants their publication. We have therefore chosen to present our first results in the form shown in Table 6: the K_M of hepatic carboxylase for a given substrate is arbitrarily represented by a double symbol. In the non-hepatic systems a single symbol indicates a K_M which is one order of magnitude higher and a triple symbol is used when the K_M is one order of magnitude lower.

As was mentioned earlier, the K_M for Phe-Leu-Glu-Glu-Leu is high (about 4 mM) in hepatic carboxylase and the same was found in the non-hepatic preparations. So we concluded that the affinity of carboxylase to this substrate is similar but low in all cases. Presumably the pentapeptide is too small to bear a sufficient recognition site in its secondary structure. In this respect it is remarkable that the K_M for the slightly larger fragment-Su is much lower in the homologous as well as in the heterologous systems. Apparently there is a structural element which is commonly recognized by all carboxylating systems. Moreover the K_M was lowest in the homologous system (0.002 mM) and about 10-fold higher in the heterologous ones.

According to our definition, all systems in which d-B.G.P. was assayed were heterologous. What we observed was that the substrate was recognized equally well in all systems, with a K_M which is comparable to that for fragment-

Su in the heterologous systems (0.015 – 0.02 mM). Again a common "recognition feature" seems to be present in this substrate. Unfortunately we do not possess the homologous system in order to test whether a specific recognition signal for bone carboxylase is eventually hidden in the amino acid sequence. When measuring the various K_M values for d-S.G.P. we observed once more rather low values for the liver (about 0.09 mM) and the other heterologous systems and an even 8-fold lower K_M for the homologous (testicular) system.

From these preliminary experiments the following conclusions have been drawn:

1. The presence of a hydrophobic signal sequence at the N-terminal side of the substrate is not an absolute requirement for the efficient recognition of a substrate by carboxylase. Whether the signal sequence is able to decrease still further the K_M remains to be seen.

2. Substrates composed of 17 aminoacid residues or more seem to contain a common structural element, which is sufficient for the recognition of carboxylatable polypeptide sequences by any kind of carboxylase.

3. Substrates of at least 17 aminoacid residues are carboxylated better in homologous than in heterologous systems. So the various carboxylases recognize their own substrates. Although this substrate specificity is not absolute, it implies that differences must exist between carboxylases from different tissues. Therefore they have to be regarded as a series of isoenzymes rather than as one enzyme which occurs in a variety of different organs.

Human carboxylase

Most of our knowledge about vitamin K-dependent carboxylase is based on in vitro model systems in which rats and cows are used as experimental animals. It is evident that the results obtained with these model systems should be compared with carboxylating systems of human origin. Therefore we have started to collect a number of organs from normal, healthy, human donors, who had died after traffic or other accidents and who had previously signed a donor-codicil. It was verified that they had not received anti-vitamin K drugs. After hospitalization they were ventilated and the organs were excised within 4 hours. In this way we have been able to obtain 3 livers, 1 spleen, 1 kidney, 2 aortas and 1 liver from a baby 2 hours after birth. Moreover we obtained a number of normal term human placentas. The microsomal fractions of all these tissues were prepared and compared with the corresponding fractions of bovine origin (Table 7). It turned out that the carboxylase content of the human livers was closely similar to that of bovine liver and, except for the aorta, we also found such a resemblance for the other organs. This indicates that conclusions drawn from our animal model systems will probably hold true for human carboxylase.

The amount of carboxylase found in human aortas was substantially lower than that in bovine ones. Also in aortas of goats and rats a comparatively high level of carboxylase was found (data not shown). The low carboxylase activity

Table 7

Carboxylase in human tissues

Origin of carboxylase	Number of donors	Relative amount of			
		Carboxylase		Endogenous substrate	
		Human	Bovine	Human	Bovine
Liver	3	100	81	100	15
Neonatal liver	1	123	—	39	—
Spleen	1	9	9	7	6
Kidney	2	14	10	14	10
Aorta	5	24	55	2	2
Placenta	5	27	—	3	—

The amounts of carboxylase and endogenous substrate were determined as described in ref. 27 and the values obtained for human liver were arbitrarily defined to be 100. All tissues were obtained from different donors. In those cases where more than one organ of the same type was used the data in this Table are means of the individual values. The data for the bovine material are means of 4 different animals.

in human aortas might be related somehow with the occurrence of atherosclerosis, a disease found almost exclusively in man. Further investigations concerning this point are in progress in our laboratory.

Returning to Table 7 we would like to discuss a second observation: the high amounts of endogenous substrates for carboxylase in the human liver. High amounts of endogenous substrate are generated when carboxylase is blocked, for instance by vitamin K-antagonists or during vitamin K-deficiency. Vitamin K-deficiency, however, is not a common feature in man and neither had the donors received anticoagulant drugs. Since it is known that the coagulation factors II, VII, IX and X are synthesized in the liver and that during warfarin-treatment the accumulated substrates for hepatic carboxylase consist almost exclusively of precursors of these coagulation factors [29], it is to be expected, that the substrates in the normal human liver are also coagulation factor precursors. So the question arose: why had these precursors not been carboxylated? This question cannot so easily be answered, but we think that the abnormally high level of clotting factor precursors in the human liver is a result of the physical condition of the donor at the moment that the livers were excised: all donors had undergone severe trauma, brain damage and massive blood loss. Since blood loss, decreased plasma concentrations of clotting factors and activation products of clotting factors all have been reported to stimulate the *de novo* synthesis of clotting factors [76, 77], it is likely that in the human livers described here, the production of clotting factor-precursors had been strongly enhanced. On the other hand the oxygen supply of the livers may have been substantially impaired. Molecular oxygen is required for the carboxylation reaction and the latter may thus be slowed down

by the lack of oxygen. In this situation carboxylation may become the rate-limiting step in maturation of the coagulation factors.

Another comment that has to be made is that the amount of endogenous substrate in the neonatal liver was lower than that in both adult livers. At this moment the presumed vitamin K-deficiency of the newborn is still a matter of debate [78] and in many countries vitamin K is routinely administered immediately after birth. Had the neonatal liver been (partly) vitamin K deficient, one might expect the level of the coagulation factor precursors to be higher than in the adult livers. The contrary was observed, however, and therefore this case does not support a vitamin K-deficiency of the newborn.

Conclusion

Vitamin K-dependent carboxylase is a very common enzyme, which occurs in many different types of tissue. Preliminary data indicate that "carboxylase" may stand for a number of isoenzymes, each having its own substrate specificity. Since vitamin K dependency is universal for all these enzyme systems, it might be expected that their vitamin K-binding sites are closely similar, if not identical. Consequently they all will be inhibited to the same extent by vitamin K-antagonists. From the medical point of view this is undesirable and therefore drugs should be developed that specifically inhibit one type of carboxylase without affecting too much the other ones. Because differences between the various carboxylases may be found at or near their substrate binding sites, these drugs should be substrate-antagonists rather than vitamin K-antagonists. Therefore it is very promising that recently the first inhibitors of this type have been synthesized [79, 80]. All inhibitors were structural analogues of Phe-Leu-Glu-Glu-Leu and they inhibited hepatic carboxylase. It is not known whether non-hepatic carboxylases were also inhibited, but at this moment it seems plausible that for the preferential inhibition of one type of carboxylase larger peptides are required.

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Myelofibrosis, Autoimmune Haemolytic Anaemia and Tn-Polyagglutinability

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A patient with myelofibrosis was studied for about four years. During this period he developed autoimmune haemolytic anaemia, a rare complication of myelofibrosis. Furthermore, about two years after the investigation started, his red blood cells, previously normal, became Tn-polyagglutinable, a change known to be due to somatic mutation in haemopoietic stem cells.

Keywords: erythrocyte autoantibodies, myelofibrosis, somatic mutation, Tn-polyagglutinability

Introduction

Myelofibrosis, a slow myeloproliferative disorder, is of interest in both haematology and immunohaematology. It may be a preleukaemic state or a sequel to leukaemia. The subject has been reviewed by Wetherley-Mein and Pearson [1]. There may be evidence of the development, by somatic mutation, of abnormal clones of haemopoietic precursor cells. As discussed below, this may result in blood grouping anomalies or in paroxysmal nocturnal haemoglobinuria (PNH).

Case Report

Clinical and haematological observations

F. J., male, 59 years, was first seen by one of us (SGNR) in July, 1980. He was referred from the Chest Clinic, which he had attended because of dyspnoea on exertion and lethargy of 3 months duration. His spleen was found to be enlarged. He had a haemoglobin level of 10.6 g/dl; a leukocyte count of 12.0×10^9/l; and a platelet count of 147×10^9/l. A blood film showed anisocytosis, poikilocytosis and a leukoerythroblastic picture with a high proportion of nucleated red cells, disproportionately higher than would be expected from the reticulocyte count which ranged between 6 and 17% over the period of preliminary investigation. Marrow aspiration resulted in a 'dry tap'. Trephine biopsy showed a hyper-

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cellular marrow with fibrosis and increased reticulin. He was treated for three months with folic acid and oxymethalone without benefit.

In November, 1980, his haemoglobin had fallen to 8.8 g/dl and he was transfused. At that time the direct antiglobulin test ('broad-spectrum' reagent only) was negative. His next transfusion was in February, 1981. Pre-transfusion tests showed that the direct antiglobulin test was strongly positive and it has remained positive since then. He was treated with steroids and, on a high dose (40 mg daily), maintained a satisfactory haemoglobin level without transfusion. Because of reluctance to keep the patient on this high dose it was decided to remove his spleen. After splenectomy he was put on a low maintenance dose of Prednisolone (10 mg daily). He was never overtly jaundiced but had a moderately elevated serum bilirubin; his haptoglobin level was consistently lower than normal. He had significant red cell rouleaux, a very high ESR, and a rising serum IgG level, but no paraproteinaemia. His anaemia appeared to be related more to myelofibrosis than to autoimmune haemolysis. No evidence of haemoglobin H was obtained. In August, 1982, his red cells, previously normal, became Tn-polyagglutinable. Serological observations are given below. In mid-January, 1984 he had a massive haematemesis, probably due to steroids. Total gastrectomy was required to control bleeding. He died of postoperative complications related to the gastrectomy and not to his haematological condition, which had remained almost unchanged in the last few months of his life, with no evidence of transformation into acute leukaemia.

Serological observations

F. J. was group A_2, Rhesus positive, CDe/CDe. The direct antiglobulin test was positive with both 'broad-spectrum' and specific anti-IgG sera, but negative with anti-IgA or anti-complement sera. His serum autoantibody reacted with normal red cells, but not with -D-/-D- or Rh_{null} red cells, and therefore had the specificity variously described as anti-nl, anti-Rh 17 or anti-Rh 18. This antibody could also be eluted from the red cells. The specificity of the autoantibody did not change during the course of the investigation.

There were no blood grouping problems until August, 1982 when his red cells were found to be polyagglutinable. Tests using a battery of selected lectins [2] showed the polyagglutination to be due to exposure of the Tn receptor in many but not all his red cells (typical 'mixed-field' agglutination). Treatment of the red cells with papain abolished the blood grouping difficulty; this is characteristic of Tn, which is a protease-labile receptor. His Tn-polyagglutinability had persisted up to the time of his death. No other red cell abnormalities, e.g. weak ABO or Rh antigens, were detected, and no evidence of PNH (positive Ham's or sucrose lysis tests) was obtained.

Cytogenetics

Lymphocyte karyotyping showed that F. J. was a normal 46, XY.

Discussion

The clinical and haematological observations were consistent with a diagnosis of myelofibrosis. The special interest in this case is the development first of erythrocyte autoantibodies, and later of Tn-polyagglutinability.

Tn-polyagglutinability

The condition known as Tn-polyagglutinability is acquired by somatic mutation in a pluripotent haemopoietic stem cell [3]. A person who acquires this condition is a blood group mosaic with a dual population of red cells, white cells and platelets: Tn and normal. The precise genetic defect of the abnormal haemopoietic precursor clone is a failure to produce the enzyme required to convert Tn to T [4]. The condition is persistent; it may, however, disappear after many years through loss of the abnormal clone either spontaneously or after cytotoxic therapy [5]. Tn has been detected in apparently healthy persons or in those with leukaemia, particularly acute myelomonocytic leukaemia [5, 6], and may be a preleukaemic state [6]. Tn polyagglutinability is therefore similar to PNH, which is also a persistent clonal condition, characterised by more than one red cell population as demonstrated by different degrees of susceptibility to complement lysis. PNH may also disappear after many years by elimination of the abnormal haemopoietic clone, and, furthermore, PNH may be associated with leukaemia.

Tn-polyagglutinability causes blood grouping difficulty. It is characterised by a "mixed-field" agglutination pattern. It is distinguished from other types of red cell polyagglutinability by use of a battery of lectins [2], and is positively identified by a Tn-specific lectin e.g. *Salvia sclarea* [7].

Autoimmune haemolytic anaemia

Autoimmune haemolytic anaemia is extremely uncommon in myelofibrosis [1]. Our patient developed typical autoimmune haemolytic anaemia of the "warm autoantibody" (IgG) type. The autoantibody had specificity within the Rhesus blood group system (see above) characteristic of many autoantibodies of this class.

Chromosomes

Chromosomal abnormalities in myelofibrosis have been reported [1]. There is no specific marker or characteristic pattern. Our patient had no apparent chromosomal abnormality of lymphocytes.

Somatic mutation

Myelofibrosis may be associated with somatic mutation in haemopoietic stem cells. There may be weakening of ABO or Rhesus antigens with evidence of dual red cell populations [8, 9], or PNH [10] or a 'PNH-like defect' [11] may

develop. Our patient had neither abnormal ABO, Rhesus, or other blood group antigens, nor PNH or a PNH-like condition. This, however, is the first report of the acquisition of Tn in a patient with myelofibrosis. This is not really surprising because of the similarities between Tn and PNH mentioned above.

Haemoglobin H

Myeloproliferative disorders of the elderly may be associated with acquired haemoglobin H disease. Many of these patients show a dimorphic red cell picture with both hypochromic and normochromic red cell populations. The subject has been reviewed by Weatherall et al. [12] who warn that any elderly patient with a dimorphic cell picture and haemoglobin H inclusions has a fair chance of developing leukaemia. F. J. had neither a dimorphic cell picture nor haemoglobin H inclusions.

Conclusion

F. J. had a rare and interesting triad of pathological states: myelofibrosis, autoimmune haemolytic anaemia and Tn-polyagglutinability. There is no indication in his previous medical history that his myelofibrosis was a sequel to leukaemia, and no evidence of transformation into leukaemia was obtained during our investigation.

*

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Effect of Dialysable Leukocyte Extract on the Mononuclear Leukocytes in Hodgkin's Disease

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Therapy with dialysable leukocyte extract repeated 6 times, had a beneficial effect on the impaired functions of mononuclear leukocytes in Hodgkin's disease. The decreased phagocytosis and chemotaxis of monocytes increased almost to the values of healthy controls. There was no significant change in the C3b receptor activity of the patients' monocytes but their pathologically increased EA rosette formation showed some correction during dialysable leukocyte extract therapy. The number of T cells bearing histamine and IgG Fc receptors was reduced initially and increased during therapy but this effect was only temporary. The results suggest that the stimulating effect of dialysable leukocyte extract on cellular immunity was due partly to the correction of mononuclear phagocyte functions rather than an effect on lymphocyte subpopulations in Hodgkin's disease. The beneficial effect of transfer factor on mononuclear phagocyte function may affect the rate of tumour progression and reduce the number of severe infections in patients with Hodgkin's desease.

Keywords: Fc-C3b-receptor function, Hodgkin's disease, monocyte chemotaxis, phagocytosis, T lymphocyte subpopulations, dialysable leukocyte extract.

Introduction

The cell-mediated immunity of patients with Hodgkin's disease (HD) is impaired even at an early stage of the illness. Progression of the disease and chemo- or radiotherapy further decrease the cellular immune reactivity [3].

T lymphocytes have been the subject of extensive studies in HD, while other cell types are also involved in cellular immunity, in particular monocyte-macrophages, which are engaged at various levels of the cellular immune response. In addition to the defensive mechanism against infective agents, they eliminate immune complexes and destroyed cells, and contribute to the suppression of the formation and dissemination of malignancies [17, 26].

Immunological abnormalities may play an important role in the pathogenesis of Hodgkin's disease [12] and therefore stimulation of deficient immune functions may be beneficial. One of the agents available for this purpose is the dialysable leukocyte extract (DLE) [10], which has antigen-dependent and antigen-independent activity in stimulating the cellular immunity of the recipient [9, 25]. Dialysable leukocyte extract has been used in patients with various malignancies, the most encouraging results were observed in osteogenic sarcoma and malignant melanoma [15].

In this paper the effect of repeated DLE therapy on monocyte phagocytosis, chemotaxis, Fc and C3 receptor activity, and on the lymphocyte subpopulations of 10 patients with Hodgkin's disease were studied.

Materials and Methods

Preparation of dialysable leukocyte extract

DLE was prepared by extraction from human tonsils [21]. The latter were homogenized in distilled water, frozen and thawed twice, centrifuged and dialysed. This extract was lyophilized and dissolved in PO_4-NaCl buffer (8.5 g NaCl/1, 0.01 mol/1 PO_4 buffer pH 7.4) 1 : 20. Fractionation was performed with chromatography equipment supplied by Messrs. LKB, Sweden, using Sephadex G-25 (fine) 50×600 mm column. Activity of the fractions was estimated by "recovery assay" [22].

Patients

The peripheral blood mononuclear cells of 10 adults (7 men and 3 women) with HD were studied. Severity of the disease was determined according to the Ann Arbor staging classification [2]. Seven of the patients had advanced HD (stages IIIB, IVA, IVB) and 3 had localized disease (IIA, IIB). All the patients had had radiotherapy and/or chemotherapy. The microscopic features were consistent with lymphocyte depletion type in 2, mixed cellularity type in 3, nodular sclerosis type in 3 and lymphocyte predominance type in 2 cases. All patients were in remission for a minimum of one year. Patients received DLE (on the 1st and 8th day and then monthly), a total of 6 injections each of $2.0. \times 10^8$ lymphocytes. Lyophilized DLE was reconstituted with 1.0 ml sterile distilled water and injected i.m. immediately.

The phagocyte capacity, Fc receptor function and chemotaxis of monocytes were examined in 17 healthy controls, voluntary blood donors of matching age and sex. Distribution of lymphocytes in peripheral blood was investigated in 50 healthy donors.

Separation of monocytes

Monocytes were obtained by sedimentation on Ficoll-Hypaque gradients [14]. After washing, the cells were suspended in Parker's medium containing 20 mM HEPES. The mononuclear leukocyte suspension contained $25-30\%$ monocytes, of which 95% were viable as estimated by nonspecific esterase staining [13] and trypan blue dye exclusion test.

Reaction of monocyte with sensitized SRBC

Of the mononuclear cell suspension containing $1-5 \times 10^5$ monocytes 0.1 ml was placed into a plastic ring fixed with wax to a glass slide. After 30 min the non-adherent cells were removed by washing and the monolayers containing

95–98% monocytes were overlaid with medium 199. Monocyte Fc receptor (FcR) activity (EA rosette formation) was assayed by the reaction of sheep red blood cells (SRBC) coated with a subagglutinating dilution (1 : 128) of an IgG fraction isolated on Sephadex G-200 from rabbit anti-SRBC serum. The adherent cells were incubated with 0.2 ml of 2% sensitized SRBC (sSRBC) in 3.0 ml of medium 199 at 37 °C for 30 min in 5% CO_2 at 100% humidity. After washing, the monolayers were stained with a solution of 0.01% crystal violet or Wright's stain, and the percentage of monocytes attaching to or ingesting three or more sSRBC was determined [7].

Phagocytosis of yeast

A monolayer technique was used as described above. The adherent cells were incubated with 25 μl of baker's yeast suspension containing 10^8 particles/ml in 0.5 ml medium 199 at 37 °C for 60 min in 5% CO_2 at 100% humidity. After washing, the monolayers were stained with a solution of 0.01% crystal violet or Wright's stain and monocytes containing ingested particles were counted. The amount of yeast ingested per cell was determined (phagocytic index).

Opsonization of sensitized yeast

Monolayers of adherent monocytes were incubated as above with 25 μl of 10^8 particles per ml of yeast, pretreated with 0.1 ml fresh AB human serum as opsonin at 37 °C for 60 min and then washed. After staining, the number of sensitized yeast particles ingested per cell was determined [6].

Monocyte chemotaxis

Chemotaxis assays were performed by the Boyden chamber technique modified in our laboratory [11]. 0.5×10^6 monocytes in 0.5 ml medium, complement derived chemotactic factor and 8 μm-pore size millipore filters (Membranfilter, Sartorius GmbH, Göttingen) were used in every chemotaxis test. After filling, the chambers were incubated at 37 °C for 90 min. The filters were stained with haemotoxylin and evaluated microscopically by measuring the distances travelled by the fast moving cells in 10 microscopic fields [28].

Lymphocyte subpopulations

Lymphocytes were separated on Ficoll-Hypaque gradients from heparinized peripheral blood.

1. *E-rosette.* Lymphocytes were tested for spontaneous rosette formation with unsensitized SRBC. The number and percentage of the active early E-rosettes [27] and the total rosettes were examined. The absolute numbers of these T lymphocyte subpopulations were calculated from the absolute lymphocyte count.

2. *Histamin receptor bearing lymphocytes* were tested by the method of Saxon et al. [20].
3. *IgG Fc receptor bearing lymphocytes* were examined by the reaction of ox red blood cells counted with subagglutinating dilution of anti-ox red blood cell serum [4].
4. *Surface immunoglobulin bearing lymphocytes* were determined using FITC-conjugated polyvalent goat anti-human immunoglobulins.

Soluble immune complexes

Immune complex (IC) assays were performed as previously described [6] on basis of the inhibitory effect of IC-s.

Results

Effect of DLE therapy on monocyte function

EA rosette formation of monocytes was significantly elevated compared to the healthy controls. After the first DLE injection there was a transient decrease in the reaction of the monocytes with sensitized SRBC and a sustained fall in monocyte Fc receptor activity to approximately normal levels was seen after the 5th and 6th DLE injections (Fig. 1).

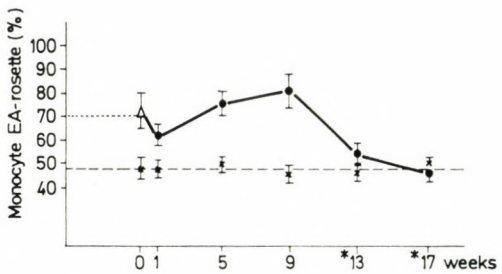

Fig. 1. Effect of DLE on EA rosette formation of the monocytes of patients with Hodgkin's disease (before DLE treatment: . . . . △); during treatment (•———•);control values (x— —x). $p < 0.01$ compared to pretreatment values

During DLE therapy the defective phagocytic activity increased significantly after the 5th week (Fig. 2).

The C3 receptor mediated opsonization of monocytes was normal in Hodgkin's disease and did not show significant alterations during treatment (Table 1).

Monocyte chemotaxis of the patients was impaired. Chemotactic responsiveness of the monocytes significantly increased after the first injection, and chemotactic function improved significantly during the whole DLE treatment (Fig. 3).

Table 1

Effect of DLE therapy on C3b receptor mediated opsonization of monocytes in Hodgkin's disease

Subjects		Phagocyte index (m ± S.D.)	Significance
Healthy donors (17)		2.71 ± 0.54	N. S.
Patients with	before DLE therapy	3.07 ± 0.58	
Hodgkin's disease	during DLE therapy		
	1. week	2.98 ± 0.52	
	5. week	2.42 ± 0.49	N. S.
	9. week	2.63 ± 0.54	N. S.
	13. week	2.78 ± 0.51	N. S.
	17. week	2.75 ± 0.58	N. S.

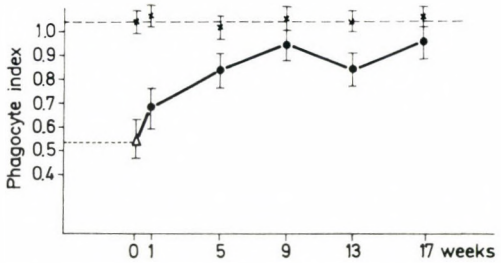

Fig. 2. Effect of DLE on phagocytic function of monocytes of patients with Hodgkin's disease (before DLE treatment: .... △); during treatment (● ——— ●); control values (x— — — x)

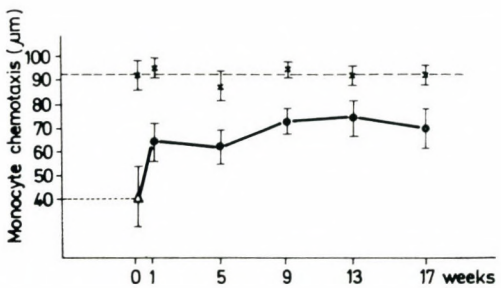

Fig. 3. Effect of DLE on the chemotactic function of monocytes of patients with Hodgkin's disease (before treatment: .... △); during treatment (● ——— ●); control values (x— — — x)

Table 2

Effect of DLE on peripheral lymphocyte subpopulations of patients with Hodgkin's disease

Cell population	Weeks						Healthy controls n = 50
	0	1	5	9	13	17	
Absolute number of ly-s	2211 ± 246	2120 ± 170	2241 ± 143	2326 ± 204	2558 ± 223	2094 ± 152	2490 ± 675
Active T-cells	480 ± 178	486 ± 142	852 ± 235*	504 ± 201	492 ± 224	418 ± 116	852 ± 418
Total T-cells	1084 ± 123	1306 ± 114	1736 ± 105*	1240 ± 172	1557 ± 144	1101 ± 102	1576 ± 547
B-cells	322 ± 95	340 ± 103	286 ± 84	359 ± 126	422 ± 147	260 ± 101	324 ± 252
T-cells bearing							
IgG-Fc-R	116 ± 23	121 ± 35	220 ± 37*	148 ± 31	149 ± 29	114 ± 24	173 ± 14
histamine-R	127 ± 29	119 ± 34	215 ± 39*	148 ± 29	151 ± 28	117 ± 22	163 ± 11

*$p < 0.001$ (compared to pretreatment values).

Effect of DLE on lymphocyte subpopulations

Patients with Hodgkin's disease had normal absolute numbers of total lymphocytes and B-cells and these values did not change significantly during DLE treatment. In contrast, the number of early rosette forming cells and total T cells (E^+ cells) was significantly decreased compared to the controls before treatment. DLE, repeated twice (5th week) resulted in a significant improvement in the number of active and total T cells but this effect was not stable. Later, during the monthly DLE therapy a not significant increase was seen. The decreased number of lymphocytes bearing IgG Fc or histamine receptors also increased significantly after two DLE injections, but later decreased to pretreatment levels (Table 2).

Effect of DLE on the circulating IC content

The immune complex level in the sera of patients with Hodgkin's disease was pathologically elevated (33.2 ± 11.1%), compared to the values of 32 healthy controls (7.7 ± 5.9%). During DLE therapy the IC level did not show significant changes (40.0 ± 12.6; 36.4 ± 10.9; 40.1 ± 12.4; 47.5 ± 13.5; 35.3 ± 9.6).

Discussion

There are no data in the literature concerning the effect of DLE on the EA-rosette formation of monocytes. In our experiments it was pathologically increased in Hodgkin's disease, similarly to the results of Saragone et al. [19]. The function returned to normal after 5 injections of DLE.

Deficient monocyte phagocytic and chemotactic function has been reported in various malignant diseases [3, 5, 24]. A serum factor was found, which acts as an inhibitor of phagocytosis and chemotaxis in vitro and of the accumulation in vivo of mononuclear phagocytes [23]. In our experiments, in Hodgkin's disease DLE has been found to improve these functions but not to influence the C3b receptor mediated opsonization of the patient's monocytes. Sander et al. [18] investigated the effects of DLE on cGMP levels in leukocytes and found an increase predominantly in the monocyte population. The increased level of cGMP went together with potentiation of the phagocytic and chemotactic activity of monocytes.

DLE vas found to convert the reactivity to skin test antigens and increase the T cell number and mitogen transformation in Hodgkin's disease [1]. In our experiments DLE did not alter the B cell number nor did it increase significantly the active and total T cell number in the 5th week of treatment. Although an enhanced suppressor activity of T cell and monocyte origin was reported in Hodgkin's disease [16], in our experiment the number of suppressor T lymphocytes (as defined by histamine receptors) decreased in the patients treated with radio- and/or chemotherapy. In the 5th week of DLE therapy the number of

lymphocytes bearing IgG and histamine receptor increased, but this effect was not lasting.

Our results suggested that repeated DLE therapy had a prolonged beneficial effect on defective monocyte functions in HD and only a slight temporary effect on lymphocytes. This may mean that the non-specific stimulating effect of DLE on cellular immunity is due to the correction of the function of mononuclear phagocytes rather than of the lymphocytes. Our findings suggest that TF may be useful in Hodgkin's disease in the control of tumour progression and/or severe infections, and that DLE may contribute to checking tumour progression and severe infections in HD.

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Bone Marrow Patterns in Chronic Lymphocytic Leukaemia Related to Various Clinical and Haematological Findings. A Preliminary Report

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In a retrospective study of 55 CLL cases subjected to bone-marrow biopsy, the histological pattern of the bone-marrow was correlated to the clinical and haematological data. Instead of the four histological patterns described by Rozman et al., five patterns have been established. The clinical staging according to the Montserrat score, compared with the bone-marrow pattern, showed a good correlation, when maintenance or replacement of normal haematopoiesis was considered. The bone-marrow pattern with maintained haematopoiesis was predicting a favourable clinical correlation; those with replaced haematopoiesis corresponded to a poor prognosis. The correlation is a useful index of severity and prognosis of CLL.

Keywords: CLL, bone-marrow biopsy, histological pattern of bone-marrow biopsy in CLL, prognosis of CLL

Introduction

Dameshek [2] assumed that chronic lymphocytic leukaemia (CLL) was a cumulative disorder of a population of functionally inactive lymphocytes with a prolonged life-span, which accumulate in the patient during evolution of the disease. Based on this concept, Rai et al. [9] proposed a clinical staging system of CLL that was unanimously accepted by haematologists.

The 5 clinical stages suggested by Rai were established according to the following criteria:

- stage 0: lymphocytosis in peripheral blood $> 15\,000/mm^2$ and in bone marrow (BM) $> 40\%$;
- stage I: lymphocytosis with lymphadenopathy;
- stage II: lymphocytosis with spleno- and/or hepatomegaly;
- stage III: lymphocytosis with anaemia (Hb < 11 g/dl);
- stage IV: lymphocytosis with thrombocytopenia (thr. $< 100\,000/mm^2$).

Subsequently, other clinical staging systems have appeared in an attempt to simplify and improve the prognostic value of the Rai staging, which was considered to be too complicated and without sufficiently well marked limits between

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the different stages: Binet et al. [1], International Committee for CLL, Binet et al. [6], Jakšič and Vitale [7], Montserrat and Rozman [8] etc. The staging system proposed by Binet et al. (International Committee for CLL) includes only 3 stages [6].

It should, however, be noted that the authors of the more recent staging systems [6] have recognized the value and utility of Rai's staging system.

In this paper we are mainly concerned with the staging system proposed by Montserrat and Rozman [8] based on points (score) (Table 1).

Table 1

The Montserrat and Rozman score

Clinical and haematological parameters	Score, points
Age $>$ 60 years	1
Lymph node involvement $\geq$ 3 territories	1
Splenomegaly $<$ 10 cm	1
Splenomegaly $>$ 10 cm	2
Lymphocytosis $> 50\times 10^9/1$	2
Anaemia (Hb $<$ 11 g/dl)	2
Thrombocytopenia $< 100\times 10^9/1$	2

A score exceeding 3 points indicates a poor prognosis with short survival; patients with a score below 3 points show a much longer survival [8].

More recently, Rozman et al. [10] have introduced a histologic classification of CLL, based on the patterns of bone marrow sections. From the prognostic point of view, four patterns were considered significant (Table 2).

Correlating these patterns with survival the authors observed that from pattern I to IV prognosis gets progressively worse; thus the BM-patterns may predict the prognosis in the course of after treatment of the disease [10]. Further-

Table 2

Bone marrow patterns in CLL, according to Rozman et al. [10]

No.	Pattern	Description
I	interstitial	Partial replacement of normal haematopoiesis by mature lymphocytes with preservation of BM-structure and fat cells.
II	nodular	Nodules of mature lymphocytes. These nodules are larger than normal lymphoid follicles and have no clear centre. No interstitial infiltration. Fat cells are preserved.
III	mixed	Combination of I (interstitial) and II (nodular) patterns.
IV	diffuse	Diffuse lymphoid infiltration with massive replacement of normal haematopoietic tissue and fat.

more, by correlating the histologic BM pattern with the clinical staging suggested by Rai et al. [9], Binet et al. [1] etc. the authors found that the histologic BM pattern represents an additional prognostic factor in CLL, but they emphasized that in some cases the correlation is not quite close [10].

The aim of the present study was to verify in our own CLL cases in which BM-biopsy was performed, the patterns described by Rozman et al. [10], and then to correlate our findings with the staging system proposed by Montserrat and Rozman [8] in order to find prognostic parameters of the disease and eventually the possibility of a better estimation of therapeutic results and survival. This preliminary paper presents only part of our investigations.

Material and Methods

The study was carried out in 55 patients (37 males and 18 females) with CLL in whom BM-biopsy was performed. The patients ranged in age between 30 and 77 years, their mean age was 58.

The total number of BM-biopsies was 60. In 3 cases biopsy was performed twice, and in one case 3 times. In these cases the BM-patterns changed according to the clinical condition.

Initially, the histologic patterns were classified according to Rozman et al. [10], but after a thorough reexamination of the slides, pattern III in Rozman's classification proved to comprise two subgroups related to haematopoiesis: indeed, in some of the slides the mixed pattern of Rozman et al. was associated with a maintained normal haematopoiesis, while in others normal haematopoiesis was replaced. This difference proved prognostically important. Consequently, we modified the histologic classification of BM-patterns by adding a fifth group (Table 3).

To find correlations for prognostic application of the results, the five histologic patterns were compared to the clinical and haematologic parameters proposed by Montserrat and Rozman [8]. The numerical and per cent distribution

Table 3

Our histologic classification of BM-patterns in CLL (see Figs 1—5)

I*	II*	III*	IV**	V+
interstitial	nodular	mixed with preserved haematopoiesis	mixed with replaced haematopoiesis	diffuse

* according to Rozman's classification of BM patterns in CLL
** our own finding
+ = pattern IV in Rozman's classification

Table 4

Numerical and per cent distribution of biopsy material as related to the BM-pattern

No. of biopsies and percentage	Histologic BM pattern
11 (18%)	I
4 (7%)	II
21 (35%)	III
12 (20%)	IV
12 (20%)	V

of our biopsy material (60 samples) related to the BM-pattern is presented in Table 4.

Table 4 shows that from the 60 cases investigated the most numerous group was represented by pattern III (mixed type with normal maintained haematopoiesis). The clinical and haematologic data of our cases according to the Montserrat and Rozman score [8] and the histologic BM-pattern are shown in Table 5.

Table 5

Numerical relationship of the clinical and haematologic data according to the Montserrat and Rozman score, and the histologic BM-pattern

Montserrat and Rozman score		Histologic BM-pattern and number of patients				
Clinical and haematologic data/points		I 11	II 4	III 21	IV 12	V 12
> 60 years	1	5	1	0	0	3
Adenopathy ≥ 3 territories	1	4	3	12	8	11
Splenomegaly < 10 cm	1	1	3	3	4	4
Splenomegaly > 10 cm	2	2	3	3	7	4
Lymphocytosis > 50×10^9/l	2	2	1	9	10	7
Anaemia: Hb < 11 g/dl	2	1	1	4	5	7
Thrombocytopenia < 100×10^9/l	2	0	0	3	5	8

Results

The correlation of the Montserrat – Rozman score and the histologic BM-pattern in our cases (Table 5) showed that no correlation could be found between age > 60 years and the BM-pattern.

Regarding the other parameters of the score, a correlation with the BM-patterns I and II was found only in a small number of cases, whereas significantly

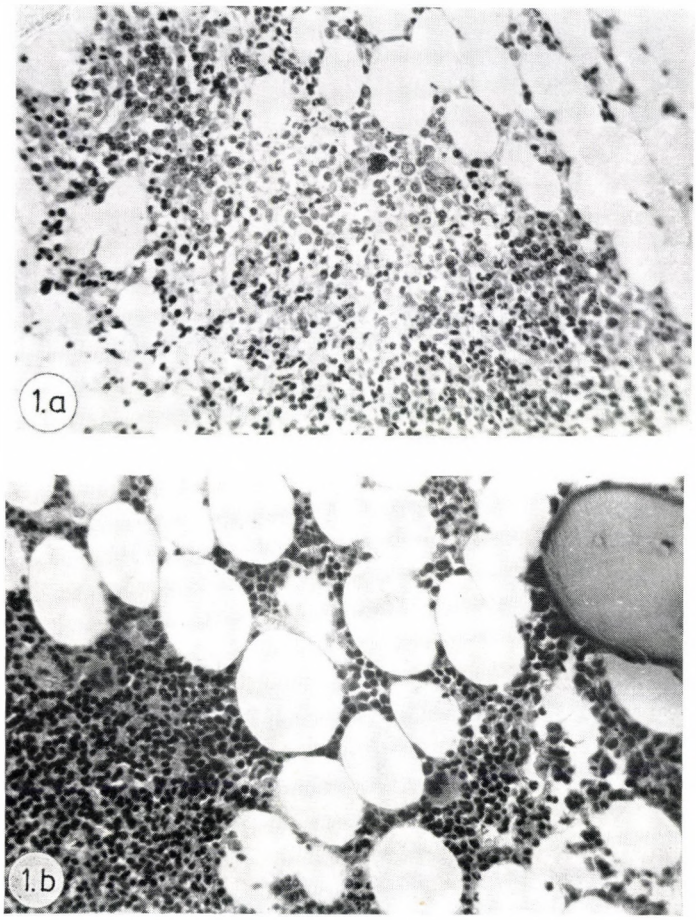

Fig. 1. a. b. (a × 10; b × 20). Pattern I: interstitial BM with slightly increased cellular density. Fat cells preserved. The lymphocytes are diffusely scattered among the cells of normal haematopoiesis (HE)

Table 6

Correlation between the Montserrat—Rozman score and the BM-pattern

Montserrat—Rozman score		BM-pattern				
No. of points	No. of cases	I	II	III	IV	V
1—3	28	9	3	14	0	2
4—5	14	2	0	3	5	4
6	18	0	1	4	7	6

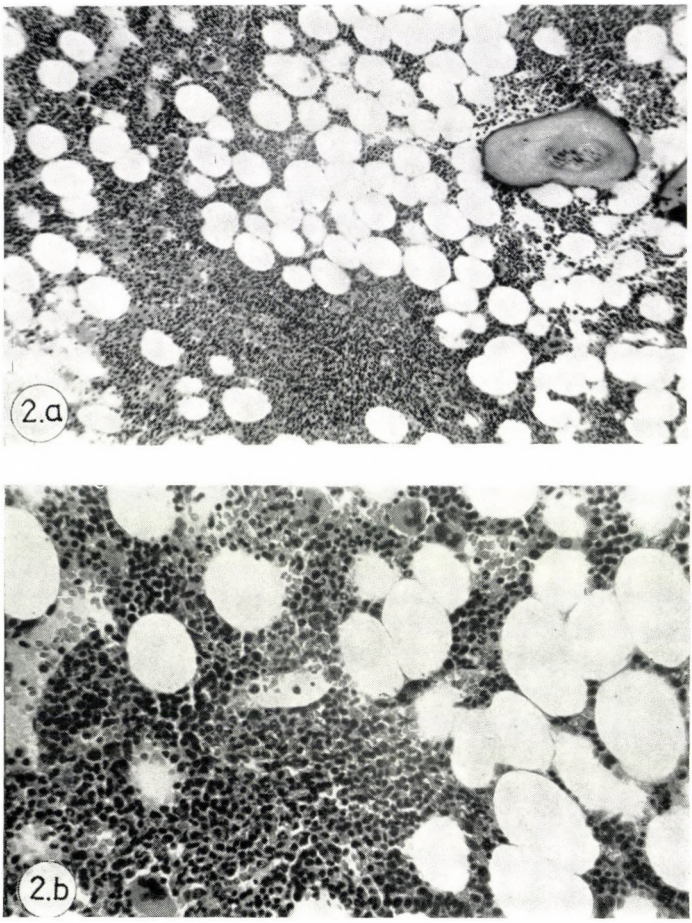

Fig. 2. a. b. (a ×10; b ×20). Pattern II: nodular. Lymphocytic proliferation with nodular disposition. Normal haematopoiesis preserved. (HE)

more cases presented a correlation with patterns III, IV and V. This would suggest that each parameter of the score is a sign of poor prognosis, but, in fact, as stated by Montserrat and Rozman, the score and not the isolated points indicate the prediction: the cases with 1–3 points showed a good prognosis and those exceeding 3 points a poor one. Thus, in the group with 1–3 points the BM-patterns I–III are prevailing; in the groups with to 6 points, patterns IV and V are predominant (Table 6).

The comparison of clinical staging according to Montserrat and Rozman with the BM pattern showed a most significant correlation especially when maintenance or replacement of normal haematopoiesis was taken into consideration (Table 7).

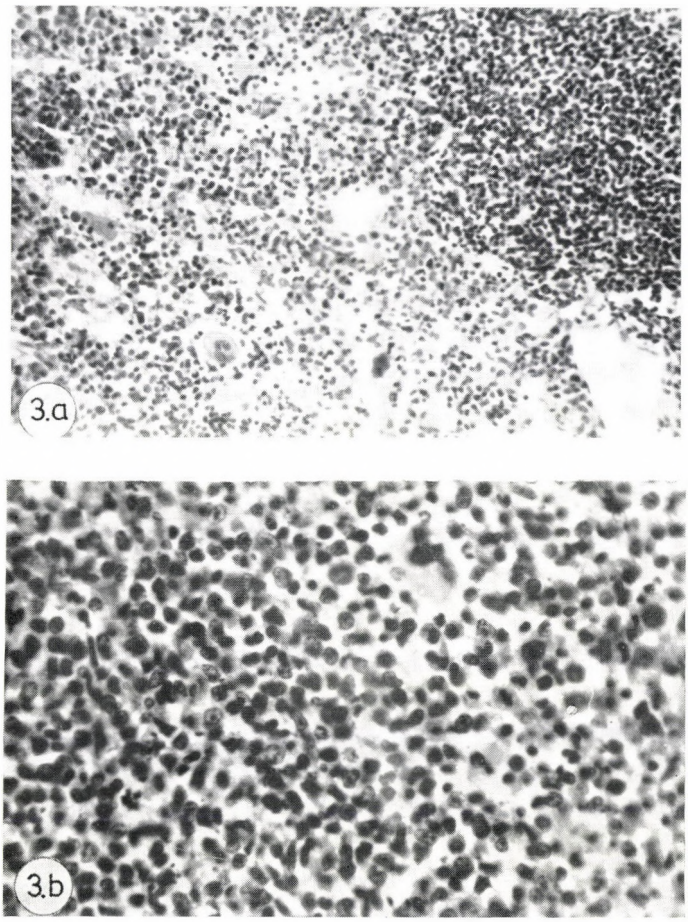

Fig. 3. a. b. (a × 20; b × 40). Pattern III: mixed with preserved haematopoiesis: important nodular and diffuse lymphocytic infiltration. Medullary haematopoiesis preserved. Fat cells scanty. (HE)

Table 7

Montserrat and Rozman score	Number of cases	
Number of points	BM-pattern with maintained haematopoiesis	BM-pattern with replaced haematopoiesis
1—3	26	2
4—5	5	9 } 22
6	5	13 }

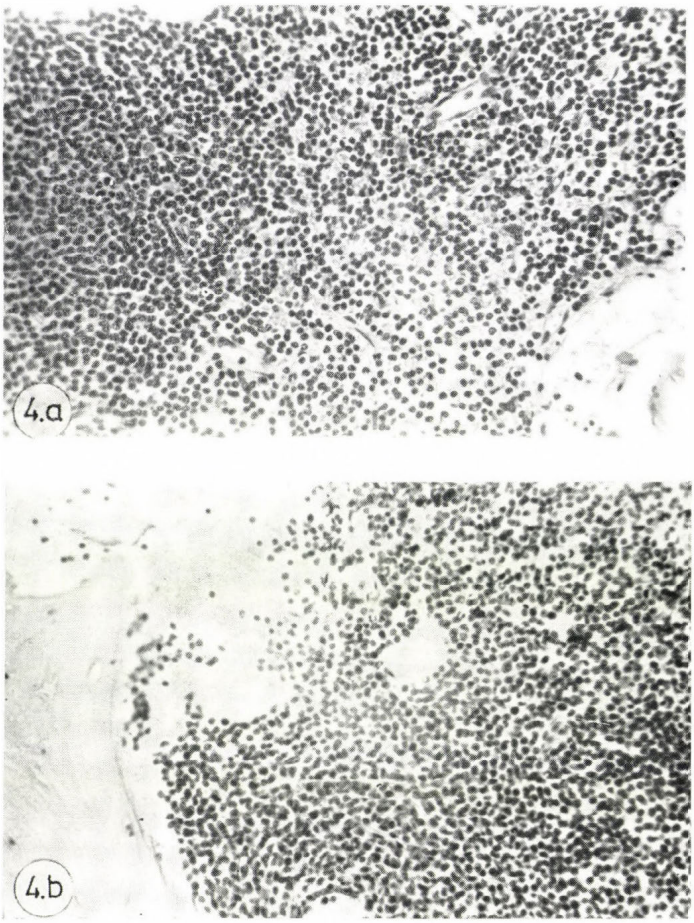

Fig. 4. a. b. (a ×20; b ×20). Pattern IV: mixed with replaced haematopoiesis: important nodular and diffuse lymphocytic infiltration with total replacement of medular haematopoiesis and fat tissue. (HE)

Discussion

In all staging systems the intention of the authors was to establish a series of clinical and/or haematologic parameters reflecting the degree of severity of the disease at a given moment of its evolution and at the same time to supply new criteria indicating the prognosis and survival. The fact that this aim has not been satisfactorily reached is proved by the increasing number of suggestions for new staging systems. This, instead of the 5 stages proposed by Rai et al. [8], Binet et al. [6] in 1981 proposed a system with 3 stages, stages A and B containing data concerning lymph node-hepato- and/or splenomegaly, and stage C based

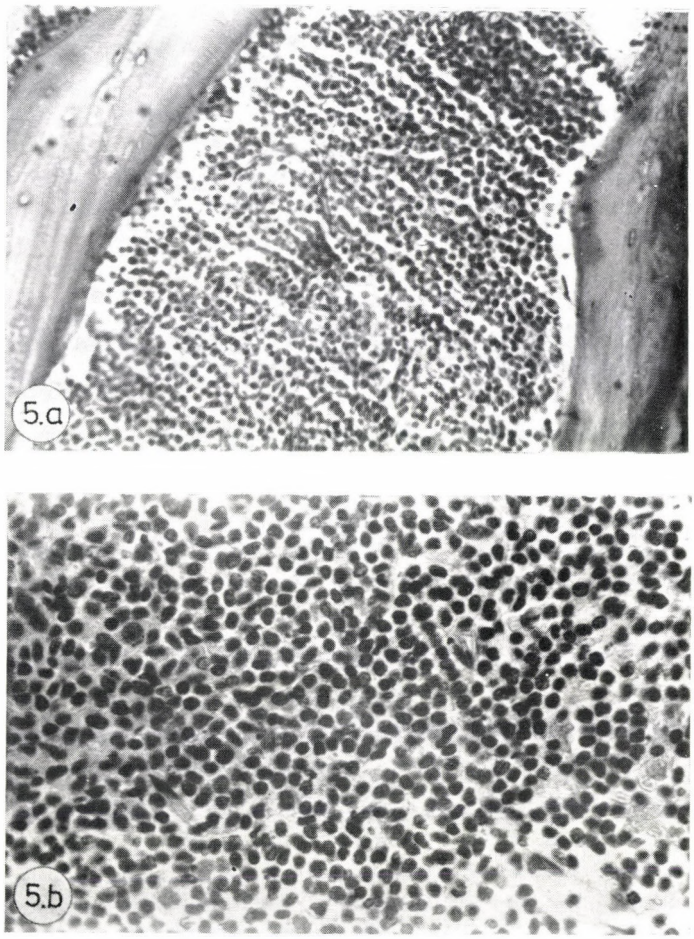

Fig. 5. a. b. (a ×20; b ×40). Pattern V: diffuse lymphocytic infiltration with total replacement of medular haematopoiesis and fat tissue. (HE)

on the degree of anaemia and/or thrombocytopenia. It should be noted that the anaemia considered in this staging system is below 10 g/dl of Hb. Comparing these two staging systems, Geisler and Hansen [3] concluded that from the therapeutic and prognostic point of view the systems with 5 stages are unpractical, and the system with 3 stages permit a greater accuracy since they depend more on quantitative than on qualitative criteria of the tumour mass. The parameters suggested by Jakšič and Vitale [7] are based on the same principle.

In an attempt to increase the criteria for prognosis, estimation of therapeutic results and prediction of survival, we have compared the histologic classification of BM-biopsy by Rozman et al. [10] with the clinical staging system of

Montserrat and Rozman [8]. In our study, instead of the four BM-patterns described by Rozman and Montserrat [10], we found five patterns, since in Rozman's pattern III we distinguished two aspects of the same histological picture (the mixed pattern), namely: one pattern with maintained normal haematopoiesis, the other with replaced haematopoiesis. The correlation of these aspects with Montserrat–Rozman score are important from the practical point of view, i.e.: *pattern III in Rozman's and in our classification predicts a favourable clinical evolution, while cases which in Rozman's classification fall to pattern III, but in our classification belong to pattern IV, pattern with replacement of normal haematopoiesis, are indicatives of a poor prognosis.*

For the present we have only given our results concerning the prognostic value of this correlation.

For statistical processing of the data, we used the χ^2 test. Significant correlations were only found between lymphocytosis $> 50 \times 10^9$/l, respectively thrombocytopenia $< 100\,000 \times 10^9$/l and the histologic patterns III–IV. It is believed that the lack of statistical significance of the other parameters was due to the small number of cases in the different categories. Subsequent investigations on a larger scale will be concerned with a better statistical estimation and a more accurate specification of survival time.

Conclusions

Unlike the four bone marrow patterns in CLL described by Rozman et al. [10], we have distinguished *five patterns* in terms of *maintenance* or *replacement* of myelopoiesis. The importance of this distinction consists in the fact that the same histologic picture of mixed (interstitial and nodular) pattern correlates with a favourable clinical evolution when normal haematopoiesis is maintained and with a poor prognosis when haematopoiesis is replaced.

In our material a significant correlation could be established between the clinical and haematologic condition of the patients, expressed by the Montserrat–Rozman score [8] and the BM-pattern, especially when an alteration of the normal haematopoiesis is considered. The BM-pattern with maintained haematopoiesis is concordant with a favourable clinical evolution (low risk); those with replaced haematopoiesis correspond to a poor prognosis (high risk). It is thought that the correlation is a useful index of the severity and prognosis of CLL.

*

We are indebted to Dr. Muresan from the Computing Centre of the Ministry of Health, for statistical calculation of the results.

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The Heparin Binding Proteins of Sarcoma Induced by Methylcholantrene in Rats

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The proteins binding and neutralizing the heparin of sarcoma induced by methylcholantrene are basic proteins. They are a mixture of at least three different proteins, possessing cathodic mobility in polyacrylamide gel electrophoresis of pH 2.7 and having a low molecular weight.

Keywords: heparin-binding basic proteins, heparin neutralizing activity, sarcoma basic proteins.

Introduction

Heparin is a naturally occurring, heterogeneous glucosaminoglucan of glucosamine and glucuronic acid, containing numerous sulfate groups giving the molecule the character of a polyanion [8, 12]. Being strongly acidic, heparin binds and forms complexes via ionic interaction with many basic molecules such as proteins, enzymes and several other substances [2, 7]. This fact causes the decrease or loss of anticoagulant activity.

Some cells and tissues, especially the experimentally induced tumours contain a number of cationic proteins and cationic polypeptides [4–6] which may be released to the extracellular fluid and to the blood serum, particularly at the final stage of tumour growth [1, 3]. They may bind and form complexes with heparin or its synthetic substitutes.

Taking the above considerations into account it was decided to investigate the interaction formed between the anionic polysaccharide heparin and the cationic proteins naturally occurring in the cytosol of sarcoma, induced by 20-methylcholantrene in rats.

Materials and Methods

Heparin (lot No. 520–2160) avarage m.w. 13 000, sulfate/hexosamine ratio, 2.65) was obtained from Sigma Biochemicals (170 USP units/mg) as standard.

Interaction of heparin in aqueous solution was studied with the sarcoma cytosol proteins at various concentrations and with the basic proteins obtained

from the fractionation of sarcoma proteins on CM-cellulose chromatography column.

Tumours were induced in male Wistar rats 3–4 months of age by subcutaneous injection of 0.1 mg of 20-methylcholantrene (MCA) dissolved in 0.1 ml of olive oil. The animals were killed six months later, when the tumour weighed about 100 g. These tumours were verified as sarcomas. The tumours were sliced and homogenized in a medium consisting of 0.25 mol/l sacharose, 0.03 mmol/l Tris-HCL and 0.2 mmol/l EDTA pH 7.4 in a volume equivalent to 10% of homogenate for 2 min in the Waring blendor. The homogenate was passed through nylon layers and then centrifuged (VAC 601 Janetzky ultracentrifuge) at 105 000 × *g* for 60 min. The supernatant has been accepted as the soluble cytosol fraction. The soluble proteins (20 mg) were dialysed against 0.05 mol/l phosphate buffer pH 4.4 and subsequently separated in a CM-cellulose chromatography column. Full details of basic protein isolation will be published elsewhere.

The soluble proteins of sarcoma and their fractions were analysed by electrophoresis on polyacrylamide gel according to the method of Ornstein [9], and basic proteins according to the method of Panyim and Chalkley [10]. The zone of proteins were stained with Coomassie brillant blue R 250. The basic proteins of sarcoma migrated at pH 2.7 to the cathode, were inhomogeneous and were mixture of at least 3 different proteins.

The ability of heparin to form complexes with sarcoma proteins was evaluated by the nephelometric method, which is based on measuring the absorbance at 670 nm of a mixture composed of:

a) various concentrations of sarcoma proteins or purified partially basic proteins with heparin at constant concentration;

b) constant concentration of sarcoma proteins or purified partially basic proteins with heparin at various concentrations mixed in equal volumes.

The second method of examining the interaction of heparin with proteins consists of measuring the thrombin induced plasma clotting time in a system with heparin [11]. The following system was used: 0.1 ml of 0.9% NaCl, 0.1 ml of heparin (0.025 mg/ml), 0.3 ml of platelet-poor citrated dog plasma and 0.1 ml of thrombin (40 u/ml). Clotting time of this system amounted to 60 s. The clotting time of the control system (without heparin) amounted to 8 s. The addition of basic proteins to the studied system instead of 0.1 ml 0.9% NaCl, caused a shortening of the clotting time.

Results

The proteins of sarcoma form with heparin insoluble complexes, and their amount is dependent on the concentration ratio of both components (Fig. 1). The formation of complexes in sarcoma protein mixture measured by the nephelometric method was not copious and its absorbance at 670 nm was low (0.15) even with high protein concentrations (10 mg/ml).

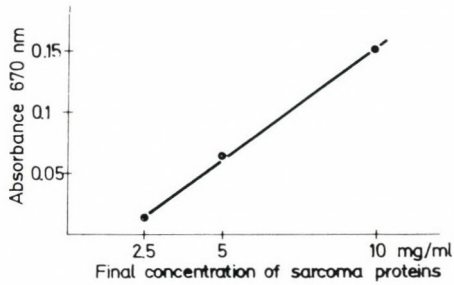

Fig. 1. Formation of insoluble protein–heparin complexes, at constant 0.2 mg/ml heparin concentration and various concentrations of sarcoma proteins

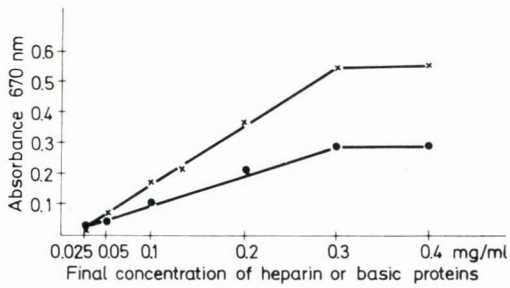

Fig. 2. Formation of insoluble protein–heparin complexes. × ——— × constant heparin concentration (0.2 mg/ml) and various concentrations of basic proteins. • ——— • constant concentration of basic proteins (0.2 mg/ml) and various concentrations of heparin

The partially purified basic proteins isolated from this mixture formed insoluble complexes with heparin in a very low proteins concentrations (Fig. 2). This absorbance was directly proportional to the concentration of basic proteins ranging from 0.025 to 0.3 mg/ml at a constant concentration of 0.2 mg heparin and

Table 1

Heparin neutralizing activity of basic proteins isolated from sarcoma proteins[1]

Basic proteins Final concentration μg/ml	Heparin-thrombin time, s
60	8
30	8
15	8
10	9
7.5	22
3.75	31
1.875	42
0.9375	68

[1] Mean values of three experiments for each protein concentration.

vice versa. It was also proportional to the heparin concentration of 0.2 mg of basic proteins.

It has also been shown that the largest amount of complexes was formed at a final concentration of about 0.3 mg/ml protein and about 0.2 mg/ml of heparin. Protein concentrations over 0.3 mg did not increase the formation of complexes, while an excess of heparin lowered their amount.

Table 1 shows that heparin was neutralized by the partially purified basic proteins isolated from sarcoma cytosol. The binding of heparin by proteins followed immediately.

On the basic of these experiments it was possible to calculate the amount of partially purified basic proteins necessary for a complete neutralization of 1 mg of heparin. It should be emphasized that about 4 mg of these proteins are needed to neutralize 1 mg of heparin completely. Thrombin induced clotting time in the presence of proteins at this concentration was shortened to the control time.

Discussion

The present results indicated the possibility of heparin inactivation by polycations other than protamine, the basic proteins of the tumour. The interaction of heparin with various model proteins revealed little specificity so far. The inactivation of heparin by proteins depends on the constellation of amino acids and their proclivity for α-helix or β-structures. Considerable amounts of these proteins in the tumour cells and tissues and their neutralization may facilitate the spread of tumour and metastatic formation. Proteins of various tumours were shown easily to penetrate into the extracellular space and from there into the blood stream [1, 2], where they play an important role in blood clotting regulation [13]. This penetration of proteins into the blood is facilitated by their cationic character and low molecular weight. The association of heparin with basic proteins in plasma may be the reason for the changing susceptibility to this substance. Thus, it is indicated before and after heparin therapy to carry out the heparin susceptibility test, in order to choose the proper dose.

The anticoagulant action of heparin is dependent on the plasma cofactor antithrombin III. It should be emphasized that the affinity of basic proteins to heparin is higher than to antithrombin III, and so is the release of antithrombin III by heparin from complexes with basic protein. Therefore, a deficiency of antithrombin III in blood plasma should by no means be considered dangerous.

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Electrochromatographic Analysis of Abnormal Serum Proteins

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An electrochromatographic method for the analysis of sera is described with particular design to studies of abnormal proteins. The method allows a simultaneous but independent evaluation of molecular weight and charge related migration of proteins. A modification which may improve the analysis of immunoglobulins by increasing their electrophoretic mobility is described.

Keywords: Electrochromatography, serum proteins

Introduction

The recognition of altered physical properties of serum proteins is an essential information for diagnosis and research. This concerns especially the change of molecular weight and charge. Most of the abnormal proteins are immunoglobulins and are thought to be associated with cancer proliferation [1]. Of the myeloma proteins, IgM and IgA more often than immunoglobulins from the IgG class, exhibit abnormal behaviour on gel filtration and electrophoresis. The changed properties arise from the abnormal amino acid composition or sequence, and/or altered carbohydrate component. This could be the effect of mutation if it concerns the sequence of amino acids, and of defective secretion in the case of abnormal carbohydrate component [2–4]. This abnormal behaviour often escapes observation in clinical tests, commonly used for analysis of serum proteins, as they are designed to optimize the separation and identification of proteins rather than to recognize their physical properties. The high resolution of serum proteins is achieved by the use of materials with sieve properties as electrophoretic beds (starch, polyacrylamide gels [5]). In these systems both charge and molecular weight affect the migration simultaneously, making the interpretation of physical properties difficult [6]. More univocal effects can be obtained by the use of electrochormatographic analysis which is a combination of a thin layer gel filtration (TLG) on Sephadex G-200 superfine [7] and paper electrophoresis. The vertically combined migration in both gel filtration and electrophoresis gives a two-dimensional distribution of the protein fractions with a defined molecular weight and charge dependent migration. The use of this method was until now limited owing to diffi-

culties which arise when Sephadex G-200 gel filtration and electrophoresis are combined [8]. These difficulties result from differences in the ionic strength required to maintain the proper conditions for electrophoresis and for gel filtration (high in filtration and low in electrophoresis). A simple modification [9] which permits to reduce the concentration of salt in electrophoresis of proteins transferred from TLG has resolved these difficulties, making more information available under standard hospital conditions.

This paper presents the use of electrochromatography for the clinical analysis of serum proteins.

Methods

The technique of this modified version of protein separation has already been published [9]. The main points are repeated in this paper.

The technique is transferring proteins separated in TLG system from Sephadex to electrophoretic paper by printing, with possible reduction of contact area between paper and gel (Fig. 1). This allows to transfer the proteins with a negligible amount of salt which interferes with electrophoresis when in excess. For this purpose, the whole Sephadex from the TLG plate with the exception of that comprising the proteins (determined by standard colour proteins: ferritin and haemoglobin) is removed before printing. The portion of Sephadex comprising the proteins, which usually covers an area of 2.5×13 cm, is then contacted with the paper strip (Whatman 3) for transferring the proteins. The paper strip, 13 cm in width, is equilibrated with buffer in electrophoretic conditions and instantly before printing partly desiccated with dry paper along the starting line for intensification of protein transfer. The desiccated fragment of paper is then contacted with Sephadex. After careful removal of stacked Sephadex grains from the paper strip – by scraping with a thin glass plate – it is placed again in an electrophoretic chamber and subjected to standard paper electrophoresis. The efficiency of protein transfer can be evaluated, when necessary, by quantitative comparison of the albumin spots of the electrochromatogram and independent electrophoresis of the same amount of serum proteins. Sephadex G-200 superfine used for TLG is swollen for three hours in a boiling water bath in 0.9% NaCl, stored in refrigerator and equilibrated with buffer before use.

The technique applied for *reversed electrophoresis* is the same as for the normal with the difference that the reversed one is carried out in veronal buffer pH 8.6 containing 1% soluble CM-cellulose (T. Schuchardt, München) and 2% glucose.

Care must be taken to avoid contact with CM-cellulose in normal electrophoresis, as even its traces affect the migration.

For analysis of sera with high protein concentration or containing sticky, aggregating fractions, electrochromatography is performed in buffer with urea added (up to 3 M).

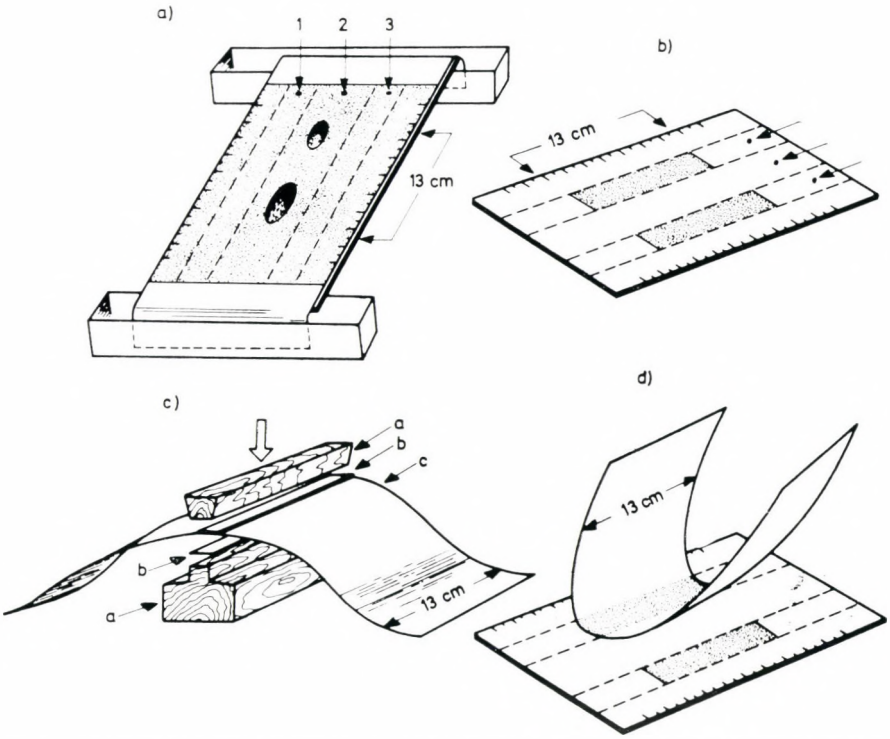

Fig. 1. The scheme of transferring the proteins from the TLG system to paper electrophoresis. a) — thin layer gel filtration; Sephadex G-200 superfine, 0.05 M Tris-HCl buffer pH 8.6 in 0.9% NaCl solution. The marks on the plate (dotted lines, scale) are to simplify the outlining of proteins comprising areas. 1. and 3. — starting points and migration paths for proteins of two serum samples; 2 — starting point and migration path for the colour protein markers — haemoglobin and ferritin — used to localize the fragment of migration path comprising all proteins and to evaluate the distance between head and end moving serum proteins. b) — glass plate after removal of the whole Sephadex, except the serum proteins comprising area (bands 2.5× 13 cm). c) — The partial desiccation of the electrophoretic paper strip along the starting line; a — wooden blocks; b — dry paper strips (Whatman 3, 2 cm wide). c — wet electrophoretic paper strip (Whatman 3, 13 cm width). Paper ribbons are changed twice for each desiccation. The desiccated fragment covers an area of 2× 13 cm. d) — Printing. Time of contact is 70 sec. The width of paper stripe (13 cm) was chosen to make it possible that the electrophoretic chamber of usual size holds two electrophoretic strips

For serological identification of protein fractions, the whole or the choosen fragments of the wet, freshly developed electrochromatogram is covered with droplets of specific antiserum, incubated for 4 hours and then washed with 0.15 M NaCl solution. After staining it is compared with electrochromatogram of the same serum sample, developed simultaneously in the same chamber.

Results and Discussion

Figure 2 presents the distribution of normal serum proteins obtained by the method described above. The deviation of molecular weight or charge may easily be noted. It is seen in Fig. 3 where four independent electrochromatograms of abnormal sera of IgA myeloma patients are compared. Electrochromatographic analysis of these sera exhibited essential differences of the properties of myeloma

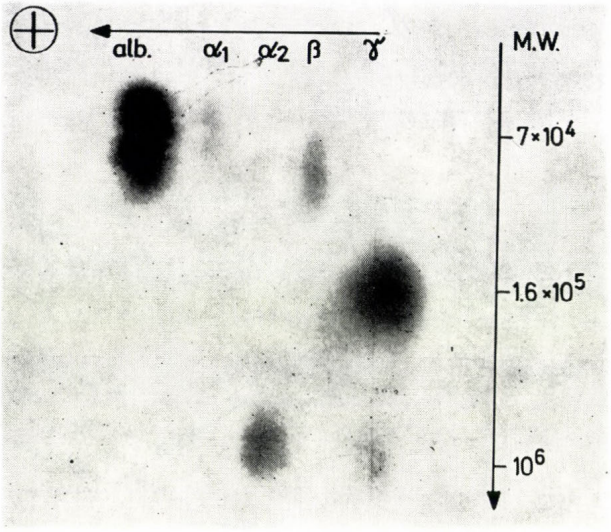

Fig. 2. Electrochromatographic separation of standard serum. Horizontal arrow: charge dependent migration (electrophoresis); vertical arrow: molecular weight dependent migration (Sephadex gel filtration)

proteins. Some of them appeared in the form of monomeric IgA, some as dimers (Figs 3A and 3B respectively), while in the cases presented in Figs 3C and 3D monomeric and polymeric IgA derivatives are simultaneously present. An interesting phenomenon is observed in Figs 3C and 3D, a mutually related alteration of molecular weight and charge which is expressed as a decrease of acidity with the increase of molecular weight. Such an effect is often observed with abnormal IgM. It seems to be the effect of the loss by assembling subunits of some glycopeptides which are believed to associate with the monomeric immunoglobulin molecules in early steps of secretion [10, 11].

A disadvantage of using this method of paper electrophoresis is the rather poor electrophoretic migration of immunoglobulins. Consequently, the separation of immunoglobulin fractions is often not sufficient. Some improvement has been obtained by using a negatively charged paper bed which induces the positive charges of separated proteins. This causes a reversal of migration as compared to

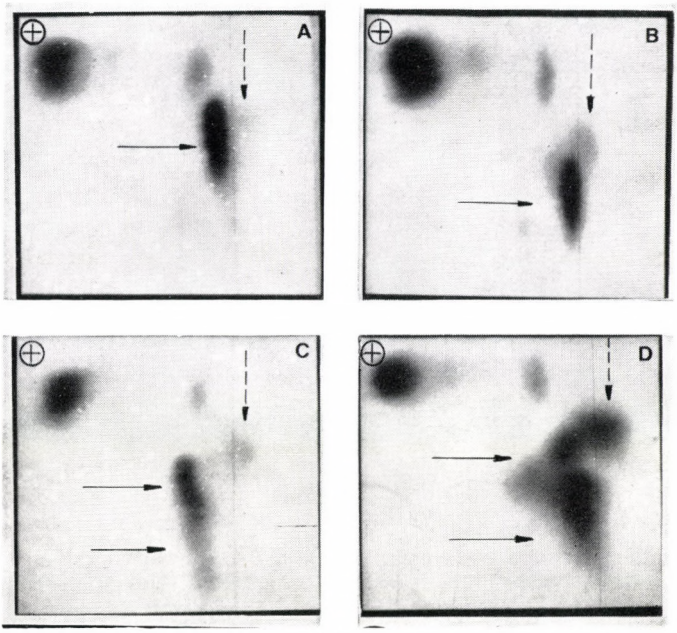

Fig. 3. Electrochromatographic separation of four pathological sera containing myeloma IgA proteins with different physical properties. A and B — monomeric and dimeric forms respectively; C and D — IgA fractions containing monomers and polymeric derivatives. There is a notable difference in the electrophoretic migration of high and low molecular weight species. Solid arrows: abnormal IgA; dotted arrows: residual polyclonal IgG

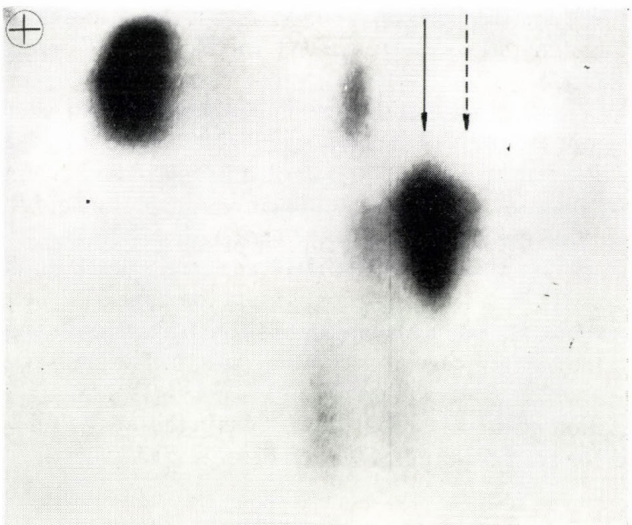

Fig. 4. Electrochromatographic pattern of IgG myeloma serum. Polyclonal IgG (dotted arrow) superimposed by monoclonal (solid arrow) protein

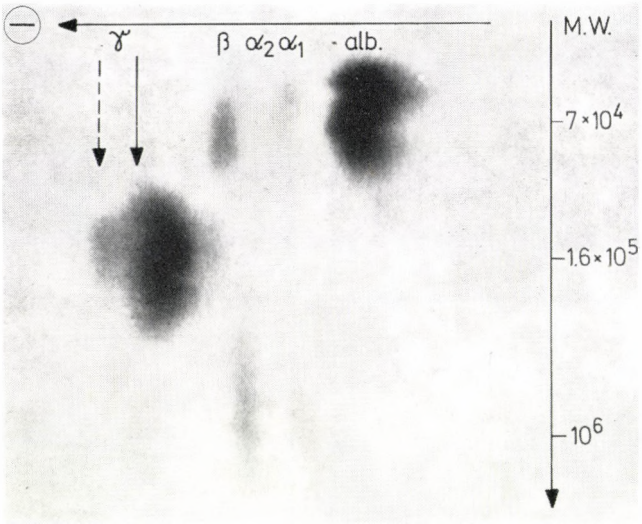

Fig. 5. Pattern of myeloma serum proteins (same as in Fig. 4) obtained by reversed electrochromatography. Improved separation of monoclonal (solid arrow) and polyclonal (dotted arrow) IgG

that obtained in the normally used systems. Such an effect was obtained by the addition of soluble CM-cellulose to the standard veronal buffer of pH 8.6 (see Methods). In this system IgG appeared to be the most mobile serum protein fraction, while the migration of albumin was the slowest. All serum proteins migrated to the cathode.

The use of the electrophoretic system in combination with gel filtration seems to be advantageous for the analysis of immunoglobulins as it is shown by a comparison of the electrochromatograms presented in Figs 4 and 5. Both are electrochromatograms of the same serum containing myeloma protein of IgG type. In such a case, evaluation of the amount of polyclonal IgG remaining in the serum is difficult because the monoclonal fraction is often superimposed on the normal IgG as it may be seen in Fig. 4, while sufficient separation appears to be possible in the reversed system (Fig. 5). The distribution of protein spots in the reversed pattern corresponds to the mirror image of the normal pattern. The main exception is IgM, the migration of which is often slower than predicted (Fig. 5).

Interpretation of the behaviour of proteins in this analytical system and use of the method for special purposes will be described elsewhere.

The authors wish to thank Mr. H. Pustkowiak for skilful technical assistance.

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ICSH/ICTH ANNOUNCEMENT

A Special Communication by ICSH *(International Committee for Standardization in Haematology)* and by ICTH *(International Committee on Thrombosis and Haemostasis)* on recommendations for reporting prothrombin time in oral anticoagulant control

To achieve harmonization in prothrombin time (PT) testing for oral anticoagulant control and thus to ensure reliable oral anticoagulant therapy, the International Committee for Standardization in Haematology and the International Committee on Thrombosis and Haemostasis have agreed on recommendations which are based on the results of international collaborative studies, published in the scientific medical literature [1 – 2] and in the proceedings of a workshop on thromboplastin calibration [3]. The recommendations are in conformity with those made by WHO using reference thromboplastins [4, 5] based on the primary international reference preparation BCT/253 [6].

It is proposed that manufacturers of thromboplastins used in oral anticoagulant control should indicate the relationship of each batch of their material to the WHO international reference preparation by a number which describes the comparative slope (c). This is at present referred to by WHO as the international sensitivity index (ISI) [4]. They should also provide a table or graph showing the relationship between the conventional terms of expression of results of the PT test and the International Normalized Ratio (INR). The INR is calculated by the equation: $INR = R^c$, where R is the PT ratio (patient PT: mean normal PT) and c is the comparative slope of the prothrombin used. Examples of how to present the relationship are given in Figs 1 and 2.

This does not preclude the development or implementation of plasma, synthetic substrates or other methods of PT standardization or oral anticoagulant control in the future. In calculating the NRI it is important to consider the effect(s) of pre-test variables and the type of instrumentation used in the PT determination.

Users of commercially available thromboplastin preparations are urged to follow the manufacturers' recommendations for use of the slope (c) of the thromboplastin to calculate the INR, and in patients receiving oral anticoagulant therapy to include this measurement along with their traditional measurement in their reports. This is especially important for the safety of any patient who is likely to be referred to another laboratory where a different modification of the PT test may be used for anticoagulant control. The referring laboratory or physician should provide such patients with the INR value of their tested plasma and

patient's prothrombin time: 18 sec
normal prothrombin time: 12 sec

ISI = 2.3

prothrombin time ratio	PT index	percent activity	INR
1.0	100	100	1.0
1.1	91	74	1.2
1.2	83	57	1.5
1.3	77	48	1.8
1.4	71	41	2.2
1.5	67	35	2.5
1.6	62	31	2.9
1.7	59	28	3.4
1.8	56	25	3.9
1.9	53	23	4.4
2.0	50	21	4.9
2.1	48	20	5.5
2.2	45	18.5	6.1
2.3	43	17.4	6.8
2.4	42	16.4	7.5
2.5	40	15.4	8.2
2.6	38	14.6	9.0
2.7	37	13.9	9.8
2.8	36	13.2	10.7
2.9	34	12.6	11.6
3.0	33	12.0	12.5

Fig. 1. Example of a manufacturer's table for translating a patient's PT into INR. The PT index is defined as $\frac{100}{\text{PT ratio}}$. Percent activity is defined as the concentration (in %) of normal plasma diluted in physiological saline. In this example the INR is obtained by $INR = (\text{PT ratio})^{2.3}$. Note that this example is valid only for one particular batch of thromboplastin. Different values will be found for other batches and brands

also, for the present, with the usual measurement (seconds, ratio, index, percentage activity) used in each laboratory where their plasma is tested.

Clinicians and investigators are urged to take into consideration INRs when dealing with intensity of oral anticoagulation. Editors and reviewers of scientific papers are urged not to accept the expression of the PT which is given only in traditional terms.

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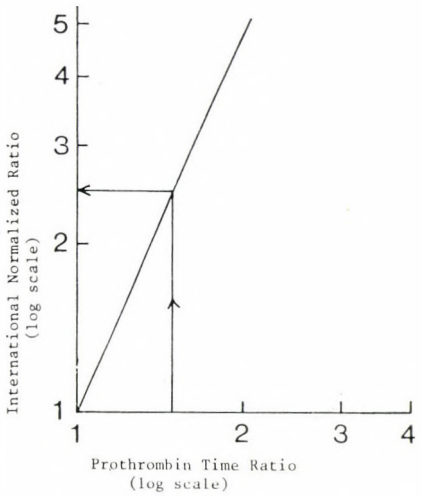

Fig. 2. Example of a manufacturer's graph for translating a patient's PT ratio into INR. In this example the value of comparative slope c is 2.3. The straight line represents the equation: log INR = log (PT ratio) × 2.3, which is equivalent to INR = antilog [(log PT ratio) × 2.3] or $(PT\ ratio)^{2.3}$. Note comment in Figure 1

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Book Review

Immunopharmacology of Endotoxicosis: Proceedings of the 5th International Congress of Immunology, Satellite Workshop, Kyoto, Japan, August 27, 1983. Editors M. K Agarval and M. Yoshida. Walter de Gruyter, Berlin—New York, 1984. 376 pp., 90 figs, 87 tabs, hard cover.

Reading the endotoxin literature of recent years, one could have the impression that interest in this topic has been decreasing. This book, however, seems to prove just the opposite. The beneficial effects of endotoxins, including nonspecific increase of the resistance to various sorts of infections, induction of interferon, antitumour activity, adjuvanticity, immunogenecity and radioprotection have been encouraging to more and more specialists in this field. In addition the study of tumor necrotizing activity of bacterial lipopolysaccharides may also help to clarify the nature of the tumor cell, which in turn could lead to some form of cancer therapy. The book — dedicated to Professor L. Joe Barry — contains 22 papers presented at the Satellite Workshop of the 5th International Congress of Immunology, in Kyoto. The papers cover tissue localization and cellular effects of endotoxins, metabolism of macrophages after endotoxin administration, LPS-induced specific and non-specific immune responses in different mouse strains, diagnostic and therapeutic uses of endotoxins in human patients and experimental animals, activation of complement-system, endotoxins and hemostasis, the role of endotoxemia in human and eyperimental pathology. The Japanese, American, Canadian, Italian, Dutch and Hungarian authors include some new informations and the papers are well documented. General discussion was divided into four subjects. This book will certainly be of interest to pathologists, pharmacologists and clinicians.

Pál Kertai

Abstracts

Rapid internalization of the transferrin receptor in K562 cells is triggered by ligand binding or treatment with a phorbol ester. R. D. Klausner, J. Harford and J. van Renswoude (Laboratory of Biochemistry and Metabolism, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda MD). *Proc. Natl. Acad. Sci. 81*, 3005 (1984)

Treatment of human K562 cells with 4β-phorbol 12-myristate 13-acetate (PMA) resulted in an approximately 50% reduction in cell surface transferrin receptors within 30—45 min as judged by binding of both ligand and antireceptor antibody. The affinity of the remaining surface receptors for diferric transferrin appeared to be unaltered. The time-dependent loss in transferrin receptors was also dependent upon PMA concentration, with a half-maximal effect observed at approximately 1 nM. The kinetic parameters for the binding, internalization, intracellular residency, and recycling of ^{125}I-labelled transferrin were unchanged by PMA treatment, as were the rate and extent of internalization of anti-receptor antibody. Moreover, despite the decrease in surface receptors, uptake of ^{59}Fe from transferrin proceeded at a rate comparable to that seen in untreated cells. Accounting for this observation was the fact that the ligand induced a reduction in surface receptors in untreated but not PMA-treated cells. Quantitative immunoprecipitation of transferrin receptors from surface-iodinated K562 cells revealed that little receptor internalization occurred in untreated cells in the absence of ligand, but internalization of ligand-occupied receptors in these cells was readily detected. In contrast, PMA treatment resulted in the rapid internalization of surface receptors irrespective of occupancy. Thus, binding of ligand appeared to trigger the internalization of receptors that were relatively static in their unoccupied state, and a signal for receptor internalization was also provided by PMA treatment. The possibility that this signal involves phosphorylation of the transferrin receptor is discussed.

A. Egyed

Kinetics of the removal of ferric ion from transferrin by aminoalkylphosphonic acids. W. R. Harris (Laboratory for Energy-Related Health Research, University of California, Davis, CA). *J. Inorg. Biochem 21*, 263 (1984).

The kinetics of ion removal at 25 °C in 0.1 M Tris, pH 7.4 by a series of phosphonic acids have been evaluated. The initial rate of iron removal is first order in ferric-transferrin, but shows a hyperbolic dependence on the concentration of the phosphonate ligand. At high ligand concentrations the reaction is clearly biphasic, and the data are interpreted in terms of nonequivalent rate constants for iron removal from the two transferrin iron-binding sites. Rate constants for three phosphonic acid ligands are ~ 0.025 min^{-1} and ~ 0.007 min^{-1} for the faster and slower binding sites. The results are discussed in relation to the conformational change mechanism for iron removal from transferrin.

A. Egyed

Effect of an anti-murine transferrin receptor-ricin A conjugate on bone marrow stem and progenitor cells treated in vitro. J. Lesley, D. L. Domingo, R. Schulte and I. S. Trowbridge (Department of Cancer Biology, The Salk Institute for Biological Studies, San Diego, CA). *Exp. Cell Res. 150*, 400 (1984).

A monoclonal antibody with specificity for murine transferrin receptor was conjugated with the toxic A subunit of ricin, The dose range, specificity, and kinetics of inhibition of protein synthesis of the conjugate were determined on the murine T-lymphoma cell line, BW5147. When toxin was present throughout the period of culture in vitro myeloid (CFUc) and erythroid (CFUe and BFUe) bone marrow colonies were inhibited by doses of conjugate comparable to those that inhibit protein synthesis in murine cell lines (IC_{50} of 5×10^{-11} M). Bone marrow exposed briefly (30 min to 6 h) to antitransferrin receptor antibody-ricin A conjugate was assayed for myeloid (CFUc) and erythroid (CFUe and BFUe) progenitors in vitro and for in vivo spleen colony formation (CFUs). Only CFUe were depleted by this pulse exposure, consistent with the higher frequency of proliferating cells and transferrin receptor expression in the CFUe population relative to other progenitors.

A. Egyed

Inhibition of erythrocyte membrane shape Change by band 3 cytoplasmic fragment. D. P. Carter and G. Fairbanks (Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Mass.). *J. Cell Biochem. 24*, 385 (1984).

The ATP-dependent transformation of crenated white human erythrocyte ghosts into smoothed disc and cup forms is inhibited by the soluble 40—45-kilodalton cytoplasmic portion of the major transmembrane protein, band 3. The band 3 fragment was prepared by chymotryptic treatment of inverted vesicles stripped of peripheral proteins. When present at $\geq$0.2 mg per mg membrane protein (i.e. $\geq$2 mol fragment per mol endogenous band 3), the fragment significantly reduced the rate of shape change but did not alter the proportion of membranes that were ultimately converted into smoothed forms (>90%). The inhibitory activity of the fragment could not be attributed to contamination of the fragment preparation by actin or proteolytic enzymes. ATP-independent shape transformation was not inhibited. The band 3 fragment may compete with endogenous, intact band 3 for an association with the spectrin-actin network required for ATP-dependent smoothing of crenated membranes.

G. Gárdos

The effect of an osmotic pressure gradient and lysophosphatidylcholine on the transient and constant potassium permeability properties of the erythrocyte membrane. S. Eskelinen and I. Bernhardt (Department of Physiology, University of Oulu, Finland and Department of Biophysics, Section of Biology, Humboldt University of Berlin, Berlin, DDR). *Biomed. Biochim. Acta 43*, 947 (1984).

The rate constant of ^{86}Rb efflux and total potassium release from erythrocytes under the influence of lysophosphatidylcholine (LPC) and osmotic pressure gradients were compared. Both osmotic pressure gradients and LPC caused a transient increase in the potassium permeability of the erythrocyte membrane. In hypotonic media without or in the presence of LPC, this sudden increase is completely reversible, especially since the rate constant of rubidium efflux from unhaemolyzed cells — an indicator for the continuous potassium release — remained constant as measured in an isotonic NaCl medium without detergents. The potassium release was more pronounced in the presence of LPC and may have a protective effect against haemolysis. In an isotonic NaCl or sucrose medium, LPC caused a transient potassium release probably due to incorporation of LPC into the membrane and vesicle release, but also an increase in the rate constant of rubidium efflux due to change in the membrane structure connected with vesicle release.

G. Gárdos

The role of ankyrin in shape and deformability change of human erythrocyte ghosts. Y. Jinbu, S. Sato, T. Nakao, M. Nakao, S. Tsukita, and H. Ishikawa (Department of Biochemistry, Tokyo Medical and Dental University, School of Medicine, Yoshima, Bunkyo-Ku, Tokyo; Japan). *Biochim. Biophys. Acta 773*, 237 (1984).

Human erythrocyte membranes (ghosts) from acid-citrate-dextrose preserved blood were digested with trypsin (protein/trypsin = 100 : 1) under hypotonic conditions and then analyzed by SDS-polyacrylamide gel electrophoresis. After digestion for about 20—30 s at 0 °C, only ankyrin had disappeared while other bands including spectrin, actin, band 4.1 and band 3 remained intact. This observation was supported by electron micrographs showing that the horizontally disposed, filamentous structure was a little apart from the lipid bilayer and its components had not been destroyed. In contrast to intact ghosts, treatment with chlorpromazine, or Mg-ATP did not induce any shape change in these trypsin-treated ghosts. The number of transformable cells correlated closely with the amount of remaining ankyrin in the SDS-polyacrylamide gel electrophoresis pattern. Furthermore, the chlorpromazine- and Mg-ATP-induced decreases in viscosity of suspensions of erythrocyte ghosts were also prevented by trypsin treatment for 20—30 s at 0 °C. These findings suggest that ankyrin plays an important role in change in shape and deformability of erythrocyte ghosts. The molecular mechanism of drug-induced shape change and the role of the undermembrane structure in regulating erythrocyte shape and deformability are discussed.

Ilma Szász

Proteolytic cleavages of cytochalasin B binding components of band 4.5 proteins of the human red blood cell membrane. M. R. Deziel and A. Rothstein (Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada). *Biochim. Biophys. Acta 776* 10 (1984).

The putative hexose transport component of band 4.5 protein of the human erythrocyte membrane was covalently photolabelled with [^{3}H] cytochalasin B. Its transmembrane topology was investigated by electrophoretically monitoring the effect of proteinases applied to intact erythrocytes, unsealed ghosts, and a reconstituted system. Band 4.5 was resistant to proteolytic digestion at the extracellular face of the membrane in intact cells at both high and low ionic strengths. Proteolysis at the cytoplasmic face of the membrane in ghosts or reconstituted vesicles resulted in cleavage of the transporter into two membrane-bound fragments; a peptide of about 30 kDa that contained its carbohydrate moicty and a 20 kDa nonglycosylated peptide that bore the cytochalasin B label. Because it is produced by a cleavage at the cytoplasmic face and because the carbohydrate moiety is known to be exposed to the outside, the larger fragment must cross the bilayer. It has been reported that the band 4.5 sugar transporter may be derived from band 3 peptides by endogenous proteolysis but the cleavage pattern found in the present study differs markedly from that previously reported for band 3. Minimization of endogenous proteolysis by use of fresh cells, proteinase inhibitors, immediate use of ghosts and omission of the alkaline wash resulted in no change in the incorporation of [^{3}H] cytochalasin B into band 4.5, and no labeling of band 3 polypeptides. These results suggest that the cytochalasin B binding component of band 4.5 is not the product of proteolytic degradation of a band 3 component.

Ilma Szász

1-Oleoyl-2-acetyl-glycerol (OAG) stimulates the formation of phosphatidylinositol-4-phosphate in intact human platelets. D. de Chaffoy de Courcelles, P. Roevens and H. Van Belle (Department of Biochemistry, Janssen Pharmaceutica Research Laboratories, B 2340 Boerse, Belgium). *Biochem. Biophys. Res. Comm. 123*, 589 (1984).

Diacylglycerol is one of the primary products formed upon activation of platelets with stimuli that induce inositol lipid turnover. Its synthetic analog, 1-oleoyl-2-acetylglycerol (OAG) is often used as a tool for studying the involvement of the lipid in platelet activation. We found that OAG induces a concomitant increase in [^{32}P]-in-

corporation in phosphatidylinositol 4-phosphate and in the 40K protein, the endogenous substrate for protein kinase C in human platelets. It is supposed that in receptor mediated platelet activation a metabolic link might exist between both processes.

Ilma Szász

ISBT ANNOUNCEMENT

JEAN JULLIARD PRIZE

The 10th Jean Julliard Prize, which was established by the International Socity of Blood Transfusion, in memory of its first Secretary General, will be awarded during the XIX International Congress of Blood Transfusion, to be held in **Sydney, Australia** from **11 to 16 May 1986.**

This prize is reserved for scientists **under 40 years of age** in recognition of recently completed scientific work on blood transfusion and related subjects.

In order to qualify, candidates must forward six (6) copies of an unpublished manuscript or recently published papers including a curriculum vitae to the Secretary General, Doctor Michel Garretta*, before: **the 10th. November 1985**

This prize will be awarded during the congress. The value of the prize is 3,000 Swiss Francs.

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Methods of Strategic Planning for Blood Services*

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(Received 29 November 1984; accepted 21 December 1984)

Among the operations research methods applied to strategic planning problems mixed integer programming has proven to be a very useful framework for a mathematical formulation of the important strategic relations in blood services. A case study shows how the method works with mathematical details omitted. The input data and the results are illustrated and discussed.

Keywords: blood services, strategic planning, mixed integer programming

Introduction

For the sake of simplicity only one regional centre (RC) will be considered with several hospitals ($H_1, \ldots, H_m$). For these organizational units Figure 1 shows the flow of information and blood products. The following remarks and especially the method used for the case study can also be applied to blood services and their strategic planning problems[1] on national as well as international planning levels.

The blood products to be considered are,

fresh blood/whole blood	(FB/WB)
red cell concentrates	(RCC)
fresh frozen plasma	(FFP)
plasma	(P)
leucocyte concentrates	(LC)
platelet concentrates	(PC)

* The strategic model of this paper has been developed in cooperation with Prof. Dr. med. A. Hässig, H. Streun and Dr. rer. pol. K. Gander of the Central Laboratories, Blood Transfusion Service, Swiss Red Cross in Berne, Switzerland. Further experience could be collected by a project of the Swiss National Funds on the disposition of blood products in regional centres. This project has been performed in cooperation with Dr. med. H. P. Burri, Basel, Dr. med. F. Kern, St. Gallen, and Dr. med. B. Wuilleret, Lausanne.

The authors are indebted to Prof. E. Brodheim, Sc. D., Vice President, The New York Blood Center, New York, N. Y., for some inspiring comments to an earlier version of this paper.

[1] For an introduction in planning problems of this type compare e.g. Hahn and Taylor [3], Hanssmann [4], pp. 253–401, and Naylor [7].

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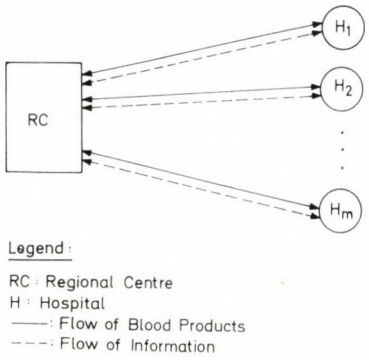

Fig. 1

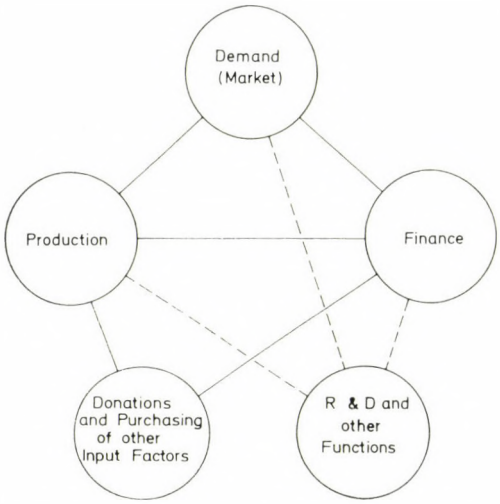

Fig. 2. Interdependences of Economic Functions

These products are the result of joint production processes, derived from the common source material "whole blood donation". In this case strategic planning has to be done by a simultaneous calculus which allows the consideration of feedback relations between different activity levels of final, intermediate and input products. Further it is important to note that in blood services the demand has to be met immediately, i.e. for strategic as well as operational planning no delay of deliveries is permitted.[2]

Most strategic planning procedures used today go step by step through sales and marketing, production, purchasing and finance. However, these economic functions are interconnected by feedback relations according to Figure 2, so that

[2] Compare, e.g. Hadley and Whitin [2].

under this aspect too simultaneous planning procedures have to be applied. During the last years experiences with simultaneous planning performed in profit-oriented companies have shown surprising improvements of efficiency measures – e.g. of the return on investment (ROI).

In future the necessity of strategic planning in blood services will increase due to the following reasons:

- changes of demand
- new products
- new production processes
- stagnation of the growth rates of donations
- alternative input factors (e.g. artificial blood substitutes)
- more financial risk by the increasing capital intensity of investments

Overview of Methods

Methods primarily used for strategic planning are:

- Heuristics[3]
- Econometric Methods[4]
- Simulation[5]
- Mathematical Programming with
  - . Linear Programming[6]
  - . Mixed Integer Programming[7]

Heuristics in most cases do not produce the optimal solution. So they should be applied only to those problems, where the calculation of exact solutions is not possible with acceptable cost.

Econometric methods are sometimes applied with success. Because of statistical constraints, however, an analysis is possible only for a limited number of variables, i.e. for complex strategic problems these methods are not suitable.

Simulation and mathematical programming offer better possibilities in this respect. Simulation requires the formulation of a mathematical model which gives a "picture" of the reality to be analysed, e.g. of the strategic planning situation of a regional centre. Then the interdependences of the variables in the model can be worked out by experiments, e.g. the interdependences of outdating, shortages and costs. For strategic planning of a regional centre Figure 3 shows a scheme for a simulation.

[3] See e.g. Krajewski and Thompson [6], pp. 384–388

[4] See e.g. Intriligator [5]

[5] See e.g. Krajewski and Thompson [6], pp. 370–409; and Schober [9].

[6] See e.g. Krajewski and Thompson [6], pp. 66–165; and Williams [10], pp. 18–119.

[7] See e.g. Coate [1]; Krajewski and Thompson, [6] pp. 212–247; Popp [8]; and Williams [10], pp. 138–214.

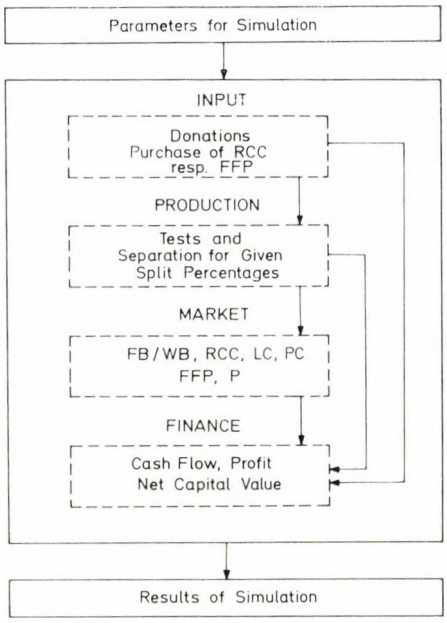

Fig. 3. Scheme for Simulation of a Regional Centre

The input factors go to production including testing and separation of donations. Then the final products go to the market. All stages have an influence on cash flow, profit and capital value. Simulation models are, however, not very suitable to consider feedback relations between variables.

A different situation arises with mathematical programming procedures, especially with mixed integer programming. The decision variables in models of this type can be continuous or integer-valued (i.e. belonging to the set of numbers 0, 1, . . .). In a strategic plan the continuous variables can represent such quantities as sales, intermediate products, input factors, capacity utilization and cash flow. Each integer variable in the model, e.g. with possible realization 0 and 1, can describe the no or yes state for a particular decision (e.g. an investment in production or in a certain marketing activity). So for production it can be of interest to find out in a dynamic planning horizon in which years and to what extent expansions of production should be scheduled. Decisions of this type must reflect relevant physical capacity factors and the financial consequences for cash flow and capital value. A mixed integer programming model consists of an objective function and a set of restrictions. The objective function defines an efficiency measure – e.g. capital value, profit or ROI – as a function of the decision variables and requires the optimization of that measure. In the set of restrictions the decision variables are submitted to the structural conditions of the problem,

defining, for example, the limitations of production and sales and the restrictive aspects during expansion steps.

The next section is devoted to a case study that illustrates the application of mixed integer programming for strategic planning of a regional centre.

Case Study

1. *Overview*

The decision variables of the case study are,

- quantities of final products
- steps of expansion for production capacities
- quantities of donations and other input factors

For an optimization of the decision variables the net capital value is the efficiency measure, and the restrictive aspects of the study are,

- demand has to be met between lower and upper bounds
- production is possible only within available capacities
- the existing production capacities can be expanded stepwise by investments
- for each year the revenues have to be larger or equal to the expenses
- costs and profits can be limited by bounds
- inputs, production and investments can be limited

Figure 4 shows a scheme allocating the decision variables to the restrictive system and to the objective function.

2. *Structure and Data*[8]

a. *Planning Horizon.* The planning horizon of the case study is eight years. It is split up into five time intervals, where the first three intervals are each of one year duration, the fourth and fifth have a size of two and three years, respectively. (Compare top part of Table 2.)

b. *Interdependence of Products.* Figure 5 shows the interdependences of the final, intermediate and input products. p_d represents the percentage of donations which cannot be used for production of FB/WB, RCC and LC, but for the production of PC, FFP and P. p_w gives the percentage of wastage for FB/WB and RCC. In the example we have $p_d = 0.15$ and $pw = 0.10$.

c. *Capacities.* Three types of capacity are considered: donor-rooms, laboratory[9] and plasma separator.[10] Table 1 shows the data for the existing capacities

[8] The data of this example are hypothetical – but not very far away from typical reality.

[9] In this example we assume automated processing based upon the capacity of the Groupamatic 360 device by Kontron.

[10] This covers the equipment and the operations that are necessary to centrifuge whole blood and to siphon off plasma.

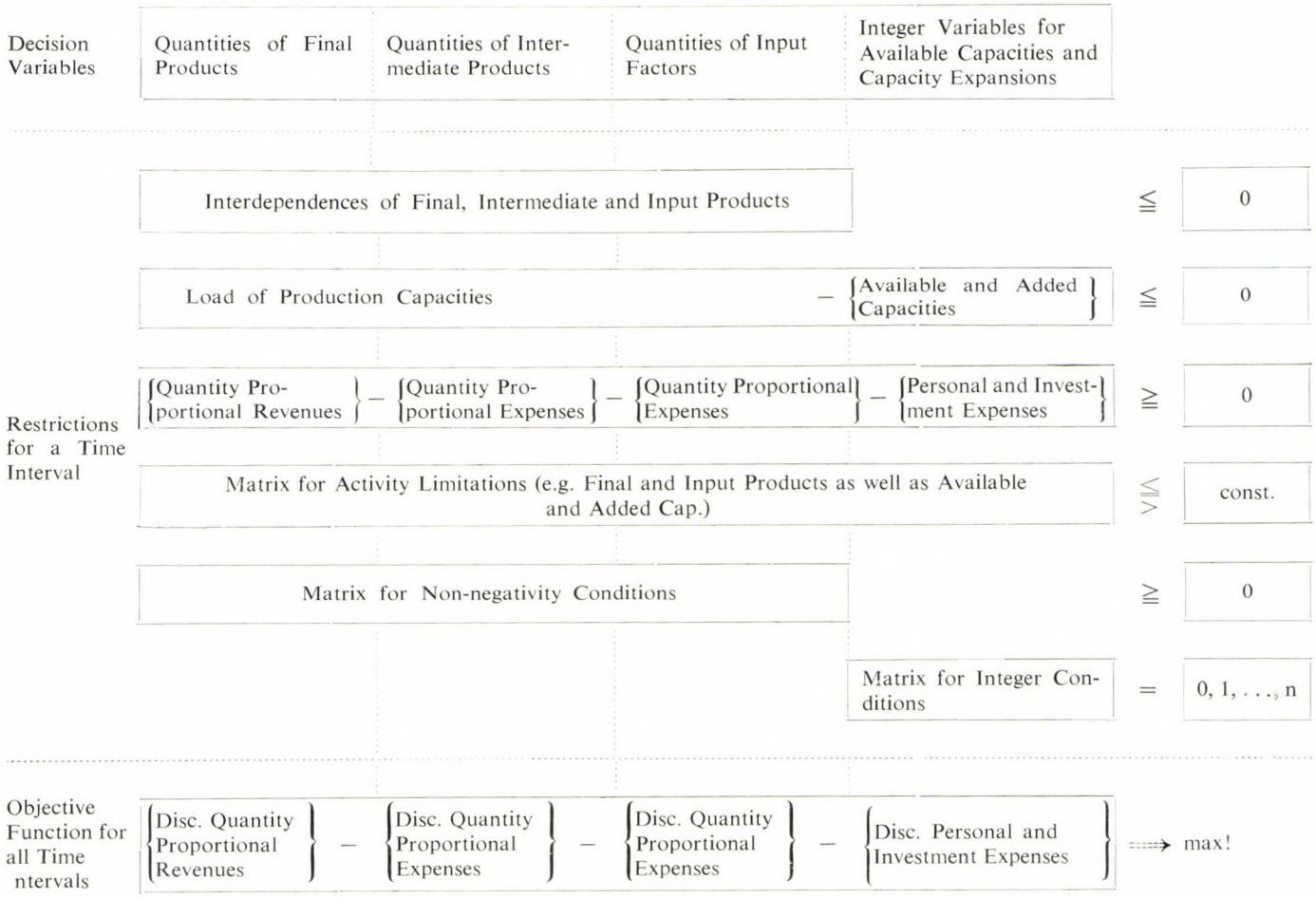
Decision Variables
Quantities of Final Products
Quantities of Intermediate Products
Quantities of Input Factors
Integer Variables for Available Capacities and Capacity Expansions
Interdependences of Final, Intermediate and Input Products
≤ 0
Load of Production Capacities
– Available and Added Capacities
≤ 0
Restrictions for a Time Interval
Quantity Proportional Revenues
– Quantity Proportional Expenses
– Quantity Proportional Expenses
– Personal and Investment Expenses
≥ 0
Matrix for Activity Limitations (e.g. Final and Input Products as well as Available and Added Cap.)
≤ ≥ const.
Matrix for Non-negativity Conditions
≥ 0
Matrix for Integer Conditions
= 0, 1, . . ., n
Objective Function for all Time ntervals
Disc. Quantity Proportional Revenues
– Disc. Quantity Proportional Expenses
– Disc. Quantity Proportional Expenses
– Disc. Personal and Investment Expenses
⟹ max!

Fig. 4

Table 1

Dominant Capacity Restrictions

	Donor Rooms	Laboratory (Groupamatic)	Plasma Separator
Number of available units	6	1	1
Maximum number of additional units	3	/	1
Maximum number of shifts per unit and day	1	3 resp. 9 (of 1/3-shifts)	3
Maximum load by donations per shift and year	26,400	85,800 resp. 28,600 (per 1/3-shift)	50,600
Expenses for employees per shift and year (sFr)	70,000	63,000 resp. 21,000 (per 1/3-shift)	54,400
Expenses for leasing of an additional unit per year (sFr)	10,000	/	/
Investment expenses of an additional unit (sFr)	/	/	35,000

Table 2

Forecasts of Expenses and Revenues [sFr]

Years in Planning Horizon		1982	1983	1984	1985/86	1987/88/89
Time Interval		1	2	3	4	5
Activity	Unit					
Expenses						
Donation	1 Donation	31	33.2	35.5	39.3	46.5
Purchase of RCC	1 RCC	37	37	41	41	41
Purchase of FFP	1 FFP (220 ml)	45	45	50	50	50
Production of RCC	1 RCC	8	8.4	8.8	9.5	10.7
Revenues						
Sales of FB/WB	1 FB/WB	65	65	70	70	70
Sales of RCC	1 RCC	42	42	46	46	46
Sales of LC	1 Buffy coat	52	51.6	61.2	60.5	59.3
Sales of PC	1 PC	27	26.6	36.2	35.5	34.3
Sales of FFP	1 FFP	50	50	55	55	55
Sales of P	1 l	75	75	75	75	75

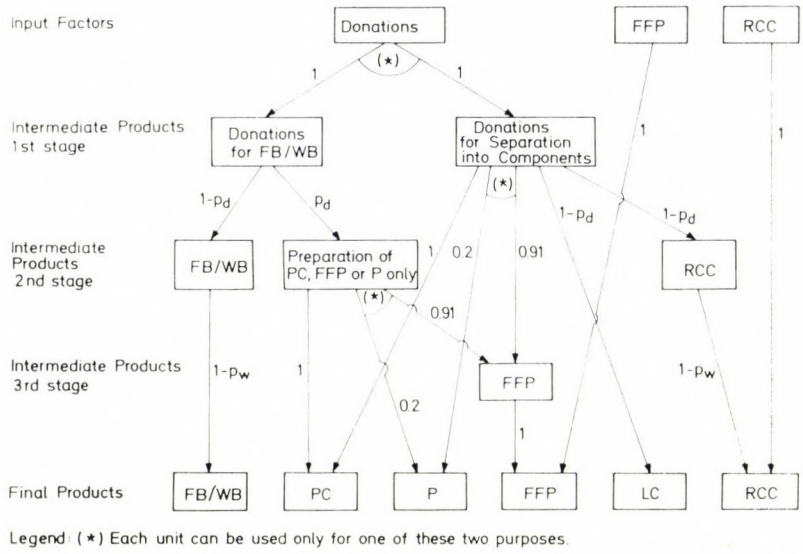

Fig. 5

Table 3

Forecasts of Upper (U) and Lower (L) Bounds

Activity	Unit	Time Interval 1	2	3	4 (per year)	5 (per year)
Donation: U	1 Donation	207,000	207,000	207,000	207,000	207,000
Purchase of RCC: U	1 RCC	2,400	3,200	4,000	5,600	8,000
Purchase of FFP: U	1 FFP (220 ml)	1,600	2,400	3,200	4,800	6,400
Sales of FB/WB: L	1 FB/WB	54,000	48,000	43,500	39,000	28,000
Sales of FB/WB: U		64,000	60,000	54,000	49,000	36,000
Sales of RCC: L	1 RCC	66,000	72,000	80,500	91,000	112,000
Sales of RCC: U		80,000	90,000	101,000	115,000	144,000
Sales of LC: L	1 Buffy coat	2,800	3,200	4,000	5,600	8,000
Sales of LC: U		4,800	6,400	8,000	10,400	12,800
Sales of PC: L	1 PC	40,000	38,000	37,000	35,000	35,000
Sales of PC: U		48,000	46,000	45,000	45,000	45,000
Sales of FFP: L	1 FFP	11,000	11,500	12,000	10,000	7,000
Sales of FFP: U		14,000	15,000	16,000	14,000	12,000
Sales of P: non restricted	1 l	/	/	/	/	/

and the possibilities of expansions. E.g. the six available donor-rooms can be expanded by up to three rooms by leasing with yearly costs of 10,000 sFr. per room. The existing capacity for plasma separation is one separator and one additional separator can be installed with costs of 35,000 sFr. For the laboratory with a capacity of one Groupamatic there is no opportunity for increase.

The available capacities for handling donations and the expenses for employees depend on the integer number of shifts (or 1/3-shifts for the laboratory) that will be scheduled. In addition it is assumed that the expenses increase by an inflation rate of 5% per annum.

d. *Expenses and Revenues of the Products.* The forecasts for expenses and revenues in the planning horizon are listed in Table 2. For calculation of the capital value a discount rate of 10% per annum is assumed.

e. *Activity Limitations.* Forecasts for upper and lower bounds of input factors and of sales volumes of final products are listed in Table 3.

They are to a large extent based on the development of the demand for FB/WB and RCC from 1977 to 1981 and forecasts from 1982 to 1989 in Switzer-

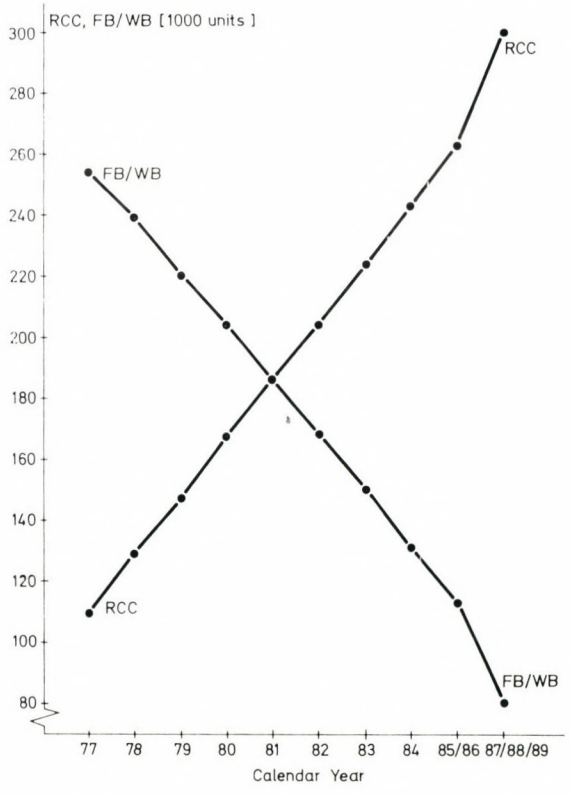

Fig. 6

land, a country with approximately 6 million inhabitants (see Figure 6). The trends (decreasing FB/WB and increasing RCC with a slight increase of the sum of both) can be seen as a consequence of the component therapy and the stabilization of the demand for blood transfusions – especially for red cells.

Results

The results show the development of purchasing, production (including the expansion of production capacities), sales and of financial aspects.

a. *Sales Projections.* The sales of FB/WB on the one hand and the sales of RCC plus FFP or P on the other hand determine donations and production activities.

The sales quantities of FB/WB in each year are equal to maximum possible values. For RCC the sales quantities in the years 1982 – 1986 lie between the lower and upper bounds of the forecasts. And from 1987 to 1989 the quantities go down to the lower bounds (see Figure 7).

LC and PC always reach their maximum sales quantities; but for them only part of the available donations is necessary. The available plasma resources are

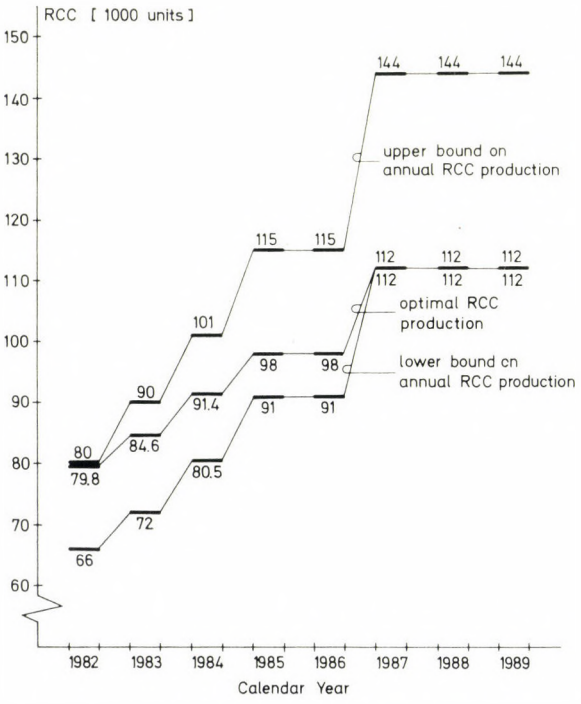

Fig. 7. Optimal RCC Production

Table 4

Causes of Restrictions

	1982	1983	1984	1985/86	1987/88/89
FB/WB			SALES		
RCC	Economics (of a second additional donor-room)				Economics (of Don. Supply)
LC					
PC			SALES		
FFP					
P	Economics (of a second additional donor-room)				Economics (of Don. Supply)

completely exhausted for sales of FFP (at the assumed upper limit) and P (non restricted).

b. *Limiting Factors.* Table 4 summarizes the factors which are limiting for the sales.

As mentioned above, the output of FB/WB, LC, PC and FFP is limited by the predicted sales potentials. Another situation can be observed for RCC and P:

i) Within the first four time periods (i.e. for the planning horizon except the last time interval) the production of RCC and P is limited by the capacity of seven donor-rooms (six existing and one additional). The other types of capacity show surplus capacities. So in the laboratory only seven 1/3-shifts are scheduled (out of nine) but not fully utilized and at the plasma separator for three shifts some idle capacity appears, too. The limitation to only one additional donor-room can be explained as follows. If a second donor-room would be added, the expenses for employees and leasing would not be covered by the additional net revenues for RCC and P. Furthermore, this capacity expansion could not be completely used without an additional 1/3-shift in the laboratory and a second plasma separator.

ii) In the last time interval the RCC and P output is restricted not by the capacity of seven donor-rooms but by economic factors. The reason for this result can be seen in the high expenses for an additional donation and for the production of the corresponding units of RCC and P; they exceed the attainable revenues. Therefore, the sales quantities of RCC and P go down to the lower bounds of the sales projections for RCC.

c. *Financial Results.* The projected net capital value (discounted at the rate of 10% per annum) and the required yearly cash flow are illustrated in Figure 8 for each planning period.

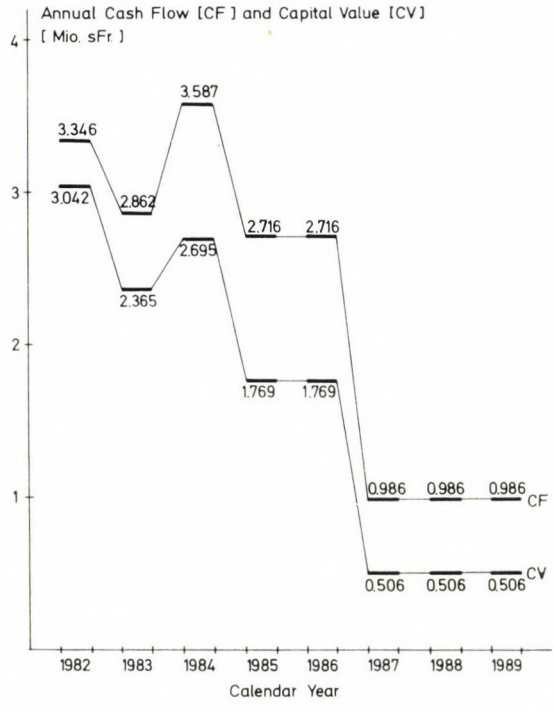

Fig. 8

The curve of the annual cash flow is strongly influenced by increasing costs (5 resp. 7% per year) and an increase of the prices in 1984 (compare Table 2). In the net capital value the development of cash flow can be observed with respect to the growing discount factors.

Discussion

A strategic planning exercise must be done in phases. Initially a relatively simple model is often used to establish confidence in the modelling process. This serves to identify areas where the data or model do not conform with the knowledge of persons familiar with the enterprise. The cause of these discrepancies can then be identified and corrected. Once confidence in the modelling procedure has been established, the implications of the model output requires significant examination and discussions between the modellers and the management to insure correct interpretation and to formulate new lines of inquiry that are suggested by the results. This will often require an extension of the possibility to be explored by the model and lead to a further round of evaluation.

This process will finally converge on a situation where management feels that they have a tool that is satisfactory in its details and provides outputs that can effectively be used in planning. The outputs need also to be reduced to standard graphs and tables, such as those illustrated in the above example, whose interpretation is well understood.

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Isoenzymes in Plasma of Acute Leukaemia Patients: Prevention of Anthracycline Cardiomyopathy

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During the diagnosis and subsequent monitoring of patients with acute leukaemia, and of those with terminal blastic crisis in chronic myelogenous leukaemia, the activity of total LDH, ALAT, AP, ASAT as well as the isoenzymes LDH-H, LDH-M were measured in the plasma.

Enzyme and isoenzyme activity of LDH, which differed in quantity at the various times of measurement, reflected the varying proliferation rate, depending upon the individual and upon the time of measurement and, consequently, the tumour cell mass.

Keywords: Haematology, isoenzyme determination, acute leukaemia, progress monitoring

Introduction

Experimental studies have shown that the high percentage of LDH-H (electrophoretic LDH_{1-2}) as compared to the total activity of lactate dehydrogenase (LDH), decreases during their maturation due to a growing synthesis of the LDH-M (electrophoretic LDH_5) isoenzyme. The result is a share of LDH-H in mature granulocytes between 50 and 65% [1–3], whereas myeloblasts, in acute leukaemia, revealed a very high percentage of LDH-H in the total activity of LDH as compared with normal cells.

A shorter life span of immature cells and treatment with antineoplastic agents increase the turnover of these cells, and therefore results in a release of LDH-H into plasma.

The aim of the present series was to study the isoenzymatic pattern in the plasma of a group of patients during the various stages of acute myelogenous leukaemia (AML) in order to make assertions about the development of the disease at a specific stage. Acute lymphatic leukaemia (ALL) patients and patients being in the terminal blastic crisis of chronic myelogenous leukaemia (CML) were also studied.

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Methods

In 18 patients aged 16–43 (average 22 years) the diagnosis was supported cytologically by a bone marrow puncture. Thirteen of them had AML (M2–M4), four ALL (L1, L2), and one erythroleukaemia (M6) according to the FAB-classification [4]. Four other patients (aged 16–62 years) were in the terminal blastic crisis of CML.

To assess the individual stages of the disease, the patients were divided in groups before therapy:

I. *Patients with acute leukaemia*

a) with a great number of peripheral leukocytes and a high percentage of blasts in the differential blood count,
b) with a normal or low number of peripheral leukocytes and a high percentage of blasts in the differential blood count,
c) with a normal or low number of peripheral leukocytes and no or sporadic blasts in the differential blood count *during observation.*

II. *Patients with terminal blastic crisis*

These patients are comparable with group I.

All venous blood samples were drawn under the same conditions, i.e.
- prior to the start of the first polychemotherapy
- during the polychemotherapy daily over a period of at least three days and at the beginning of a new course of treatment.

Plasma was separated immediately after blood sampling, rapidly frozen and stored at −20°C until analysis.

In each case the isoenzymes of LDH (LDH-H and LDH-M) (reference range: $x \pm 2S$; LDH 26.5–52.5 U/l, LDH-H 11.5–36.5 U/l, LDH-H/LDH-M 1.0–2.1) were analysed. The results were compared with the enzyme activity of alanine aminotransferase, alkaline phosphatase, cytosolic and mitochondrial aspartataminotransferase and gamma-GT. The creatine kinase-MB enzyme activity was determined only in patients with strongly elevated LDH-H.

After diagnosis polychemotherapy was initiated immediately in the acute stage. According to the diagnosis, most patients received a TAD regimen (thioguanine, alexan, daunorubicin) and few a COAP regimen (cyclophosphamide, oncovin, alexan, prednisone), administering individual doses of daunorubicin on the average of 40 mg/m^2 body surface [5, 6] and also the calcium antogonist verapamil for prophylactic treatment of anthracycline cardiomyopathy.

Patients in complete remission received a COAP regimen at intervals of three months.

Results

First treatment

Group Ia patients with an elevated number of peripheral leukocytes and blasts (Fig. 1) showed a 2.5–35fold elevation of LDH and LDH-H activity as compared with the reference range before the start of polychemotherapy, whereas the normoleukaemic patients of groups Ib and Ic (Fig. 2) showed no significant elevation of LDH-H values.

After the beginning of polychemotherapy, LDH and LDH-H increased in each patient of group I. However, the quantitative change of the enzyme activity in group Ia with elevated enzymes was less than in patients with a normal number of peripheral leukocytes.

Increased plasma activity of LDH-H after the start of polychemotherapy could be seen in various subjects. In subgroup Ia patients the LDH-H levels had been markedly elevated before polychemotherapy, increased to a multiple in the first two or three days of polychemotherapy, then decreased significantly and approached the reference range towards the end of the first course of treatment. In subgroup Ib and Ic the increase of enzyme activity persisted only during the first two or three days after which LDH-H values were within the reference range.

At the beginning of the second course of treatment of first-time patients this subgrouping was no longer valid. Only few patients (n = 3) showed increased peripheral leukocyte values, whereas the percentage of blasts in the differential

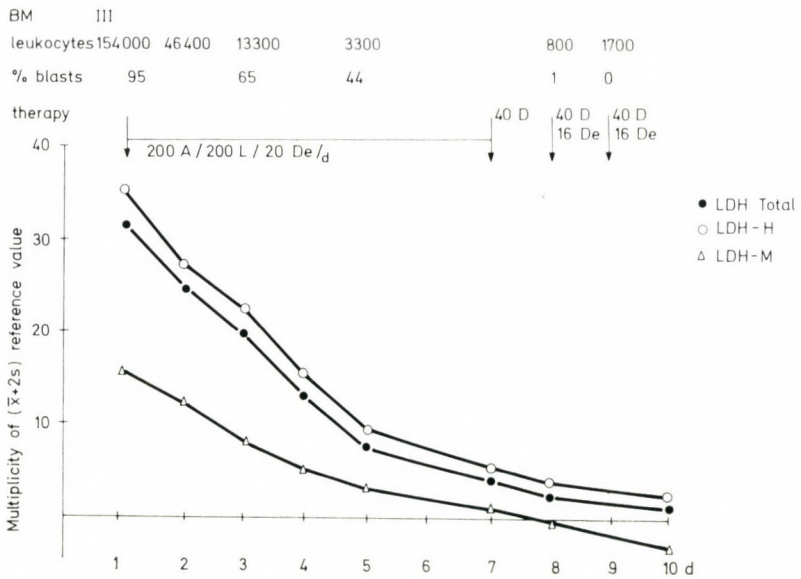

Fig. 1. Monitoring of LDH isoenzyme pattern in a first-time acute myelogenous leukaemia patient (M. R., aged 18), subgroup Ia

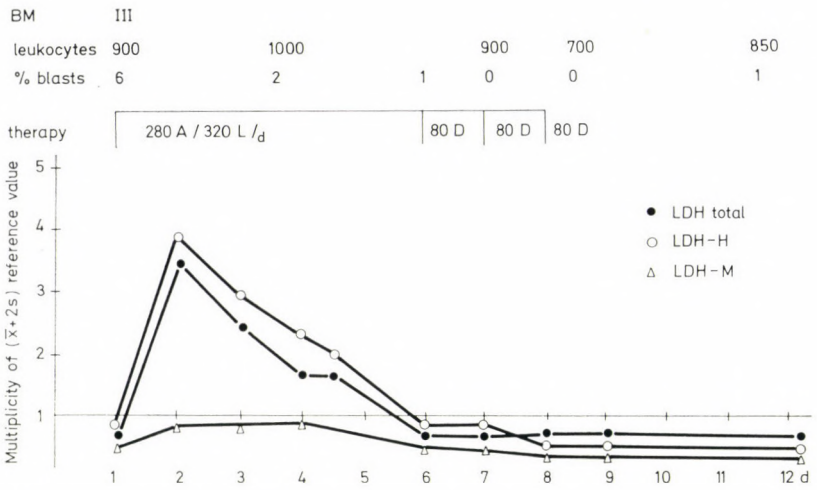

Fig. 2. Monitoring of LDH isoenzyme pattern in a first-time acute myelogenous leukaemia patient (T. S., aged 17), subgroup Ic. Therapy: first-time treatment with thioguanine and alexan (over 7 days) and daunorubicin

blood count either decreased considerably or, in most cases, disappeared. Consequently, the response to polychemotherapy could not anymore be distinguished for those groups.

Second treatment

Before resuming polychemotherapy, an elevated (up to 2.5fold) LDH-H activity could be observed in four patients. The rest were within the reference range. In the first two days of polychemotherapy those four patients (subgroup Id) showed an elevation and four more patients (subgroup Ic) a recurrent increase of LDH activity which, however, in the latter four, then decreased to the reference range, whereas in the first four patients enzyme activity also decreased but did not reach the reference range at the end of treatment.

During the third course of treatment only three subgroup Id patients did still show an increase of LDH-H activity.

If we compare the results of bone marrow puncture after the second and third courses of treatment with the LDH-H activity changes in the various stages of therapy, it could be observed that those patients who showed no change of LDH-H activity during the second and third courses of treatment had already achieved complete remission. The four subgroup Ie patients finally also achieved a complete remission (according to the bone marrow finding) after the third course of treatment. Further intensive treatment of sugroup Id patents even after the third course was found to be necessary, since no complete remission of the basic disease had been obtained.

Additional determination of isoenzymes ASAT-C and ASAT-M showed no change of enzymatic activity during polychemotherapy, nor did the enzymes ALAT, alkaline phosphatase and gamma-GT show any change that could have been attributed to some specific stage of the disease. Only alkaline phosphatase had increased to twice the upper limit of its standard value in three subgroup Ie patients, but continued to normalize under polychemotherapy.

In the state of complete remission (Fig. 3) no change of LDH-H activity was found during reinduction therapy confirmed by long-term bone marrow

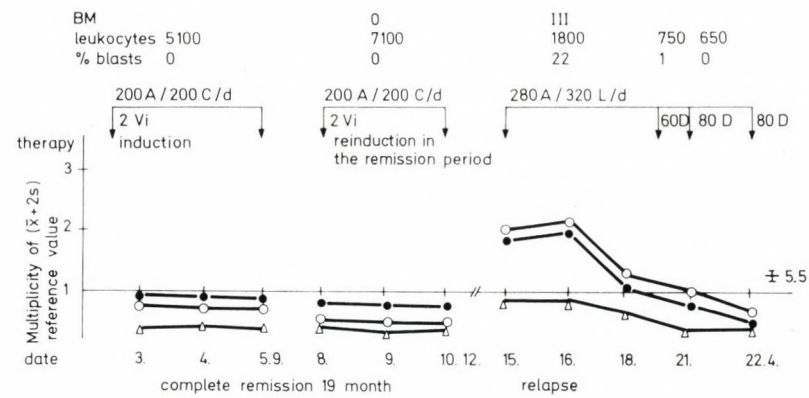

Fig. 3. Monitoring of LDH isoenzyme pattern during reinduction therapy at complete remission of an acute erythroleukaemia and during first relapse (Patient R. L., aged 29)

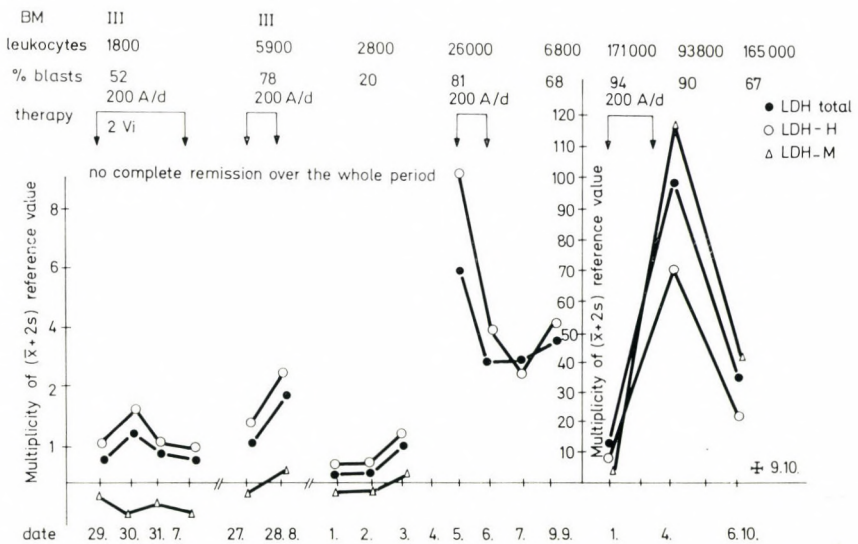

Fig. 4. Monitoring of LDH isoenzyme pattern of an acute lymphatic leukaemia patient (H. T., aged 27) with neither partial nor complete remission during various stages of treatment

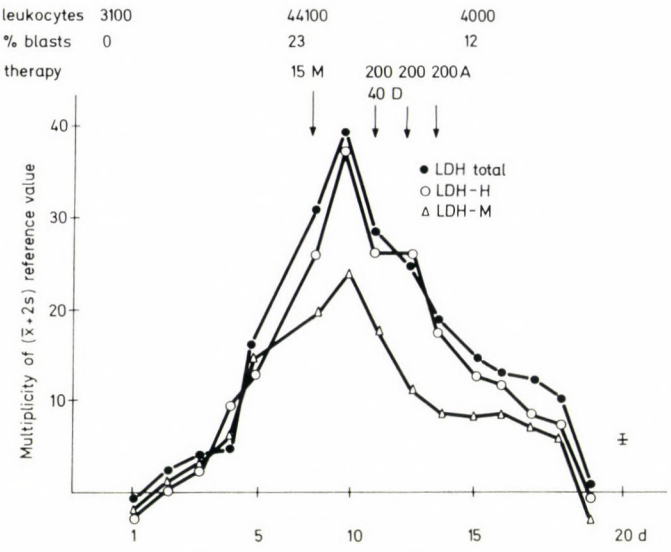

Fig. 5. Monitoring of LDH isoenzyme pattern of a patient (L. B., aged 18) with terminal blastic crisis under chronic myelogenous leukaemia during repeated stages of treatment

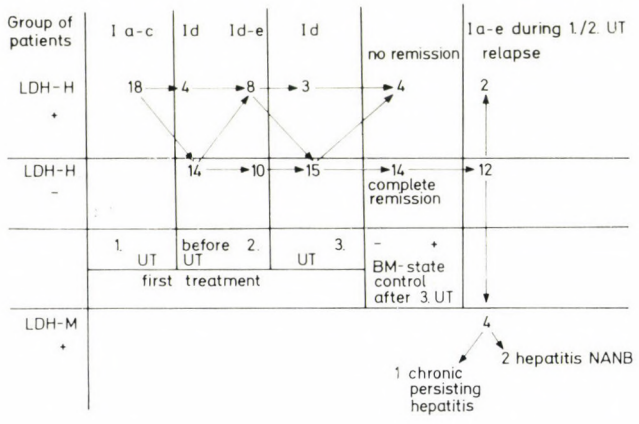

Fig. 6. Patients with acute leukaemia and grouping during first-time treatment and reinduction therapy monitoring

follow-up analyses. In two patients a relapse of acute leukaemia was diagnosed in the therapy-free stage (Fig. 3), in which recurrence of LDH-H activity increases was identified as had been the case in the acute stage of first-time treatment.

Four patients showed increased LDH-M activity as well as a change of the LDH-H/LDH-M quotient which, in three of them, led to an increase in total LDH activity. At first, the LDH-M changes occurred only under reinduction ther-

apy. Changes, however, continued to exist even during the therapy-free interval in three patients. After different periods of time two of them developed non-A-non-B hepatitis, which could be confirmed enzymatically on the basis of a marked increase in total LDH activity and an increase in LDH-M, ALAT and gamma-GT.

Depending upon the state of activity of the basic disease (differential blood count, percentage of blasts) without remission after three treatments (Fig. 4) revealed a quantitatively more or less pronounced elevation of LDH-H even before the new course of treatment, which in the first stage of polychemotherapy continued to increase, but then decreased significantly to reach, at least in some cases, the reference range after polychemotherapy.

The same was the case with patients in the terminal blastic crisis (Fig. 5), where, depending upon the state of activity, a more than 40fold increase in enzyme activity was measured during polychemotherapy.

Discussion

Repeated determinations of the isoenzymes of LDH (LDH-H, LDH-M) supplied additional information to differential blood counts and bone marrow analyses of acute leukaemic patients. This additional information became apparent in certain groups of patients after systematic monitoring.

Patients with high peripheral leukocyte values and a high percentage of blasts in the differential count (Ia) could be divided in those showing a significant, and those revealing a moderate increase of LDH-H. The basis for this distinction was provided by the different proliferation kinetics of immature cells, which became indirectly measurable by increased plasma enzyme activity.

For patients with normal peripheral leukocyte values and no identification of blasts in the differential count (Ic), LDH-H determination under polychemotherapy gave, beside the bone marrow finding, additional information about the responsiveness to chemotherapy of the malignant cells.

Kronberg and Polliak [7] studied first-time patients with ALL and acute non-lymphatic leukaemia only when diagnosing them prior to the first course of treatment. Seventy-five per cent of their ALL patients had an increased total LDH activity (1.8 to 5.1fold of $x + S$); in 25% the increase was moderate, and in four patients suffering from acute blastic crisis total LDH activity was also elevated (1.9 to 3.9fold of $x + S$). Isoenzymes of LDH were determined in only four patients (increased LDH-2 and LDH-3 activities). In general, no significant relationship could be shown for these patients between total LDH activity and the percentage of blasts prior to the institution of therapy.

In contrast to our approach with patients with ALL, AML and terminal blastic crisis, Kronberg and Polliak [7] did not categorize their patients according to the peripheral leukocyte count and its relation to the percentage of blasts prior to therapy. Our categorization of patients followed this principle in assessing LDH isoenzyme activity, disregarding the kind of acute leukaemia as a primary

criterion. The value of LDH isoenzyme determination was limited, because it was only done in a few patients. Monitoring of the various groups of patients including the dynamics of total LDH and isoenzyme activity in the various stages of the disease is illustrated by two examples, but nothing can be said with regard to the whole group of patients or to the individual phases of therapy. However, it is only through the measurement of total LDH or isoenzyme activities in the course of monitoring that the specific information gained from these measurements becomes obvious in combination with other methods.

The fact that in patients in complete remission no change in LDH-H enzymatic activity could be found during polychemotherapy in the framework of reinduction therapy confirms at the same time the assumption that there is a close relationship between the increase of LDH-H and the increased proliferation kinetics of immature cells, and that no other tissue is involved in the LDH-H release even during polychemotherapy. The same holds true for LDH-M.

The increase of LDH-M activity and the associated decrease of the LDH-H/LDH-M quotient and, partly, the increase of LDH total activity constitute specific changes taking place during the observation period which indicate liver parenchyma injury due to the therapy (polychemotherapy, targeted cell substitution) at an early date. Simultaneous determination of other specific enzymes supports this indication.

Repeated determinations of LDH-H enzyme activity in the plasma of acute leukaemia patients in neither partial nor complete remission and of patients with terminal blastic crisis during the follow-up permitted rapid information about the state of activity of the basic disease and, consequently, immediate therapy checkups providing the conditions for an extension of therapy at short notice or for drug substitution.

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Abbreviations

Leuko: leukocytes in peripheral blood count
%Bl: percentage of blasts in differential blood count
BM: bone marrow puncture finding according to stages (group I: 5%, group II: 5–50%, and group III: >50% of blasts out of the total of nucleated bone marrow cells
Vi: vincristin
A: alexan
D: daunorubicin
L: thioguanine (Lanvis)
M: methotrexate
C: cyclophosphamide
De: dexamethason
200 A: 200 mg alexan
ALL: acute lymphatic leukaemia
AML: acute myelogenous leukaemia
UT: unit of treatment
LDH: lactate dehydrogenase

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Inhibitory Effect of Monocyte Reactive Antibodies on Monocyte Chemotaxis in Systemic Lupus Erythematosus

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Presence of different types of autoantibodies is a basic feature of systemic lupus erythematosus (SLE). Though monocytes, macrophages play an important role in cellular immunity, autoantibodies against monocytes have not been sufficiently studied. The authors used automatic fluorochromatic assay to detect monocyte reactive autoantibodies in the sera of SLE patients. Of SLE 35.5% sera showed complement-mediated monocytotoxic activity against healthy monocytes. Monocyte reactive SLE sera as well as monoclonal antibodies against human monocytes inhibited chemotaxis of control monocytes. The results suggest that monocyte reactive autoantibodies may play a role in the decreased monocyte number and defective monocyte functions observed in SLE.

Keywords: systemic lupus erythematosus, monocyte, autoantibodies, chemotaxis, immune complex, cytotoxicity.

Introduction

The presence of different types of autoantibodies in the sera of patients with systemic lupus erythematosus (SLE) is a basic feature of this autoimmune disease. Cytotoxins against lymphocytes were extensively studied and demonstrated in the sera of more than 50% of the patients [13, 15, 16]. There are only few and contradictory data concerning monocyte reactive autoantibodies [1, 13]. In these studies monocyte reactive antibodies were examined by means of dye exclusion tests. Bruning et al. [3] worked out a carboxyfluorescein fluorochromasia assay to detect cell-mediated lympholysis. The leakage properties of the carboxyfluorescein-diacetate made it applicable as a vital stain for lymphocytes in cytotoxicity assays.

In this study the carboxyfluorescein fluorochromasia assay was applied to detect monocyte reactive antibodies in the sera of SLE patients. As in SLE a low number of monocytes was observed in the circulation [2] as well as an impaired monocyte chemotaxis in vitro [9] and in vivo [12], we examined the possible role of monocyte reactive antibodies in these alterations. The effect of monocyte

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reactive antibody positive SLE sera as well as monoclonal anti-human antibody against monocytes on the chemotaxis and spontaneous migration of healthy monocytes were studied.

Materials and Methods

Patients

Sera of 45 patients with SLE were collected. 18 of the patients were in the active, 27 in the inactive clinical stage on basis of the ARA criteria [5]. After clotting at room temperature, the separated serum samples were decomplemented by heating at 56 °C for 30 min, then stored until using them in the experiments. Sera of 35 healthy volunteers served as controls.

Separation of monocytes

Blood from normal donors was drawn in heparin and mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation. The cells were washed twice and resuspended at a concentration of 5×10^6 mononuclear cells/ml in Parker's medium supplemented with 20 mM HEPES. 1.5 ml of this cell suspension was placed into each well of tissue culture plates (Linbro, 3.5 × 1.0 cm bottom wells). After 1 h incubation at 37 °C, the non-adherent cells were removed by washing. Adherent monocytes were detached by adding 2 ml ice-cold Parker's medium containing 20 mM HEPES buffer and 2.5% FCS and leaving the cells at 4 °C for 30 min. The resuspended monocytes were readjusted at a concentration of 5×10^6/ml. By nonspecific esterase staining 85–90% of the resuspended cells were monocytes with over 95% viability.

Monocyte labelling

Carboxyfluorescein-diacetate (CFD) was synthetized as described previously [3]. 10 mg CFD was dissolved in 1 ml acetone and stored at 4 °C. 1 ml monocyte suspension containing 10^7 monocytes/ml was incubated for 15 min at 37 °C with 20 µl CFD solution diluted with 5 ml Parker's medium just before use. After labelling the cells were washed twice with protein-free Parker's medium and resuspended at a concentration of 5×10^6/ml. 50 µl of labelled cells were used in each test.

Detection of monocyte reactive antibodies

50 µl of 5×10^6/ml labelled monocyte suspension was incubated with 50 µl of 1:2 dilution of decomplemented human sera at 37 °C and 4 °C, each for 30 min. Then 0.2 ml of fresh rabbit serum as a source of complement was added to each plastic tube and incubated at 20 °C for 90 min. In the tubes containing monocyte reactive antibody positive SLE sera, due to the complement mediated membrane damage,

CFD was released from the prelabelled monocytes during the incubation period. After adding 2.7 ml PBS to each tube to obtain a tenfold dilution, the cells were centrifuged at 400 g for 10 min. Fluorescence in the supernatants, and in separate experiments in the disrupted centrifuged monocytes was measured by Hitachi fluorometer. Cytotoxicity was expressed as per cent label release calculated by the formula:

$$\frac{\text{Exp.} - \text{spontaneous release}}{\text{Max.} - \text{spontaneous release}} \times 100.$$

Heat inactivated rabbit serum was applied to determine the spontaneous release. Maximum release was determined after ultrasonic disruption labelled monocytes.

Circulating immune complexes

Immune complex was precipitated by 4.16% polyethylene-glycol dissolved in 0.1 N sodium-hydroxide and subjected to photometry at 280 nm [7].

Chemotaxis test

Monocyte chemotaxis was measured in modified Boyden's chamber using 5 μm millipore filter (Sartorius Membranfilter GmbH, W. Germany) by the leading front method, as described previously [9]. 10^6 monocytes were preincubated with 2 ml heat-inactivated patients sera as well as monoclonal anti-human monocyte antibody (Anti-human monocyte-lx, Research Laboratory Inc., Bethesda, MD, USA) with 2 mg/ml immunoglobulin concentration diluted 30, 60 and 120 times with Parker's medium. After washing and readjustment, 0.5×10^6 monocytes in 0.5 ml were filled into each chemotactic chamber and tested by zymosan-activated serum as a chemotactic factor (1 mg/ml serum diluted 5 times with Parker's medium). Control cells were incubated without addition of antibody containing serum.

Results

The spontaneous leakage of carboxyfluorescein-diacetate from living monocytes did not exceed 25% by the end of the incubation period under our experimental conditions (Fig. 1). The sum of residual cellular fluorescence and fluorescence measured in the supernatants was standard in each experiment (Table I). On the basis of these data only the fluorescence of supernatants was measured in later experiments.

45 tested SLE sera caused 15.0 $\pm$ 2.4% (mean $\pm$ S. E.) specific CFD release from normal monocytes; the values changed between 0 and 72.9%. In spite of this we found only 5.3 $\pm$ 0.79% specific CFD release caused by the sera of 35 healthy controls at 37 °C. Similar results were observed at 4 °C (Table II).

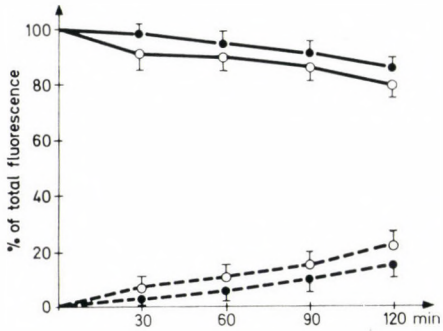

Fig. 1. Fluorescence activity of the supernatants (– – –) of living monocytes (living cell ≥ 95%) prelabelled with carboxyfluorescein-diacetate and that of the disrupted monocytes (———) after incubation at 37 °C (○) and 4 °C (●) for 30 min, respectively and then at 20 °C for 90 min

Table I

Release of CFD from labelled monocytes

Assay	*Fluorescence of	
	Supernatants	**Cells
Monocytes + SLE serum + complement	85.8±5.1	42.0±3.5
Monocytes + SLE serum + heat-inactivated complement	28.5±2.8	120.4±9.2
Monocytes + control serum + complement	29.4±3.1	118.2±8.4
Disrupted monocytes + SLE serum + complement	124.0±5.3	2.8±0.5

* Fluorometer Exm = 495 Ratio = 520 S = 10

** Monocytes were incubated with heat-inactivated SLE sera for 30 min at 37 °C and after addition of fresh rabbit serum as a source of complement for 90 min at 37 °C. Then cells were centrifuged at 400 *g* for 10 min, and supernatants were removed and measured. The cells were resuspended in the original volume, disrupted ultrasonically and the residual cellular fluorescence was also measured.

Table II

Specific CFD release from labelled normal monocytes during incubation with SLE sera and complement

Temperature	Specific CFD release % caused by	
	SLE sera n = 45	Control sera n = 35
37 °C	15.0±16.2	5.3±4.4
4 °C	14.5±12.6	5.1±3.8

$$\text{Specific release} = \frac{\text{Exp.} - \text{spontaneous}}{\text{Max.} - \text{spontaneous}} \times 100$$

Spontaneous release was measured using heat-inactivated complement, max. release after ultrasonic disruption of labelled monocytes.

Specific release caused by 16 SLE sera exceeded the mean value ± 2 S. D. of the healthy controls, showing a complement-mediated monocytotoxic effect at 37 °C or 4 °C (Table III). 9 out of the 16 positive SLE sera contained both warm- and cold-reactive antimonocyte antibodies, 6 of the positive SLE sera had antilymphocyte activity as well. Only 1 of the tested 35 control sera showed a monocytotoxic effect (Table IV).

Table III

Cytotoxic effect of SLE sera

SLE sera	Complement-mediated cytotoxicity against		
	Monocytes		Lympho-cytes
	4 °C	37 °C	37 °C
1.	+	+	+
2.	+	+	+
3.	+	+	+
4.	+	+	−
5.	+	+	−
6.	+	+	−
7.	+	+	−
8.	+	+	−
9.	+	+	−
10.	+	−	+
11.	+	−	−
12.	+	−	−
13.	+	−	−
14.	−	+	+
15.	−	+	+
16.	−	+	−
17–45.	−	−	n. m.

SLE sera causing specific CFD release exceeded the mean +2 S.D. ($5.3 \pm 8.8 = 14.1\%$) of healthy control values marked as positive.

Table IV

Antimonocyte-antibody in SLE sera

Antimonocyte-antibody positive sera	Number	Per cent
Cold or warm reactive	16/45	35.5
Cold reactive	13/45	28.8
Warm reactive	12/45	26.6
Cold and warm reactive	9/45	20.0

Table V

Relationship between the clinical activity and monocyte reactive antibody positivity in SLE

SLE sera	Active SLE	
	Number	Per cent
Monocyte reactive antibody positive	11/18	61.1
Monocyte reactive antibody negative	7/18	38.8

Table VI

Effect of SLE sera on the monocyte chemotaxis

Sera	*Chemotaxis (μm)	Significance
Control n = 17	68.2±1.7	$p < 0.001$
**SLE monocyte reactive n = 9	51.3±1.9	$p < 0.05$
SLE monocyte reactive antibody negative n = 15	64.9±2.6	

* Chemotaxis was evaluated by the leading front method. 1 ml 1×10^6 of monocytes were incubated with 0.2 ml of heat-inactivated sera for 30 min at 37 °C and washed before filling into the chemotactic chambers.

** SLE sera containing both cold and warm reactive monocyte antibodies.

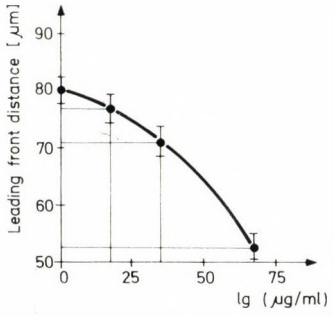

Fig. 2. Effect of monoclonal anti-human monocyte reactive antibody (Ig) on the chemotaxis of healthy monocytes

Table VII

Effect of SLE sera on spontaneous migration of monocytes

Sera	Spontaneous migration (μm)	Significance
Control n = 17	26.2± 2.9	N. S.
Monocyte reactive antibody positive SLE sera n = 9	27.4± 4.2	

10^6 monocytes in 1 ml were incubated with 0.2 ml heat-inactivated sera at 37 °C for 30 min then washed and filled into chemotactic chambers. Instead of chemotactic factor, Parker's medium was placed into the lower part of the chambers to test the spontaneous migration of monocytes.

The presence of antimonocyte antibodies in the sera of SLE patients showed a correlation with the activity of the disease. Most of the antimonocyte antibody positive sera originated from patients with active SLE (Table V). SLE sera containing both cold- and warm-reacting antimonocyte antibodies caused a strongly significant chemotaxis inhibition compared to the effect of the control sera (Table VI). In spite of this, antimonocyte antibody negative SLE sera had a weak chemotaxis inhibitory effect, the difference between the two SLE groups was significant. At the same time, SLE sera had no effect on the spontaneous migration of monocytes (Table VII).

Similarly to the antimonocyte antibody positive SLE sera, monoclonal antibody against human monocytes inhibited the chemotaxis of monocytes (Fig. 2). The inhibitory effect of the monoclonal antibody was dose-dependent.

The immune complex content of SLE sera was also tested. They contained significantly more immune complex than the control sera (optical density 0.074 ± ± 0.016) but there was no difference between the antimonocyte antibody positive (0.163 ± 0.03) and negative (0.165 ± 0.03) sera.

Discussion

Bruning and his colleagues [3] found that the leakage properties of CFD made it applicable as a vital stain for lymphocytes in cytotoxic assays. Our results showed that the carboxyfluorescein fluorochromasia assay was a suitable method to detect monocytotoxic effects as well.

In our experiments 16 out of 45 SLE sera showed monocytotoxic effect at 37 or 4 °C. The monocyte membrane damage must have been due to a complement-mediated antimonocyte effect, which was not seen when heat-inactivated complement was applied.

Arend and his colleagues [1] found cold-reactive antibodies cytotoxic for monocytes only in the sera of two (8%) of 25 patients with SLE. In spite of this Pruzanski et al. [13] showed that 14% of SLE sera contained cold-, and 63% warm-reacting cytotoxins against monocytes. Both studies were performed on adherent monocytes using dye exclusion to detect the cell damage. Tested by an automated fluorochromasia assay, 35.5% of SLE patients had cold- or warm-reacting cytotoxins against monocytes. The percentage of cold- and warm-reacting sera were similar.

Antimonocyte antibody positive sera as well as monoclonal antibody inhibited monocyte chemotaxis in a dose-dependent manner. Cotter and his colleagues [6] showed that monoclonal antineutrophil antibody inhibited the neutrophil chemotaxis and lysosomal enzyme release. The mechanism of chemotaxis inhibition is not known. Our antimonocyte antibody positive sera did not influence the spontaneous migration of monocytes, suggesting that inhibition of chemotaxis may be the result of an inhibition of the ligand-chemotaxis receptor interaction. In contradiction with this Cotter et al. [6] found that monoclonal antibody did not have any effect on the binding to neutrophils of the radiolabelled chemotactic factor.

The monocyte reactive antibodies in the circulation of SLE patients may be an important factor playing a role in the decrease of the monocyte count [2] as well as in the altered monocyte functions [8, 9, 14] and in the decreased in vivo immune complexes clearance [10, 11].

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T-Lymphocytes Bearing Fc-Receptors for IgG in B-Cell Chronic Lymphocytic Leukaemia

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Peripheral venous blood lymphocytes of 10 untreated B-CLL patients at stages Rai 0–III and of 10 healthy individuals were studied. In B-CLL the absolute number of T lymphocytes present in peripheral blood was similar to that in healthy controls; however, the ratio of T lymphocyte subpopulations in B-CLL was changed in favour of TG cells, the absolute number of which was found to be increased. In B-CLL DNA synthesis of the T_G cells in response to PHA stimulation was more intense than in healthy individuals. These observations are discussed in the light of the conflicting reports on the numerical and functional changes in B-CLL T-cells.

Keywords: Chronic lymphocytic leukaemia, suppressor cells, T-lymphocytes

Introduction

Chronic lymphocytic leukaemia of B cell type (B-CLL) is defined as a clonal, neoplastic proliferation of B cells arrested in their differentiation at an early stage [1, 16, 31]. It is known that B lymphocytes from healthy individuals, forming rosettes with mouse red blood cells (M-rosettes) are incapable of producing Ig after PWM stimulation (B). Since a considerable proportion of peripheral blood B cells in B-CLL form M rosettes, perhaps B-CLL is a clonal expansion of such a B-cell subpopulation. However, the possibility of physiological stimuli inducing this B cell population to differentiate into plasma cells cannot be excluded [17].

The humoral immune response, like the synthesis of immunoglobulins (Ig) after an antigen stimulus, is known to be regulated qualitatively and quantitatively by certain subpopulations of T lymphocytes [34]. The absolute number of T lymphocytes in the peripheral blood of patients with B-CLL is similar to that of healthy individuals and there are observations to show that T lymphocytes do not differ from normal T cells in their functions and accordingly the development of the pathological process may be due to B lymphocytes [9, 18, 32, 36].

Other reports, however, have described numerical alterations and functional deficiencies of T lymphocyte subpopulations in B-CLL. These reports mention an elevated T_G cell count [6, 23], a reduced T_M cell count [22] and thus an altered

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ratio of the subpopulations [19]. It is conceivable that this defect may be connected with the functional activity of T lymphocyte subsets [5, 20]; either the insufficiency of T helper cells [24] or the increased activity of T suppressor cells may play a role in blocking the maturation of B cells into plasma cells [7, 10]. Normal reponses of B-CLL T lymphocytes to mitogen stimulation have been reported [35], although a decreased colony formation [14] or a decreased OKT3 reactivity of B-CLL T-cells were also described [26]. After stimulating B lymphocytes of B-CLL patients co-cultured in the presence of PWM with normal T cells, differentiation into plasma cells was found by Fu et al. [15], and interpreted as being indicative of a deficiency of a subpopulation of T lymphocytes in B-CLL.

Remarkably, some of their cases, in which there was paraproteinaemia, were possibly immunocytomas, i.e. malignant lymphomas with highly differentiated cells. Thus, the differences in the classification of lymphocytic leukaemias make it difficult to evaluate some observations. According to other reports, B-CLL B-cells were found to be defective, showing a low rate of Ig synthesis and the helper activity of B-CLL T-cells seemed slightly increased [13].

Yet, immunoglobulin synthesis was detected in B-CLL B cell cultures by other mitogens such as anti-immunoglobulin antibodies, phorbol esters and T helper factors [35].

These conflicting reports have prompted us to study peripheral blood T-lymphocytes of untreated B-CLL patients. The IgG Fc rosetting cells and stimulation of separated IgG Fc positive and negative cells by phytohaemagglutinin were examined. According to our observations the absolute number of IgG Fc rosetting cells was higher in B-CLL and showed a stronger stimulation to PHA than the corresponding normal cells.

Materials and Methods

Lymphocytes from the peripheral venous blood of 10 patients with B-CLL and 10 healthy controls were investigated. Diagnoses were based on routine haematological, immunological (EAC, M rosettes, sIg) and histological examinations. Cases with lymph node biopsies (5 cases) showed pseudofollicular types of CLL (CLL-PF). At the time of our studies stage Rai 0 was found in five patients, Rai I in one patient, Rai II in three patients and stage Rai III in one patient. Immunoelectrophoresis for paraproteins was negative in all patients. None of them had received cytostatic or steroid therapy within the previous five months.

T cell isolation. Peripheral venous blood lymphocytes were isolated by centrifugation over a Ficoll-Hypaque gradient [3]. The monocytes were removed by their plastic-adherence at 37 °C in 5% CO_2 for 30 minutes. T cells were separated by rosette formation with 2-aminoethylisothiouroniumbromide hydrobromide (AET) treated sheep erythrocytes and by their subsequent separation by a Ficoll-Paque gradient [28]. The attached red blood cells were haemolysed by a 0.83% NH_4Cl solution. T cell purity was 89–96% in the B-CLL group

and 92 – 97% in the control group. The cells were tested for viability by trypan blue exclusion and a value of over 97% was obtained for both groups.

T_G cell isolation. Positive cells were obtained by rosette formation with anti-ox-IgG antibody treated ox red blood cells from previously isolated T cells and subsequent separation of the rosette-forming cells by a Ficoll-Paque gradient [27]. The attached red blood cells were haemolysed by 0.83% NH_4Cl solution. In both groups more than 95% of the cells were found viable as tested by trypan blue exclusion.

Cell cultures. T cells (2×10^5/ml) were cultured in RPMI-1640 (Wellcome) supplemented with 25 mM Hepes (Wellcome), 15% FCS and 50 μg/ml gentamicin with 0.02 mE/ml of phytohaemagglutinin (PHA, Wellcome), in 100 μl microtitre plates (Nuch) at 37 °C in a humidified atmosphere of 5% CO_2 for 96 hours. After 90 hours, 1 μCi of ^3H thymidine was added to each culture and then, after six more hours of incubation, the cells were collected with glass fiber filter paper (MA Bioproducts). Radioactivity was measured in a liquid scintillation counter (Beckmann LS-230), the stimulation indices were calculated from the counts/min (cpm/min) of the cultures.

Student's *t*-test was used for statistical analysis of the results.

Results

Peripheral blood lymphocyte counts of the patients varied between 2 and $4.7 \times 10^4/\mu$l.

Figure 1 shows the numerical distribution of isolated T lymphocytes and T lymphocyte subpopulations from B-CLL patients and normal controls. The percentages of T lymphocytes were decreased in the total lymphocyte counts in all patients with B-CLL, as compared with normal controls. However, there was no significant difference between the mean absolute numbers of T lymphocytes in the blood of the patients and the controls. T cells isolated by their Fc receptors

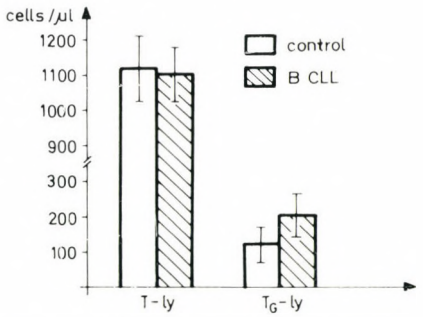

Fig. 1. Numerical distribution of total T lymphocyte counts (T ly), and T lymphocytes isolated by their Fc receptors for IgG (T_G ly) in the peripheral venous blood of patients with B-CLL and healthy controls. N = 10 – 10 cases

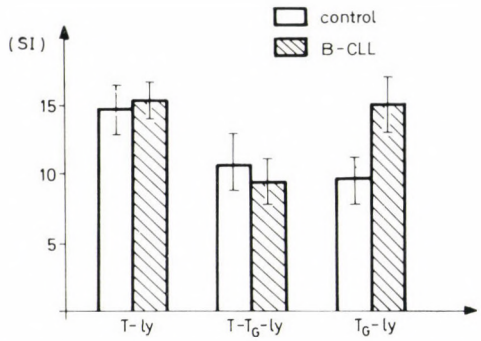

Fig. 2. Proliferation of T cells (stimulation index, SI) of B-CLL patients and controls after incubation with PHA. The three isolated cell fractions are:
Total T lymphocyte count (T ly), IgG Fc receptor negative T lymphocytes (= T$-$T$_G$ ly) and IgG Fc receptor positive T lymphocytes (= T$_G$ ly)

for IgG were present in a significantly elevated number ($p < 0.01$) in the peripheral blood of patients with B-CLL as compared with normal controls.

T cells from patients with B-CLL and from normal controls were tested for their capacity to differentiate into blasts after stimulation with PHA, in three separated T-cell fractions (Fig. 2):

(1) total T lymphocytes (T),
(2) IgG Fc receptor positive T lymphocytes (T_G) and
(3) IgG Fc receptor negative T lymphocytes ($T - T_G$).

Figure 2 shows the results of our experiments with PHA. The total lymphocyte population (T) gave the highest stimulation index (SI), while the lowest value was obtained for the T_G cells. On comparing the stimulation index of T lymphocytes of B-CLL patients, we found that their total T cells and T_G cells proliferated at a similar rate and the lower values were observed in the $T - T_G$ population. Stimulation indices (SI) of normal T_G cells compared with T_G cells from B-CLL patients showed more intense DNA synthesis after stimulation with PHA of B-CLL T_G cells than that of normal T_G cells.

Discussion

In the peripheral blood of patients with B-CLL the absolute number of T cells is similar to that of normal controls [36]. This would suggest that T cells do not participate in lymphoid proliferation; however, qualitative and quantitative characteristics of T cell subpopulations markedly differ from those of normal controls [5, 22, 35]. A decreased T helper effect or lack of soluble helper factors are thought to be responsible for the impaired differentiation of B-CLL cells [15, 37]. According to other observations, this may be due to an excessive suppressor effect [10, 12, 21].

In the present study the number of cells with Fc receptors for IgG (T_G) of patients with untreated B-CLL was significantly higher ($p < 0.01$) than in normal controls. The proportion of IgG Fc receptor negative cells ($T - T_G$) was decreased.

A marked elevation of the number of T_G cells was reported by Kay et al. [22]. The functional significance of the elevated T_G number in B-CLL is unknown. Besides anti dependent cytotoxicity, the suppressor function is attributed to T_G cells [2, 33]. The elevated number of T_G cells observed in B-CLL patients may result in an increase of these functions.

This assumption was further substantiated by our observations of mitogen stimulation of B-CLL T-cells.

Mitogen stimulation is a widely applied method for the investigation of T cell functions. Some authors reported a weak response of T cells to mitogens in B-CLL [25], while others state that the mitogen reactivity of T lymphocytes is normal [11, 36]. Our experiments with PHA showed that the mitogen reactivity of unseparated T lymphocytes is similar to that of normal cells; a significant difference was, however, observed in the response of T lymphocyte subpopulations. T_G cells responded to PHA mitogen stimulation with more intense DNA synthesis than did the TP cells of normal controls. The consequences in B-CLL of the increased number and PHA stimulation of T_G cells are unknown.

We agree with Han and Dadey [18], Prochal et al. [29] and Wolos et al. [35] in that although the T cells are not primarily involved in malignant cell proliferation, an alteration in their proportion and functions may play some role in the pathomechanism of cell proliferation in B-CLL.

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Screening of Toxic-Haemolytic Anaemia in Laboratory Rats: A Model of Phenylhydrazine-Induced Haemolysis

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An increase of the plasma haemoglobin level was found to be a reliable characteristic of toxic-haemolytic anaemia induced by a single dose of phenylhydrazine in laboratory rats. Very high doses of the substance increased the changes in many other characteristics to an extent that was up to now observed only for haemolytic events.

Keywords: rats, toxic-haemolytic anaemia, screening detection

Introduction

Haemolytic anaemia is one of the haematological adverse drug reactions. Its occurrence can be dangerous for human health [7]. Thus, it is necessary to include detection of eventual haemolytic anaemia into the haematologic evaluation of newly developed drugs.

The first steps may be performed on laboratory rats which are commonly used in tests for adverse effects of new drugs. We have found that it is not always possible to use an equal diagnostic scheme for preclinical tests in laboratory rats and in human clinical haematology.

Phenylhydrazine was used as a reference substance. It is well known to cause erythrocytic destruction in many species, e.g. in rats [9, 12, 14], rabbits [11] and human subjects [8, 10].

We compared the fundamental haematological characteristics used for detection of haemolysis in laboratory rats.

Materials and Methods

One hundred and twenty-four male Wister rats WIST/RIPB from the breeding station of our Institute, received at least two weeks before use, were caged in groups of three per one 15 l cage, and fed pelletted Velaz DOS 2b diet and water, both ad libitum. The animals were maintained in a room with automatically regulated temperature of 21 – 23 °C, and illuminated for 12 h daily.

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Blood was drawn under slight ether anaesthesia from the orbital plexus, into Wintrobe mixture, 30–60 min after the lights were switched on, and marrow, spleen and hepatic samples were prepared immediately from rats killed by bloodless cervical traction. Erythrocyte and leukocyte counts and blood haemoglobin levels were determined in Coulter Counter ZF6 using Isoton II® and Zapoglobin.® Plasma haemoglobin levels were determined by the Crosby and Furth method [6], haematocrit in capillary tubes and differential counts of panoptically stained blood smears or organ imprints. Spleens were weighed after 4 h fixation in 10% aqueous solution of formaldehyde. Marrow cellularity was estimated by a haemocytometer [2]. Reticulocytes and erythrocytes with Heinz bodies were stained with brilliant cresyl blue, and osmotic fragility was determined in NaCl solutions following 2 h incubation at 37 °C as a proportion of released haemoglobin.

All rats were 8 weeks old when they were given phenylhydrazine (Lachema, Neratovice) in a dose of 90 mg/kg, i.e. 5 ml/kg body weight. The substance was dissolved in 0.90% saline and injected 30–45 min after the light had been switched on.

Table 1

Red cell count following experimentally induced toxic-haemolytic anaemia in the rat

Characteristic	Dose	Days after injection			
		1	3	7	14
RBC (10^{12}/l)	Control	6.59±0.11	6.65±0.12	7.00±0.09	7.04±0.06
	PHZ	4.60±0.17[a]	2.98±0.11[a]	4.10±0.13[a]	6.06±0.06[a]
RET (10^9/l)	Control	364.3±14.0	370.1±14.8	413.8±33.6	375.5±19.2
	PHZ	528.4±37.4[a]	1117.8±118.9[a]	1962.0±85.2[a]	454.6±12.3
RET (%)	Control	5.53±0.19	5.57±0.21	5.89±0.42	5.37±0.27
	PHZ	11.53±0.82[a]	37.17±3.20[a]	47.78±1.01[a]	7.63±0.13[a]
MCV (fl)	Control	64.3±1.4	63.7±1.8	63.9±0.6	65.2±0.6
	PHZ	57.9±1.8[a]	77.5±2.1[a]	107.6±2.2[a]	79.7±1.0[a]
HTC	Control	0.43±0.01	0.43±0.01	0.45±0.00	0.46±0.00
	PHZ	0.27±0.01[a]	0.23±0.01[a]	0.44±0.01	0.48±0.00[a]
MCH (pg)	Control	21.8±0.3	21.8±0.3	21.6±0.3	20.0±0.5
	PHZ	29.3±0.5[a]	35.7±0.5[a]	33.7±1.0[a]	25.5±0.7[a]
EHJ (10^6/l)	Control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	PHZ	125.3±20.9[a]	600.0±172.3[a]	1723.2±515.5[a]	39.1±15.9[a]
HEINZ (10^9/l)	Control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	PHZ	1.7±1.7	106.9±40.0[a]	52.8±6.2[a]	0.0±0.0
NBL (10^9/l)	Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	PHZ	3.84±1.14[a]	9.98±3.13[a]	1.65±0.83[a]	0.00±0.00

RBC – red blood cell counts, RET – reticulocyte counts, MCV – mean cell volume, HTC – haematocrit, MCH – mean cell haemoglobin, EHJ – counts of erythrocytes with Howell–Jolly bodies, HEINZ – counts of erythrocytes with Heinz bodies, NBL – circulating normoblasts, PHZ – phenylhydrazine 90 mg/kg s.c.,
[a] statistically significant as compared with the control

Four experimental groups were used: (i) sham-injected controls (saline 5.0 ml/kg b. w.), (ii) phenylhydrazine treated animals (90 mg/kg), (iii) sham-treated controls for haemorrhagic effects (anaesthesia and injury of orbital plexus), and (iv) rats after 25 ml blood loss per kg body weight within 30 s. For comparison between haemorrhagic and haemolytic effects, haemorrhage was induced as reported previously [3, 4].

Results were expressed as mean ± S. E. M. for 6–18 animals. Comparison of data was performed using the Mann-Whitney two-tailed U test at 0.05 significance level.

Results and Discussion

Phenylhydrazine induced distinct anaemia: markedly decreased erythrocyte counts, haematocrit (Table 1) and blood haemoglobin levels (Fig. 1), increased counts of reticulocytes, MCH, erythrocytes with Howell–Jolly or Heinz bodies (Table 1) and plasma haemoglobin levels (Fig. 1). MCV increased after a temporary decrease on day one (Table 1). On day 14, increased values of blood heamoglobin levels and haematocrit were caused by the persistence of the increase of MCH and MCV when erythrocyte counts were already almost normal. Karyorrhexis in circulating normoblasts was found in all animals on day one, while later this phenomenon started to disappear, and on day 14 it was already very rare. Polychromasia and anisocytosis were moderate on day 1, intensified on day 3, decreased on day 7, and disappeared within the 2nd week after injection. Osmotic fragility of erythrocytes (Fig. 2) decreased within the first 3 days following the phenylhydrazine injection.

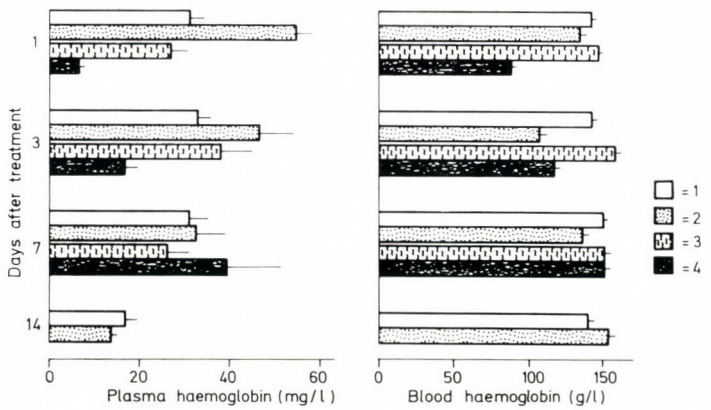

Fig. 1. Plasma haemoglobin (left) and blood haemoglobin (right) levels following induction of haemolytic anaemia by phenylhydrazine. 1 = controls (saline), 2 = phenylhydrazine, 3 = controls for haemorrhage (sham-operated), 4 = acute blood loss, ✳ statistically significant difference versus controls ($p < 0.05$), + statistically significant difference versus controls for haemorrhage ($p < 0.05$)

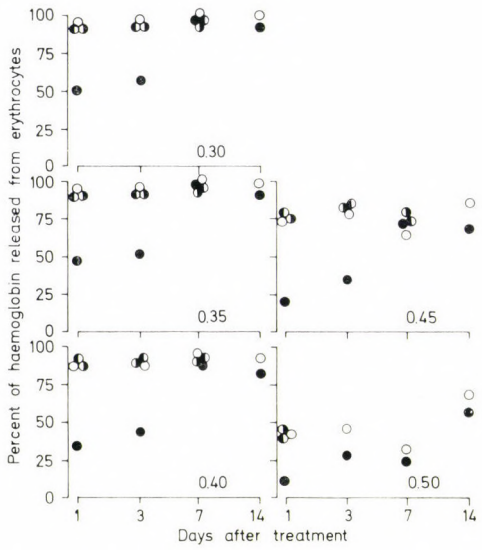

Fig. 2. Osmotic fragility of rat erythrocytes in 0.30, 0.35, 0.40, 0.45, 0.50% NaCl. ○ controls (saline), ● phenylhydrazine, ◐ controls for haemorrhage (sham-operated), ◑ acute blood loss

The marked increase of total circulating leukocyte counts was caused by the increment of both segmented neutrophil and lymphocyte counts. This increment was accompanied by release of band neutrophils into blood (Table 2). Hypersegmented neutrophils were observed rarely on day 3. Blood lymphocytes

Table 2

White cell count following experimentally induced toxic-haemolytic anaemia in the rat

Characteristic	Dose	Days after injection			
		1	3	7	14
LEU (10^9/l)	Control	8.86±0.62	8.66±0.69	7.59±0.33	9.80±0.60
	PHZ	49.23±3.14[a]	82.28±14.89[a]	37.93±4.54[a]	8.33±0.53
SN (10^6/l)	Control	730±200	560±90	650±100	950±120
	PHZ	19,020±2,420[a]	25,62±8,580[a]	2,090±430[a]	1,550±130[a]
BN (10^6/l)	Control	10±10	0±0	10±10	0±0
	PHZ	940±100[a]	1080±380[a]	60±60	70±30
LY (10^6/l)	Control	8,040±610	8,050±710	6,870±290	9,700±670
	PHZ	24,000±2,140[a]	43,360±6,640[a]	34,250±3,610[a]	7 070±760[a]

LEU — total circulating leukocyte counts, SN — segmented neutrophils, BN — band neutrophils, LY — lymphocytes, PHZ — phenylhydrazine (90 mg/kg s.c.)
[a] statistically significant versus the control group

with phagocyted particles (perhaps destroyed erythrocytes) appeared on day 1, were frequent on day 3 and were already very rare on day 7 (Fig. 3). On day 3 and 7, sporadic lymphocytes with butterfly-shaped nucleus were found in the smears.

The cellularity of femoral marrow increased after phenylhydrazine as compared with controls maximally on day 3 (Table 3), absolute and relative weights of the spleen were measured on day 7 (Table 4). Granulopoiesis was moderately higher in the bone marrow on day 3, but splenic granulocytopoiesis did not

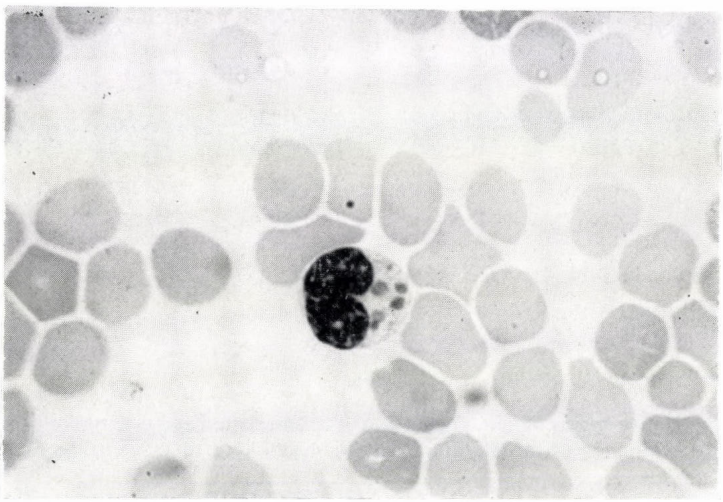

Fig. 3. Blood lymphocyte with phagocyted particles

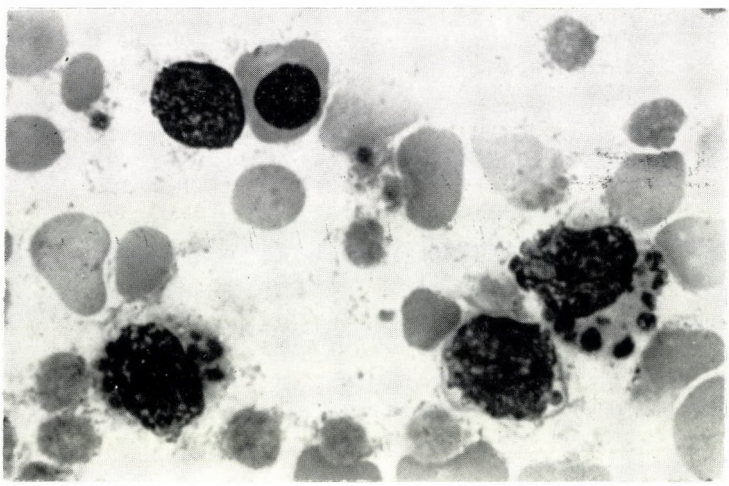

Fig. 4. Panoptically stained imprint of the liver with phagocyting hepatic cells

Table 3

Femoral marrow morphology after induction of haemolytic anaemia

Characteristic	Dose	Days following injection			
		1	3	7	14
Cellularity (10^6/femur)	Control	140.9±5.1	137.4±5.3	136.3±5.5	172.0±16.2
	PHZ	169.0±4.1[a]	184.0±10.8[a]	172.2±16.5	212.3±11.0
Red nucleated cells (10^6/femur)	Control	49.1±3.2	47.6±3.4	41.4±5.7	54.7±4.7
	PHZ	71.9±6.7[a]	62.6±3.2[a]	91.1±8.3[a]	60.9±7.5
Myeloid cells (10^6/femur)	Control	46.5±2.8	48.0±3.1	44.0±1.6	55.8±4.9
	PHZ	36.9±2.1	62.2±5.8[a]	42.8±6.4	62.0±4.2
Lymphoid cells (10^6/femur)	Control	29.2±2.9	26.5±2.0	28.1±1.7	38.3±7.2
	PHZ	30.0±3.8	16.3±5.1	7.9±2.1[a]	77.8±4.1[a]
E : M	Control	1.11±0.10	1.03±0.09	0.96±0.16	1.00±0.09
	PHZ	1.94±0.13[a]	1.06±0.13	2.33±0.34[a]	0.97±0.08

Symbols as in Table 1

Table 4

Absolute and relative spleen weight, proportion of red nucleated and myeloid cells in the spleen following experimentally induced toxic-haemolytic anaemia in the rat

Characteristic	Dose	Days after injection or blood loss			
		1	3	7	14
Absolute weight, mg	Control	778±45	779±53	635±26	738±39
	PHZ	806±30	1641±146[a]	1924±353[a]	1131±209
	C−H	682±54	647±29	762±21	
	H25	758±29	1213±55[b]	963±53[b]	
Relative weight, mg/100 g b.w.	Control	306.8±15.1	302.6±17.7	246.0±11.9	246.0±8.3
	PHZ	361.0±11.9[a]	758.8±63.8[a]	933.5±40.5[a]	474.0±14.4[a]
	C−H	264.7±20.3	284.1±11.8	276.4±7.5	
	H25	308.0±10.2	516.1±20.6[b]	343.8±16.5[b]	
Red nucleated cells, per cent	Control	1.0±0.4	1.1±0.3	1.4±0.9	0.1±0.1
	PHZ	2.0±1.0	8.1±2.4[a]	25.0±4.9[a]	0.0±0.0
Myeloid cells, per cent	Control	1.6±0.3	1.7±0.4	1.0±0.4	0.9±0.3
	PHZ	1.3±0.4	0.9±0.5	1.3±0.4	0.7±0.6

PHZ = phenylhydrazine, 90 mg/kg s.c.
C−H = controls for bled animals
H25 = bled 25 ml/kg b.w.
[a] statistically significant versus controls
[b] statistically significant versus the C−H group

increase. Erythropoiesis increased in both marrow and spleen. The slight temporary lowering of nucleated marrow red cell counts on day 3 may have been connected with the increment of splenic erythropoiesis as well as with the increase of marrow myelopoiesis in the limited bone cavity volume.

No erythropoiesis was found in the hepatic imprints of control animals. Following phenylhydrazine injection the proportion of normoblasts among other nucleated cells on hepatic imprints was $2.83 \pm 1.99\%$ (24 h after phenylhydrazine), $9.33 \pm 3.02\%$ (on day 3), $2.00 \pm 0.26\%$ (on day 7), and $0.00 \pm 0.00\%$ (on day 14). Phagocyting cells were frequent on hepatic imprints in the 1st week of the experiment (Fig. 4).

Boelsterli et al. [5] suggested that a combination of several haematological characterictics can provide comprehensive information about the haemolytic potential of xenobiotics. However, only four (erythrocyte and reticulocyte counts, haematocrit, MCV) of the nine characteristics used by Boelsterli et al. [5] are commonly applied in safety drug evaluations on rats, and the osmotic fragility of erythrocytes was examined in these studies casually. We have demonstrated that it was necessary to add only one haematological characteristic, i.e. plasma haemoglobin assessment to the obvious scale [1, 15] of haematological tests for toxic haemolytic anaemia to be detected.

The incidence of erythrocytes with Heinz bodies, phagocyting blood lymphocytes and hepatic cells, the increment of MCV and MCH, and the decrease of osmotic fragility of erythrocytes may significantly support the diagnosis of haemolytic anaemia in laboratory rats. It also seemed that very high doses of the substance could induce such changes in reticulocyte, erythrocyte, leukocyte, circulating normoblast and nucleated marrow erythroid cell counts, proportion of normoblasts in hepatic imprints, and the relative and absolute weight of the spleen, that it was easier to differentiate between haemolytic and haemorrhagic anaemia.

Our unpublished results on oxidative haemolysis induced by repeated administration of xenobiotics indicated that the plasma haemoglobin level may also detect chronic intravascular haemolysis in laboratory rats.

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Coexistence of Red Cell Aplasia and Autoimmune Haemolytic Anaemia in a Family

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Red cell aplasia is thought to have an immunological basis and has been reported to exist in association with various immunological abnormalities including autoimmune haemolytic anaemia in the same patient. A case is reported of a patient with pure red cell aplasia probably of acquired primary variety; but laboratory tests revealed only slight immunological abnormalities. On the other hand, the mother of the patient had autoimmune haemolytic anaemia. It is believed that such a familial coexistence of the diseases may further support the immunological basis of red cell aplasia.

Keywords: Anaemia, red cell aplasia, autoimmune haemolytic anaemia

Introduction

Immunological factors are thought to be implicated in the pathogenesis of pure red cell aplasia (RCA). Patients with acquired primary RCA are known to have immunoglobulin inhibitors of erythroblasts [1, 2] or erythropoietin [3]. The remainder are idiopathic. ANA positivity, gammaglobulin alterations and autoimmune haemolytic anaemia (AIHA) occasionally observed with RCA and the development of RCA in systemic lupus erythematosus (SLE), rheumatoid arthritis and thymoma support the immune pathogenesis of RCA [4]. We describe here a case of RCA and AIHA in a daughter and her mother, respectively, with a possible aetiological significance.

Case report

A 21 year Palestinian female was admitted in January, 1983, with weakness, fever, cough and ear discharge for two months during which she received cephalosporin without relief and had also required blood transfusion. Born to consaguineous parents, her mother was anaemic and her sister had died 3 years ago follow-

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ing a blood transfusion. Previous history was not significant. Examination showed a febrile, pale, young woman having throat and left ear infection, right basal pneumonia and hepatosplenomegaly. Ampicillin and cloxacillin controlled her infections. The haemoglobin content was 7.5 g/dl, RBC count, 2.5×10^{12}/l; and the reticulocyte count was 0.2%, with normocytic normochromic red blood cells and high ESR. Leucocytes and platelets were normal. Anaemia persisted after the control of infection and bone marrow aspiration was done. It showed normal granulopoiesis and megakaryocytes but severe erythroid hypoplasia, so pure RCA was diagnosed. No thymic mass was demonstrated by X-rays, serum gammaglobulin was high, and the IgG level was raised to 2600 mg/dl; lupus erythematosus (LE) cells, antinuclear antibodies (ANA), Coomb's test and rheumatoid factor were negative with a normal complement level. After she had been given prednisolone 40 mg/day, the reticulocyte count rose to 12%, the haemoglobin content increased and her general condition improved.

The patient's mother, a woman of 50 years, was admitted in January 1981, and for re-examination in February 1983. Six weeks prior to first admission, following a normal delivery, she had received a blood for transfusion indicated by vaginal bleeding without gynaecological cause. She had no fever but was pale with 4 cm splenic enlargement. Haemoglobin was 5.5 g/dl. RBC 1.6×10^{12}/l with 15% reticulocytes. The platelet count was 90×10^{9}/l and the leucocyte count, normal. Bleeding and clotting time were also normal. Coomb's test (direct and indirect) was positive. Bone marrow revealed erythroid hyperplasia (M/E = 0.3/1). Her haemolytic anaemia and thrombocytopenic bleedings were considered autoimmune in origin and she was put on 75 mg prednisolone daily. This resulted within 10 days in a reticulocyte response of 18%, a haemoglobin of 9.9 g/dl, RBC 2.8×10^{12}/l and a platelet count of 463×10^{9}/l. Prednisolone was decreased to 10 mg/day. ANA and rheumatoid factor were positive, with elevated serum IgM, depressed C4 complement level, positive VDRL and 1 : 1280 Trepanoma pallidum haemagglutination titre. The re-examination in 1983 still indicated a mild autoimmune haemolytic anaemia due to noncompliance of treatment. Prednisolone was again raised to 60 mg/day and so the haemoglobin content increased to 14 g/dl.

Discussion

Our patient with RCA presented during infection, but her anaemia persisted after the control of infection. In the absence of causally related diseases such as SLE, thymoma or some tumour, she was considered a case of acquired primary variety of pure RCA which is thought to have an autoimmune basis. This patient, however, had normal immunological tests except a raised gammaglobulin and IgG level. Erythroblastic cytotoxic antibody was not measured but with presence of AIHA in her mother, RCA in this patient could be a component of the syndrome of multiple immunological abnormalities known to occur in more than one member of a family. Reports on the association of AIHA and RCA in the same indi-

vidual have described the immunological background to these conditions. A similar immunological basis may be operative in the rare association of the same disorders in two different individuals of the same family, as reported here.

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Genetic Polymorphism of the Gypsy Population in Hungary as Based on Studies of Red Blood Cell Antigens

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Based on the examination of blood groups (ABO, Rh, MNSs, Duffy, Kell-Cellano, P, Kidd, Lewis, LW) the genetic polymorphism of the Gypsy population living in Hungary has been studied. Comparisons were made between Hungarian Gypsy and non-Gypsy populations, and significant differences were found in the incidence of most of the tested blood groups.

Keywords: Gypsies, blood groups, genetical polymorphisms

Introduction

Gypsies living in Hungary originate from North India [1, 2]. The beginning of Gypsy migration dates back to the period between the 5th and 10th centuries. Gypsies settled down in Hungary in and after the 15th century, but there are some data on even earlier settlements [3]. Even today the way of life, traditions and somatic characteristics of Gypsies and of the Gypsy population involved in our study differ from those of other inhabitants of Hungary.

The Gypsy population of Hungary is not homogeneous. Many facts are unknown yet: the original composition of the Gypsy population migrating from North India; the extent of mixing between Gypsies and other nations they had met during their migration; and, finally, we do not exactly know to what extent the different Hungarian Gypsy groups are isolated from each other, neither do we know the degree of their genetic differentiation.

At least two types of heterogeneity must be taken into account: heterogeneity which originates from the time when Gypsies migrated from North India; and their heterogeneity due to genetic isolation. The latter seems to be linked with the fact that the Gypsies living in Hungary have their own separate language. Based on linguistic differences they fall into three main groups, *viz.* whose native language is Hungarian, or Gypsy (in the majority Wallachians), and whose native language is Roumanian [4].

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Our study consisted of two main parts. The first involved the examination of the genetic differences between Gypsy and non-Gypsy inhabitants. Our findings explain certain facts in the process of differentiation within the major Europid races. The second part of our work was aimed at determining the degree of genetic differentiation among Gypsy groups and we have tried to find some original components characteristic of Gypsies.

The facts that the Hungarian Gypsy population is of considerable size, that they are different from other populations, and that a certain isolation according to their marriages can also be observed, have been a great aid to our work.

The present paper reports on the results of the first part of our study.

Materials and Methods

Collection of data was performed within the scope of the national Gypsy study program of the National Institute of Haematology and Blood Transfusion, Budapest.

First we had to decide on the site of sampling and the sample size. Districts representing different Gypsy groups were selected. Naturally we had to take into consideration areas where there were health workers interested and willing to participate in the programme.

In Hungary, Gypsies are registered as an ethnic minority [5], and not separately listed in the official census; this has made it difficult to establish the sample

Table 1

Tested blood groups and number of tested subjects

Blood group	Subjects, No.
ABO (tested with anti-A, anti-B and anti-A + B sera)	583
Rh/D (tested with anti-D serum)	582
Rh-system (tested with anti-D, anti-C, anti-E, anti-c and anti-e sera)	539
MN (tested with anti-M and anti-N sera)	304
MNSs system (tested with anti-M, anti-N, anti-S and anti-s sera)	74
Duffy (tested with anti-Fy^a and anti-Fy^b sera)	49
Kell—Cellano system (tested with anti-K and anti-k sera)	81
P (tested with anti-P_1 serum)	74
Kidd (tested with anti-Jk^a serum)	81
Lewis (tested with anti Le^a and anti Le^b sera)	81
LW (tested with anti-LW^a serum)	128

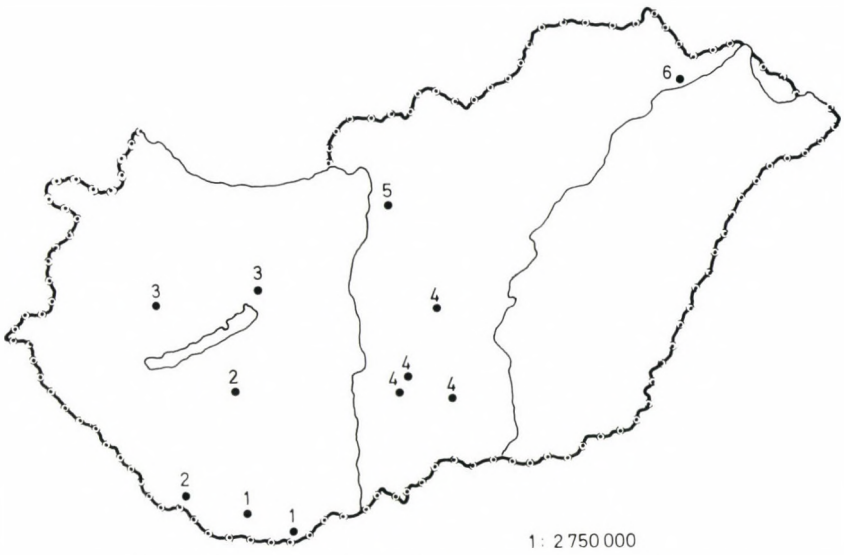

Fig. 1. Sites of sampling of Gipsy populations in Hungary

Legends:

1	County	Baranya
2		Somogy
3		Veszprém
4		Bács-Kiskun
5		Pest
6		Borsod-Abaúj-Zemplén

size. The following sources were used for estimating the number of Gypsies living in Hungary:

– a representative survey performed in 1971 [4]; according to this, the number of Gypsies was 320 000. The native language of 224 000 of these (71%) was Hungarian; of 61 000 (21.1%) Gypsy; of 2500 (7.6%) Roumanian; and of 0.2 it was miscellaneous,

– the survey report of the Inter-departmental Committee of the Cabinet Council responsible for Gypsy affairs gave the number of Gypsies in 1977–78 as 325 000 [6].

In our opinion, the order of these number is acceptable and using these sampling levels, blood groups listed in Table 1 can be well established. So far studies have been performed in 6 counties (Somogy, Baranya, Bács-Kiskun, Veszprém, Pest, and Borsod-Abúj-Zemplén). A total of 583 persons was involved, i.e. approximately a sample size of 0.2% for the whole country. Figure 1 demonstrates the places of sampling. This figure also indicates which other areas must be involved in our future studies.

Besides, the blood group systems listed in Table 1, the Gm-Km and HLA-A, B, C, DR were also tested. Some results of the latter have been presented previously [7].

Due to technical reasons, the number of determinations is not identical in the different blood group systems. Therefore Table 1 also indicates the number of tests, and the number of sera used for testing. The results for the Gm-Km blood group system will be published later.

For comparison of our results we have used literary data concerning Hungarian Gypsies, data demonstrating blood group distribution of the blood donor population in Hungary, and other adequate data related to blood groups in Hungary. The gene frequency calculations of ABO blood groups were performed according to Berstein's method. Other gene frequencies were calculated according to Mourant [8].

Results

Table 2 demonstrates the ABO blood group frequencies of Hungarian Gypsies, on basis of the present results, previously published literary data and those of the National Institute of Haematology and Blood Transfusion. It is seen that the frequency of O blood group was comparatively low. Determinations in county Vas differed from those of the other two. The calculated mean of these three results was 29.4%. The low incidence of blood group A was striking, with the calculated mean of 29.7%. In contrast, the incidence of blood group B was about

Table 2

Incidence of ABO blood groups in some Hungarian Gypsy populations and in non-Gypsy donors registered in the national blood service

Site / Phenotypes	Gypsy population				Non-Gypsy donors****
	1*	2**	3***	$\bar{m}$	(data for the whole country)
O	138/583	126/335	162/600	29.43	29–33
per cent	23.67	37.61	27.10		
A	179/583	81/335	206/600	29.74	40–44
per cent	30.70	24.18	34.33		
B	195/538	99/335	168/600	30.33	16–20
per cent	33.45	29.55	28.00		
AB	71/583	29/335	64/600	10.50	8–10
per cent	12.18	8.66	10.66		
per cent	100	100	100	100	

* Data of present study (N = 583 subjects), samples taken in 6 counties (Bács-Kiskun, Veszprém, Somogy, Baranya, Borsod-Abaúj-Zemplén and Pest).

** Data collected by Ilse Teubl and L. István in 1979 in county Vas (N = 335 subjects).

*** Data collected by Rex-Kiss et al. in 1975 in county Pest (N = 6000 subjects)

**** Data reported by T. Tauszik et al. since 1980 (N = 254 000 subjects).

Table 3

Gene-frequency of ABO blood groups in Gypsy population and non-Gypsy blood donors in Hungary, based on phenotype incidence presented in Table 2

Gene frequency per cent \ Site*	Gypsy population				Non-Gypsy donors (data for the whole country)	
	1	2	3	$\bar{m}$	min.	max.
r	48.16	61.95	51.96	54.02	53.4	58.0
p	25.01	17.35	26.35	22.90	27.8	31.3
q	26.83	20.70	21.69	23.07	12.7	17.3
	100	100	100	100		

* Sites of sampling are identical to those listed in Table 2.

30% in all three studies, the calculated mean was 30.3%. The frequency of AB was also high, the calculated mean of the three data was 10.5%.

Based on data of Table 2, the gene-frequency of ABO blood groups was calculated or adapted from our previous studies (Table 3). Thus, p/A gene frequency is low, 17–26% in Gypsy populations, while the q(B) frequency is over 20%. Data of r-gene frequency were uncertain, like those related to blood group O frequency, as the values covered a wide interval.

From practical point of view the Rh(D) antigen is the most important antigen of the Rh system. Of 582 subjects 37 were Rh(D) negative (6.36%). In county Pest the incidence of Rh(D) negative 10% was also low [4]. In Gypsy inhabitants of county Vas a ratio of 16.42% was found, approximately the same as that of in the total non-Gypsy population (Table 4). The three studies of 1424 Gypsies gave 144 (10.11%) for Rh(D) negativity, while in the total population it amounted to 15–17%.

The incidence of the antigen pairs (C, c, E, e) often determined in the Rh system also differed from the average frequency in the Hungarian population. This was supported by data demonstrated in Tables 4 and 5, showing the distribution of phenotypes and gene frequency.

Table 5 draws attention to three correlated phenomena:

– the frequency or r chromosomes (cde) was comparatively low in Gypsy populations: 27.68%, as compared to the 38–40% in non-Gypsy population;

– the frequency of R_1 (CDe) chromosomes was high in Hungarian Gypsies, 50–60% as compared to the 40–45% observed in non-Gypsy population;

– the frequency of R_2 chromosomes (cDE) was low in Gypsies (5–9%) in contrast to the 13–15% found in non-Gypsy population.

All in all, 74 subjects were typed for MNSs blood groups and 304 for MN blood groups. Table 6 demonstrates our results and data reported in the Hungarian literature. Frequency of M was somewhat higher and that of N somewhat

Table 4

Phenotype frequency in Rh-system

Site* / Phenotypes	Gypsy population				Non-Gypsies, samples from Budapest
	1	2	3	$\bar{m}$	
cc dd ee	29/539	55/335	49/507		286/1686
per cent	5.38	16.42	9.66	10.49	16.96
cc dd Ee	2/539	0	0		8/1686
per cent	0.37	0.00	0.00	0.12	
cc dd EE	0	0	0		0
per cent	0.00	0.00	0.00	0.00	0.00
cc D ee	1/539	1/335	2/507		16/1686
per cent	0.19	0.30	0.39	0.29	0.95
cc D Ee	28/539	18/335	18/507		189/1686
per cent	5.19	5.37	3.55	4.70	11.21
cc D EE	4/539	3/335	0		37/1686
per cent	0.74	0.90	0.00	0.55	2.19
Cc dd Ee	3/539	0	3/507		16/1686
per cent	0.56	0.00	0.59	0.38	0.95
Cc dd Ee	0	0	0		0
per cent	0.00	0.00	0.00	0.00	0.00
Cc D ee	178/539	132/335	190/507		542/1686
per cent	33.02	39.40	37.48	36.63	32.15
Cc D Ee	58/539	39/335	43/507		231/1686
per cent	10.76	11.64	8.48	10.29	13.70
Cc D EE	0	0	0		1/1686
per cent	0.00	0.00	0.00	0.00	0.06
CC dd ee	0	0	0		0
per cent	0.00	0.00	0.00	0.00	0.00
CC D ee	236/539	87/335	202/507		357/1686
per cent	43.78	25.97	39.84	36.53	21.17
CC D Ee	0	0	0		3/1686
per cent	0.00	0.00	0.00	0.00	0.18
	100	100	100	100	100

* Sites of sampling are identical to those listed in Table 2.

Table 5

Gene-frequencies of the Rh-system

Site* / Phenotypes	Gypsy population				Non-Gypsy population samples from Budapest	
	1	2	3	$\bar{m}$	(a)	(b)**
c d e	24.91	38.76	31.08	31.58	39.29	38.74
C d e	1.20	0.00	0.95	0.72	1.11	1.06
c d E	0.81	0.00	0.00	0.27	0.58	1.19
c D e	0.42	0.35	0.63	0.47	1.08	2.37
C D e	64.76	51.49	60.39	58.88	43.48	40.46
C^W D e	—	—	0.74	—	—	2.42
c D E	7.91	9.40	5.22	7.51	14.27	13.42
C D E	0.00	0.00	0.22	0.07	0.18	0.34

* Sites of sampling are identical to those in Table 2 except b** which are data collected by Rex-Kiss and Horváth 1970 [12] used in our study for comparison.

Table 6

Incidence of MN blood groups in some Hungarian Gypsy populations and a large donor population

Site* / Phenotypes	Gypsy population				Donor population (Budapest and suburbs**)
	1	2	3	$\bar{m}$	
MM	157/304	122/335	166/536		6520/20 000
per cent	51.64	36.42	30.97	39.68	32.60
MN	107/304	106/335	267/536		9800/20 000
per cent	35.20	47.76	49.81	44.26	49.00
NN	40/304	53/335	103/536		3680/20 000
per cent	13.16	15.82	19.22	16.07	18.40

* Sites of sampling are identical to those listed in Table 2.
** Data reported by Rex-Kiss and Horváth (1970)

lower than in the whole Hungarian population. The situation was similar in the frequency of Ss blood groups; S frequency was lower and that of s higher in Gypsies than in the Hungarians (Table 7).

Comparison of MNSs blood groups showed important facts. A survey of our data indicated that Mss had a high (37.8%) incidence, while MSS a 4.05% low one; the frequency of Nss was 9.5%, and that of the NSS was 0% (Table 8). Similar characteristics were found in the calculated means of Gypsy data: high

Table 7

Incidence of S – s blood groups in some Hungarian Gypsy populations and in blood donors

Site* / Phenotypes	Gypsy population 1	Gypsy population 2	Gypsy population $\bar{m}$	Donor population (Budapest and suburbs** County Vas***)
SS	3/74	32/335		137/1110
per cent	4.05	9.55	6.80	12.34
Ss	19/74	149/335		483/1110
per cent	25.68	44.48	35.08	43.51
ss	52/74	154/335		490/1110
per cent	70.27	45.97	58.12	44.14

* Sites of sampling are identical to those listed in Table 2.
** Data reported by Rex-Kiss and Horváth (1970)
*** Data reported by Ilse Teubl and L. István (1979)

Mss (21%), and low MSS (4%), 6% Nss and 2% NSS frequencies, in contrast with these of the Hungarian population, where the frequencies were Mss 9%, MSS 7%, Nss 14% and NSS 1%. These differences explain the significant difference between the two distributions $x^2 = 73.71$, d. f. = 8, $P > 99.9$). The gene frequencies of the MNSs system showed similar differences (Table 9).

For Duffy blood group 49 subjects were tested (Table 10). This smaller group displayed the same features as the pooled group of 384 subjects, thus in both groups Duffy a (Fy^a) positivity was higher and Fy^b positivity was lower than in the Hungarian population. The gene-frequency showed similar characteristics ($Fy^a = 0.5365$; $Fy^b = 0.4635$).

The distribution of Duffy genes was:

	Gypsy N = 384	Hungarian[1] N = 891	Hungarian[2] N = 140
Fy^a	0.5365	0.4540	0.3906
Fy^b	0.4635	0.5460	0.6094

H^1 = donors of county Vas and donor panel of National Institute of Haematology and Blood Transfusion
H^2 = data published by Rex-Kiss and Horváth (1970) tests performed with anti-Fy^a.

Comparing the Duffy blood group, the difference between the phenotype groups of the pooled Gypsy and non-Gypsy groups was significant.

Table 8

Incidence of MNSs-blood group system in some Hungarian Gypsy populations and blood donors

Site* / Phenotypes	Gypsy population				Blood donors
	1	a**	b***	$\bar{m}$	(Budapest and suburbs County Vas****)
Mss	28/74	54/283	5/52		102/1110
per cent	37.84	19.08	9.62	22.18	9.19
MSs	10/74	44/283	7/52		187/1110
per cent	13.51	15.54	13.46	14.17	16.85
MSS	3/74	9/283	3/52		77/1110
per cent	4.05	3.18	5.77	4.33	6.94
MNss	17/74	63/283	15/52		237/1110
per cent	22.97	22.26	28.85	24.69	21.35
MNSs	9/74	56/283	13/52		247/1110
per cent	12.16	19.78	25.00	18.98	22.25
MNSS	0	11/283	2/52		45/1110
per cent	0.00	3.88	3.85	2.58	4.05
Nss	7/74	17/283	0		151/1110
per cent	9.46	6.00	0.00	5.15	13.60
NSs	0	23/283	6/52		49/1110
per cent	0.00	8.12	11.54	6.55	4.41
NSS	0	6/283	1/52		15/1110
per cent	0.00	2.12	1.92	1.35	1.35

* Data of present study
** Torony (County Vas). Data reported by Ilse Teubl and L. István (1979)
*** Pankasz (County Vas). Data reported by Ilse Teubl and L. István (1979)
**** County Vas. Data reported by Ilse Teubl and L. István (1979)
Budapest and Suburbs data reported by Rex-Kiss and Horváth (1970).

Table 9

Gene-frequencies of the MNSs blood group based on data presented in Table 8

Site* / Phenotypes	Gypsy populations				Blood donors
	1	a	b	$\bar{m}$	(Budapest and suburbs County Vas)
Ms	57.88	44.34	34.64	45.62	30.10
MS	15.09	16.44	23.05	18.19	26.70
Ns	25.95	24.47	27.38	25.93	36.34
NS	1.08	14.75	14.93	10.25	6.86

Symbols are identical to those used in Table 8

Table 10

Distribution of Duffy blood group in some Hungarian Gypsy populations and in blood donors

Site* / Phenotypes	Gypsy populations				Blood donors
	1	a	b	m	(County Vas (donors)** Donor panel of NIHBT***)
Fy (a + b −)	21/49	84/283	12/52		201/891
per cent	42.86	29.68	23.08	31.87	22.56
Fy (a + b +)	16/49	135/283	27/52		407/891
per cent	32.65	47.70	51.92	44.09	45.67
Fy (a − b +)	12/49	64/283	13/52		283/891
per cent	24.49	22.61	25.00	24.03	31.76

* Sites of sampling of Gypsy population are identical to those listed in Table 8
** Data related to Vas megye reported by the authors at sites listed in Table 9
*** Unpublished data
NIHBT = National Institute of Haematology and Blood Transfusion

Table 11

Incidence of Kell-Cellano blood groups in some Hungarian Gypsy populations and blood donors

Site* / Phenotypes	Gypsy populations				Blood donors
	1	a	b	m	(donors of County Vas** donor panel of NIHBT***)
KK	1/81	1/283	0		2/894
per cent	1.23	0.35	0.00	0.53	0.22
Kk	6/81	48/283	3/52		79/894
per cent	7.41	16.96	5.77	10.05	8.84
kk	74/81	234/283	49/52		813/894
per cent	91.36	82.69	94.23	89.42	90.94

Symbols are identical to those used in Table 10

For Kell-Cellano blood group, 81 subjects were tested. Results were not in agreement with those published previously. As the number of samples was low, the distribution in Gypsies was examined on the basis of our own and other data. The mean value of homozygotes was 0.53% in Gypsy samples, in the same group the heterozygote ratio was 10.05% (Table 11).

A significant difference was found when the mean of Gypsy data was compared to that of the non-Gypsy population ($x^2 = 7.92$, d. f. $= 2$, $95 < P < 99$).

Table 12

Data for P blood groups tested in some Hungarian Gypsy populations and blood donors

Phenotypes / Populations	P_1^+	P_1^-	Gene frequency
Gypsy populations	60/74	14/74	$p_1^- = 0.4350$
per cent	81.08	18.92	
Non-Gypsy populations (donor panel of NIHBT)	71/93	22/93	$p_1^- = 0.4864$
per cent	76.34	23.66	

Table 13

Data of Kidd blood group tested in some Hungarian Gypsy populations and blood donors

Phenotypes / Populations	Jk^{a-}	Jk^{a+}	Gene frequency
Gypsy population	25/81	56/81	$jk^{a-} = 0.5556$
per cent	30.86	69.14	
Non-Gypsy population (donor panel of NIHBT)	17/70	53/70	$jk^{a-} = 0.4928$
per cent	24.29	75.71	

Table 14

Data of Lewis blood group tested in some Hungarian Gypsy populations and blood donors

Phenotypes / Populations	Le(a+b−)	Le(a−b+)	Le(a−b−)	Gene frequency
Gypsy population	26/81	36/81	19/81	le = 0.4843
per cent	32.10	44.44	23.46	Le = 0.5157
Non-Gypsy population (donor panel of NIHBT)	14/91	62/91	15/91	le = 0.4060
per cent	15.38	68.13	16.48	Le = 0.5940

Samples of 74 Gypsy and 93 non-Gypsy subjects (National Institute of Haematology and Blood Transfusion donor panel) were examined with anti-P_1 serum (Table 12). In the Gypsy group P_1-antigen negativity was slightly lower than in the other group. The difference was not significant.

Samples of 81 Gypsy and 70 non-Gypsy subjects from the donor panel were examined with Kidd serum (anti-JK^a) (Table 13). The phenotype distribution did not differ significantly.

Samples of 81 Gypsy and 91 non-Gypsy subjects were examined with anti-Le^a and anti-Le^b sera. The results are only preliminary ones as they have not yet been confirmed by saliva test. The phenotype distribution of the two populations differed significantly (Table 14).

Anti-LW^a serum was used for LW blood group testing. As the test serum originated from a patient of "A" group and anti-B was not absorbed, subjects with "AB" or "B" group were excluded from the study. All 128 tested subjects were LW^a positive. One case was suspected to be a LW^aLW^b heterozygote, but this could not be confirmed as no anti-LW^b serum was at our disposal.

Discussion

Previous Hungarian [10] and other [13] data have demonstrated that the frequency of blood groups "B" R_1(CDe) chromosome and M phenotype is higher in Gypsy then in other European populations. Our present findings are in agreement with these reports.

In our studies scarcely examined blood groups have also been tested (MNSs-system, Duffy, Kell-Cellano and P blood groups), and blood groups not yet studied in Gypsy population have also been examined (Kidd, Lewis and LW blood groups).

We tested the phenotypes (Rh and Duffy) determined by two different genes linked to chromosome 1, and the phenotypes linked to chromosomes 2, 4, 6, 9 and 19. These revealed several differences in the gene frequencies in the Gypsy and non-Gypsy populations in that the incidence of gene alleles on the same site of the respective chromosomes differed significantly. This observation could be extrapolated to a number of other chromosome sites. On the basis of this hypothesis, a population of rather varying genetic constitution inhabits Hungary.

The question arises whether the price of survival of the populations of various genotypes living in similar circumstances was always identical. Naturally, this problem has many aspects to be considered. One of these is the social aspect which will not be discussed in this paper.

The medical-biological aspect can be approached by comparison of the incidence of genetically determined diseases and pathologies and their linkage with genetic markers in Gypsy and non-Gypsy populations. Some reports have already discussed this subject [14, 15, 16].

Another way of approaching the problem is to analyse the demographic data of Gypsies. Several Hungarian authors have found that the higher birth-rate of the Gypsy population is associated with the higher incidence of infant deaths [17, 18, 19]. It appears that the problem of high infant death and low life expectancy of the Gypsy population is due not only to socioeconomic factors, but genetic factors may also be responsible. The latter problem needs further investigations.

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Abstracts

Oncogenes, ions and phospholipids. I. G. Macara (Department of Radiation Biology and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.). *Am. J. Physiol. 248*, C3 (1985).

Recent discoveries in tumor virology, lipid biochemistry, and ion transport studies promise to revolutionize our understanding of cell proliferation, differentiation, and tumorigenesis. A model is proposed, based on similar schemes presented recently by others, that incorporates these discoveries and provides a focus for future research on the functions of oncogene proteins. The model suggests that the early (competence) events in the initiation of cell proliferation are triggered by activation of phosphatidylinositol (PI) turnover which releases two second messengers, 1,2-diacylglycerol (1,2-DG) and inositol-1,4,5-trisphosphate (IP_3). PI turnover is proposed to be regulated by the oncogene protein kinases (src, ros, abl, fps) either directly (acting as PI kinases) or indirectly (as tyrosine kinases). The IP_3 triggers Ca^{2+} release from internal stores, and the elevation of cytosolic Ca^{2+} acts synergistically with 1,2-DG to activate the Ca^{2+}- and phospholipid-dependent kinase C. Kinase C copurifies with the receptor for the tumor-promoting phorbol esters. It is suggested that kinase C then activates the $Na^+ - H^+$ exchange system, resulting in an elevation of cytosolic pH and Na^+, and that these ionic signals (including the change in Ca^{2+}), either in concert or individually, induce further events, including expression of the protooncogene c-myc, which together commit the cell to initiate replication. Evidences in support of this model are reviewed, together with complications indicating its present inadequacies, particularly recent data suggesting that 1,2-DG may activate tyrosine kinases independent of kinase C.

Ilma Szász

Temperature effects on osmotic fragility, and the erythrocyte membrane. G. V. Richieri and H. C. Mel (Department of Biophysics and Medical Physics, University of California, Berkeley, Cal.). *Biochim. Biophys. Acta 813*, 41 (1985).

Results are reported on the temperature-dependence of intact-cell surface area, isotonic volume, hemolytic volume, and ghost steady-state surface area and volume, using several techniques of resistive pulse spectroscopy. Temperature was found not to alter the intact cell surface area permanently: the area remains constant at 130 ± 1 μm^2, at temperatures ranging from 0 to 40 °C. Temperature does alter the steady-state volume of the cells, with a colder temperature inducing swelling by about 0.29 μm^3/deg. C. Such a temperature-induced volume change is sufficient to explain only approximately half of the fragility differences which result from temperature changes. The remainder was found to result from higher temperatures enabling a substantial transient increase in surface area of intact cells (up to at least 14% at 40 °C), with a corresponding increase in the cell's hemolytic volume (up to 21%). The hemolytic volume apparently increases linearly with temperature, since

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steady-state ghost volumes are found to increase linearly with the temperature at which the ghosts were produced. In the steady-state (at high temperature), the membranes of electrically-impermeable released ghosts can remain extended by more than 10%, compared with membranes of the corresponding unhemolyzed, intact red cells.

G. Gárdos

The osmotic response of human erythrocytes and the membrane cytoskeleton. P. Heubusch, C. Y. Jung and F. A. Green (Departments of Medicine, Biophysical Sciences, and Microbiology, State University of New York at Buffalo and Veterans Administration Medical Center Buffalo, New York). *J. Cell Physiol. 122*, 266 (1985).

The volumes of human erythrocytes suspended in solutions of varying concentrations of sodium chloride and sucrose were measured by a Coulter Channelyzer Model H4 with appropriate corrections. The cells showed greatly restricted volume changes at osmolarities between 200–700 mOsm. At osmolarities outside this limit, on the other hand, the cells showed nonrestricted volume changes following essentially the predictions of an ideal osmometer. This unexpected volume response was not spuriously due to changes in shape or to a changing orientation of the cells as they traversed the aperture. The restricted volume change observed was abolished when the cells had previously been treated with diamide or had been heated for 60 minutes at 50 °C, conditions that are known to disturb the spectrin-actin network. The possibility must be considered that the osmotic behavior of human erythrocytes may be nonideal and that this nonideal behaviour is primarily due to mechanical restriction provided by the spectrin-actin network of the membrane cytoskeleton.

Ilma Szász

The effect of ethanol on the passive Ca permeability of human red cell ghosts measured by means of arsenazo III. D. R. Yingst, P. M. Polasek and P. Kilgore (Department of Physiology, School of Medicine, Wayne State University, Detroit, Mich.). *Biochim. Biophys. Acta 813*, 277 (1985).

Ethanol in the range of 0.76–2.40 M caused an immediate increase in the Ca permeability of the plasma membrane of resealed human red blood cell ghosts in which intracellular free Ca could be continuously monitored by means of the Ca chromophore arsenazo III. At a given concentration of ethanol, the Ca permeability increased markedly a few minutes following the mixing of the ghosts and the ethanol, and continued to increase over at least the next 30 min. Preincubating the ghosts in ethanol for 15,60 and 120 min before measuring the rate of free Ca accumulation, progressively increased the effect of a given concentration of ethanol. These results indicate that the effect of a given concentration of ethanol is a complex function of concentration and exposure time. The effects of ethanol in this concentration range were completely reversible. The resealed ghosts used in these experiments were depleted of ATP to avoid interference from the Ca pump and all experiments were carried out with 150 mM KCl on both sides of the membrane to minimize changes in either the volume or membrane potential associated with activation of the Ca-dependent K channel.

G. Gárdos

Stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase activity in human erythrocyte membranes by synthetic lysophosphatidic acids and lysophosphatidylcholines. Effects of chain length and degree of unsaturation of the fatty acid groups. A. Tokomura, M. H. Mostafa, D. R. Nelson and D. J. Hanahan (University of Texas Health Science Center, Department of Biochemistry, San Antonio TX, USA). *Biochim. Biophys. Acta 812*, 568 (1985).

Synthetic lysophosphatidic acids and lysophosphatidylcholines were examined for their effects on the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocyte membranes. Addition of these compounds to erythrocyte ghosts caused significant changes in ATPase activity. The degree of unsaturation and the length of the sn-1 long chain hydrocarbon moiety were both contributing factors. All

lysophosphatidic acids tested stimulated (Ca^{2+} + Mg^{2+})-ATPase activity. Of the species having a saturated acyl group, the most active was the myristoyl derivative. Linoleoyllysophosphatidic acid was the most potent of the unsaturated species. Saturated lysophosphatidylcholines with a short chain fatty acyl group (C_{10} to C_{14}) exhibited only a moderate stimulatory activity, whereas the longer chain homologues, i.e. C_{16} and C_{18}, were inhibitory at high concentrations. On the other hand, unsaturated lysophosphatidylcholines had stimulatory activities comparable to the unsaturated lysophosphatidic acids. These results suggest that the acidic moiety of lysophosphatidic acid is not an important structural determinant for expressing ATPase stimulatory activity in ghosts. Rather the nature of the hydrocarbon chain as well as the lyso structure of these compounds appear most critical under these conditions. The stimulatory effects of lysophosphatidic acids or lysophosphatidylcholines were additive to that induced with calmodulin, suggesting that these lysophospholipids affect the (Ca^{2+} + Mg^{2+})-ATPase by a mechanism which is different from that seen with calmodulin.

Ágnes Enyedi

Kinetics of water transport in sickle cells. C. T. Craescu, R. Cassoly, F. Galacteros and C. Prehu INSERM U-91 Hopital Henri Mondor, Créteil and Institut de Biologie-Physico-chemique, Paris, France). *Biochim. Biophys. Acta 812*, 811 (1985)

This paper reports the results of stopped-flow studies on differences in the kinetics of osmotic water transport of sickle and normal erythrocytes. The kinetics of inward osmotic water permeability are similar in sickle and normal red blood cells. In contrast, the kinetics of outward water flux are significantly (approx. 38%) decreased in sickle cells. Deoxygenation does not modify the water influx kinetics in either type of cells, but accelerates considerably the rate of water efflux in sickle cells. No significant variation of water transport kinetics was observed in density-separated cell fractions of either type. The results suggest that membrane-associated hemoglobin may decrease the outward water permeability and that in deoxygenated sickle cells the fraction of hemoglobin S near the lipid bilayer is lower than in oxygenated conditions.

G. Gárdos

Molecular defect in the sickle erythrocyte skeleton. Abnormal spectrin binding to sickle inside-out vesicles. O. S. Platt, J. F. Falcone and S. E. Lux (Division of Hematology and Oncology, Children's Hospital and Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts). *J. Clin. Invest. 75*, 266 (1985).

Although functional abnormalities of the sickle erythrocyte membrane skeleton have been described, there is little quantitative data on the function of the proteins that compose the skeleton. We have examined the association of spectrin, the major skeletal protein, with ankyrin, its high-affinity membrane binding site, and found sickle erythrocytes to have markedly reduced binding. Binding is assayed by incubation of purified ^{125}I-spectrin with spectrin-depleted inside-out vesicles (IOVs) and measurement of the label bound to IOVs. Sickle IOVs bind $\sim 50\%$ less ankyrin than do control IOVs ($P < 0.001$). Control experiments show that this reduced binding is not a function of faulty composition or orientation of sickle IOVs, or of reticulocytosis per se. Our least symptomatic patient has the highest binding capacity, suggesting that this abnormality may be related to clinical severity. This trend is supported by experiments showing that asymptomatic subjects with sickle trait, sickle cell anemia and high fetal hemoglobin, and sickle β^+-thalassemia have normal binding, whereas a symptomatic patient with sickle β^0-thalassemia has abnormal binding. In contrast to what we see with ankyrin in situ on the IOV, when isolated and studied in solution, sickle ankyrin binds normally to spectrin. This discrepancy may be related to preferential purification of the normal ankyrin species or to an abnormal topography of the membrane near the spectrin attachment site. We hypothesize that sickle hemoglobin or perhaps the metabolic consequences of sickling damage the protein

skeleton. This damage may alter the surface of the erythrocyte and result in abnormal cell-cell interactions which may be related to clinical severity.

Ágnes Enyedi

Intracellular calcium redistribution and its relationship to fMet-Leu-Phe, Leukotriene Bf, and phorbol ester induced rabbit neutrophil degranulation. P. H. Naccache, T. F. P. Molski, P. Borgeat, and R. I. Sha'afi (Departments of Pathology and Physiology, University of Connecticut Health Center, Farmington, Connecticut). *J. Cell Physiol. 122*, 273 (1985).

The addition of low concentrations ($<10^{-7}$ M) of the calcium ionophor A23187 to rabbit neutrophils releases the intracellular pool of calcium previously shown in radioactive steady-state and chlortetracycline fluorescence studies to be mobilized by chemotactic factors. A23187 at these concentrations elicits no functional responses from these cells. However, A23187, added before chemotactic factors such as fMet-Leu-Phe and leukotriene B_4, inhibits the ability of the latter stimuli to induce, in the presence of cytochalasin B, and exocytotic release of the neutrophil's cytoplasmic granules. These results imply that the chemotactic-factor-induced release of intracellular calcium is a necessary event for the optimal activation of the neutrophils. Phorbol ester-induced neutrophil degranulation on the other hand is unaffected by exposure to A23187, thereby completely dissociating its mechanism of action from rises in cytoplasmic free calcium.

Ilma Szász

Chemical modification of the Na^+/H^+ exchanger of thymic lymphocytes. Inhibition by N-ethylmaleimide. S. Grinstein, S. Cohen and A. Rothstein (Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada). *Biochim. Biophys. Acta 812*, 213 (1985).

A Na^+/H^+ exchanger is involved in the regulation of cytoplasmic pH and cellular volume in a variety of cells. Little is known about the molecular nature of this exchanger. The purpose of this study was to survey a variety of group-specific covalent reagents as potential inhibitors of the exchanger. Na^+/H^+ countertransport activity was assayed as the amiloride-sensitive rate of Na^+-induced alkalinization in acid-loaded lymphocytes, or as the rate of swelling in cells suspended in sodium propionate medium. Activity was not affected by proteinases or by carboxyl-group and amino-group specific reagents. A significant inhibition was produced by diethylpyrocarbonate, a histidine-specific reagent and by N-ethylmaleimide, a sulfhydryl group reagent. A similarly reactive but nonpermeating sulfhydryl agent, glutathione-maleimide, failed to inhibit Na^+/H^+ exchange. Moreover, the reaction with N-ethylmaleimide was sensitive to changes in the cytoplasmic pH. The data suggest that the chemically reactive groups of the Na^+/H^+ exchanger of lymphocytes have limited exposure to the extracellular medium but that an internally located sulfhydryl group is critical for the cation-exchange activity.

B. Sarkadi

Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. A. Truneh, F. Albert, P. Golstein and A. Schmitt-Verhulst (Centre d'Immunologie ISERM-CNRS de Marseille-Luminy, Marseille, and Faculté de Pharmacie, Université d'Aix-Marseille II, Marseille, France). *Nature 313*, 318 (1985).

Although it has been proposed that the activation of T lymphocytes is mediated by an early rise in cytosolic calcium concentration, it has not been possible to mimic antigen- or mitogen-induced mouse lymphocyte activation by calcium ionophores that bypass receptor-mediated processes. There is now evidence from other systems that the rise in cytosolic calcium which follows receptor triggering is preceded by the breakdown of phosphatidylinositol bisphosphate into 1,2-diacylglycerol and inositol trisphosphate. The latter is known to cause release of calcium from intracellular stores. The cellular target for the former is now widely accepted to be protein kinase C. Therefore, ligand-

induced cellular response follows a rise in cytosolic calcium concentration and protein kinase C activation. Here we confirm that the calcium ionophores A23187 and ionomycin do not activate mouse T lymphocytes. However, either one in combination with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), which is structurally related to 1,2-diacylglycerol, induces in lymphoid cell populations the expression of receptors for interleukin-2 (IL-2), the secretion of IL-2 and cell proliferation as measured by ^{3}H-thymidine uptake. The growth-promoting effect of IL-2 on an exogenous IL-2-dependent clone could not be substituted for by ionomycin either alone or with TPA. Thus, the combination of calcium ionophores and TPA bypasses the requirement for antigen- or lectin-induced signal at the onset of lymphocyte activation.

B. Sarkadi

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Steroid Sensitivity of Chronic Uraemic and Renal Transplant Patients Measured by the Antibody Dependent Cellular Cytotoxicity Reaction

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The steroid (methylprednisolone) sensitivity of chronic uraemic and renal transplant patients was examined on the basis of the extent of inhibition of the antibody-dependent cellular cytotoxicity (ADCC) reaction, and via the effect on the ADCC capacity test (ADCC-C). Individuals with an inhibition of 30% or more were classified as steroid-sensitive.

Immunopharmacological tests and the clinical picture showed 67%, 12 of the 18 renal transplant patients to be steroid-sensitive. In 92% of the cases the transplanted kidney was functioning well one year or more postoperatively. In 5 of the 6 steroid-resistant patients rejection necessitated removal of the transplanted kidney.

The method is simple to perform and gives reproducible results, and appears suitable for application in clinical practice.

Keywords: steroid sensitivity, uraemic-renal transplant patients, ADCC, ADCC-C

Introduction

In 1981, Dumble et al. reported on the measurement of steroid inhibition on the basis of changes in killer activity [3]. They found a correlation between the survival of renal allografts and the extent of steroid inhibition measured in the antibody-dependent cellular cytotoxicity (ADCC) reaction. The method seemed promising for clinical practice, but an in vitro tumour cell line was used as target cell and its maintenance caused difficulties.

In the present work we have measured the steroid effect in an ADCC reaction, but in a substantially simpler system, where human erythrocytes were used as target cells. In addition, the steroid effect was measured in the traditional ADCC reaction (number of effector cells in excess) and in the ADCC capacity test (excess of target cells, ADCC-C).

Patients

The average age of the chronic uremic patients was 32 years (range 17–51 years). They participated in haemodialysis on 2–3 occasions per week on average. The total number of haemodialyses they had received was, 36–200 times: 12

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patients; 201 – 500 times: 14 patients; 501 – 1000 times: 4 patients. On the average, the patients received 400 ml washed red blood cell mass monthly.

In renal transplant patients, uniform immunosuppression with prednisolone and azathioprine was carried out by administering 1 g steroid on the first postoperative day, and 50 mg steroid daily from the second postoperative day on. Rejection crises were treated with prednisolone booster therapy or by local X-ray irradiation of the transplant.

The results of examinations of healthy blood donors were used as controls.

Methods

Cytotoxic capacity of lymphocytes (ADCC-C)

In case of a high number of target cells the cytotoxic activity of the lymphocytes increases, and thus the maximum cytotoxic activity of the cell population may be measured by titration of the target cells [5].

A three times washed suspension of freshly taken Rh (D, C)-positive red blood cells was used as target cells. ^{51}Cr labelling was performed in the presence of sodium citrate, by the addition of 100 μCi $Na_2{}^{51}CrO_4$ (Poland, spec. act. 4 – 8 TBq/g Cr) for 60 minutes at 37 °C. After labelling, the cells were washed 3 times, and the cell count was adjusted to 10^7/ml. The effector lymphocytes were isolated on a Ficoll-Uromiro gradient after treatment of the whole blood with colloidal iron powder (GAF, USA).

50 μl quantities of stepwise 1 : 2 dilutions of the target cells were added per culture, in microtiter plates (Greiner), together with 50 μl of a 2×10^6/ml suspension of effector lymphocytes. Thus, while the effector cell count remained constant (10^5 lymphocytes), the target cell count decreased due to the dilutions.

Fifty μl of a 1 : 20 dilution of incomplete anti-D antibody was added per well and the volume was made up to 200 μl with RPMI-1640 (Serva) culture medium containing 10% calf serum (Human, Budapest). The cultures were incubated in CO_2 for 18 h at 37 °C, and the radioactivity of 100 μl supernatant was measured. The steroid (methylprednisolone) was added together with the supplementary culture medium, in a final concentration of 5 – 10 μl/ml.

For determination of the total activity, measurements were made of the radioactivity of 25 μl labelled red blood cells. The spontaneous activity was given by the count rates for steroid-containing cultures without anti-D antibody.

The means of the count rates for 3 parallel samples were used for evaluation.

$$\text{No. of target cells destroyed:} \frac{\text{No. of target cells added} \times \text{cytotoxicity}}{100}$$

The steroid sensitivity studies were carried out by means of target cell excess cytotoxic capacity investigations, together with the effector excess traditional

cytotoxic reaction, ADCC [12]. In the latter reaction all conditions were similar (culture medium, target cell, time of incubation) to those described in the ADCC–C test, the only difference was that here the effector/target ratio was 10 : 1.

Results

The mean ADCC capacity for normal blood donors, chronic uraemic patients and renal transplant patients is presented in Table 1. The values mean the red blood cells killed by 10^5 lymphocytes. Variance analysis by the Scheffe

Table 1

ADCC capacity of patients (ADCC–C)

Normal blood donors	Chronic uraemic patients	Renal transplant patients
n = 16	n = 23	n = 35
23 593.74	30 500.00	15 489.13
± 20 396	± 19 507 ←——→	± 10 395
	$p < 0.01$	

Values are given as numbers of red blood cells (± SD) killed by 10^5 lymphocytes. ADCC–C reaction: target/effector cell ratio $5\times 10^5 - 1.56\times 10^4/10^5$

Table 2

A. *Steroid sensitivity in ADCC reaction*

	Percentage inhibition					
	20	21–30	31–40	41–50	>50	>30
Normal blood donors n = 20	0	3	5	6	6	85
Chronic uraemic patients n = 22	7	6	1	4	4	41

B. *Steroid sensitivity in ADCC capacity test, per cent*

	20	21–30	31–40	41–50	>50	>30
Normal blood donors n = 20	0	4	7	4	5	80
Chronic uraemic patients n = 22	9	7	6	4	4	47

A.: ADCC-reaction = target/effector cell ratio 1 : 10
B.: ADCC–C reaction = target/effector cell ratio $5\times 10^5 - 1.56\times 10^4/10^5$

method revealed a significant difference ($p < 0.01$) between the mean values for the two patient groups, but neither patient group displayed a significant difference from the normal values.

Table 2 gives the results of steroid sensitivity examinations of 20 normal blood donors and 22 dialysed patients with chronic uraemia. The steroid effect of patients was studied both in the effector excess ADCC reaction and in the target cell excess ADCC capacity test. Almost the same inhibition values were found in the two different systems. In evaluation, individuals displaying an inhibition of 30% or more were classified as steroid-sensitive, and the remainder as steroid-resistant. 40–47% of the chronic uraemic patients proved steroid-sensitive in the two in vitro tests. The steroid sensitivity measured in vitro by the

Table 3

Distribution of steroid sensitive and resistant patients with respect to number of haemodialyses

Total No. of dialyses	Inhibition of ADCC–C reaction in steroid sensitive and resistant patients		% of sensitive patients
	resistant	sensitive	
36–200	3	56	
	29	50	
correlation	10	50	5/12
coefficient*	14	38	
−0.083	25	50	42%
	19		
	10		
201–500	18	55	
	26	64	
correlation	28	41	
coefficient*	24	51	
0.121	22	84	8/14
	27	35	
		42	57%
		38	
501–1000	23	43	
correlation	24		
coefficient*	5		1/4
0.038			25%
		Total:	14/30
			47%

* Spearman's correlation coefficient between number of dialyses and steroid sensitive patients

ADCC–C reaction = target/effector cell ratio $5\times 10^5 - 1.56\times 10^4/10^5$

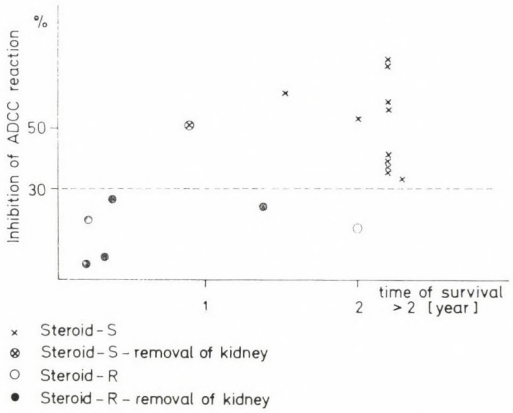

Fig. 1. Correlation between steroid sensitivity and survival of transplanted kidney in renal transplant patients

inhibition of the ADCC-C reaction did not exhibit a correlation with the number of haemodialyses performed (Table 3).

The results of tests prior to kidney transplantation and in the postoperative weeks are shown in Fig. 1. 67%, that is, 12 of the 18 renal transplant patients proved to be steroid-sensitive (inhibition $\geq 30\%$); there was only one case of rejection necessitating removal of the transplanted kidney in this group, 10 months after operation.

Table 4

Repeated steroid sensitivity testing by ADCC—C reaction in chronic uraemic patients

J. I. ♀	Sz. I. ♂
(978) 82.09.29. 43%	(148) 82.06.01. 51%
(990) 82.10.27. 33%	(180) 82.09.29. 47%
(1029) 83.01.19. 54%	
Sz. M. ♀	**K. H. ♂**
(288) 82.10.26. 10%	(510) 82.06.01. 8%
(307) 82.12.07. 38%	(562) 82.10.26. 29%
	(580) 82.12.06. 10%

Number of dialyses in parentheses.
ADCC—C reaction: target/effector cell ratio $5\times 10^5 - 1.56\times 10^4/10^5$

In contrast with the steroid-resistant group where there was only one patient with a functioning graft, in the other 4 patients kidney removal because of rejection occurred after 3 months and in that of one patient after 15 months.

We next attempted to establish the reproducibility of the steroid effect in the patients. A repeated test series was carried out in 2 male and 2 female chronic uraemic patients (Table 4). The extent of steroid inhibition was in most cases the same when the tests were repeated after an interval of 2–3 months: the first two patients remained steroid-sensitive, while one out of the two steroid-resistant patients remained resistant and the other one showed a slight change from 10 to 38 % inhibition.

Discussion

During the rejection reaction following organ transplantation, and in general clinical practice too, glucocorticoids are widely used as general immunosuppressive agents. In the course of treatment it emerged that a number of patients are steroid-resistant and not even prednisolone booster therapy prevented rejection.

As to the inhibitory effect of glucocorticoids on lymphocyte functions, it has been demonstrated that, in various mitogen-stimulated lymphocyte cultures, mitogenesis inhibition depended on the mitogen and on its dose [1, 8]. Crabtree et al. [2] showed that the glucocorticoids exerted their suppressive effect via inhibition of the production of the "T cell growth factor". This observation was later confirmed by other authors [11].

Since the interleukins (IL) play an important role in lymphocyte proliferation and in the cytotoxic reactions mediated by lymphocytes, the question arose whether the in vivo steroid sensitivity could be monitored with an in vitro lymphocyte function test. In our experience, the mitogen-induced lymphocyte proliferation is not suitable for study of the steroid effect, as even a low concentration of steroid results in strong inhibition, independently of the individual in contrast with the latest results of Langhoff et al. [10]. They found that both control and uraemic lymphocyte cultures have a reproducible individual lymphocyte response to the immunosuppressive effect of steroid. Dumble et al. [3] were the first to report on a steroid effect measured with a cytotoxic test that can be used in clinical practice. We have slightly modified and simplified the method, and applied it to determine the ADCC capacity and steroid inhibition in chronic uraemic and renal transplant patients.

It was not possible to distinguish between the chronic uraemia group and the normal group by the mean values of the ADCC capacity test. If the individual values are considered, there were large differences in both groups (as indicated by the S.D.). During immunosuppressive treatment, the ADCC capacity of the renal transplant patients was significantly lower than in the uremic patients.

The steroid effect was examined by the traditional ADCC reaction and the ADCC capacity test. The ADCC capacity examinations are themselves sufficient

for measurement of the steroid effect; further, fewer lymphocytes are needed for the method, which means that less blood must be taken from the patient.

Our investigations indicated that neither the number nor the duration of haemodialyses influence the steroid sensitivity. The steroid effect is well reproducible in time, nearly the same values being obtained after an interval of a few months.

Palacios et al. [11] described the inhibitory effect of steroids during the interaction of IL-2 and T lymphocytes. Since IL-2 production plays no role in immunosuppression following blood transfusions [6], it is probable that the steroid sensitivity too is independent of the effect of transfusions.

Our results with renal transplant patients showed that, if tests before transplantation or 2–3 weeks postoperatively reveal steroid resistance, it is advisable to carry out the immunosuppression with other drugs, CyA or ATG. Further, in the resistant cases a positive result is not to be expected from prednisolone booster therapy, even if the maximum dose is administered.

Fassbinder et al. [4] applied ATG or plasmapheresis treatment with good results in the event of rejection in steroid-resistant patients. Gordon et al. [7] demonstrated the synergistic effect of CyA and glucocorticoids; the two agents act at two different points of lymphocyte activation.

Since mainly the IgG Fc receptor bearing lymphocytes take part in the ADCC reaction [9], it is conceivable that the differences in steroid sensitivity or resistance between individuals may be correlated with a shift in the proportion of the T_g lymphocyte subpopulation. We plan such investigations in the future.

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Vesicle Release from Erythrocytes during Storage and Failure of Rejuvenation to Restore Cell Morphology

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During storage, discocyte-echinocyte transformation of erythrocytes occurs progressively and microvesicles release from the spicules in the form of membrane-encapsulated red cell fragments. After rejuvenation part of the membrane-deficient cells, mainly spheroechinocytes, transform into spherostomatocytes because of their inability of restoring the surface area to volume ratios of normal discocytes. The proportion of spherostomatocytes formed on rejuvenation increases with storage time. Numerical indices for characterizing the "level of reversion" and the "quality of reversion" were developed. Osmotic fragility curves and light scattering histograms also reflected a partially irreversible increase of inhomogeneity of stored cell populations.

Keywords: erythrocyte, fragmentation, membrane, morphology, rejuvenation, reversibility, storage

Introduction

The alterations of red blood cells (RBC-s), which occur progressively during liquid preservation of blood at 4 °C, deteriorate oxygen-transport function and viability of reinfused erythrocytes [1]. That is, the therapeutic effect of RBC-s decreases with each day of storage until their use for transfusion is no longer admissible. Therefore red cells of a certain number of donations are lost as a result of outdating. Rejuvenation of stored erythrocytes is a relatively new method in blood banking by means of which outdated RBC-s can be salvaged [2]. Rejuvenation means a process of biochemical modification of cells *in vitro* to restore the decreased levels of intracellular ATP and 2,3-DPG.

Gabrio et al. [3] reported results on the importance of nucleosides for the resynthesis of organic phosphate compounds in stored erythrocytes. Nakao et al. [4] published observations on the relationship between ATP content and the shape of RBC-s. These findings initiated extensive investigations in this field, and as a result improved preservative media were introduced for liquid storage [5, 6] and methods of rejuvenation were elaborated. The systematic laboratory and clinical studies on the rejuvenation of human RBC-s carried out by Valeri

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and associates demonstrated the safety and therapeutic effect of reinfused rejuvenated erythrocytes and the usefulness of the method in specific clinical situations [1, 7–9].

Rejuvenation results in significant improvements of a number of red cell properties, which can be detected by *in vitro* methods and oxygen-transport function may be estimated from measurements of 2,3-DPG and oxygen dissociation values. However, *in vitro* parameters have little if any predictive values as regards viability of RBC-s [1]. Nevertheless, red cells suffer damages during storage and some consequences of these lesions are reflected even in some properties of rejuvenated cells. Thus further informations on the relationship between the degree of storage lesions and the parameters of rejuvenated cell populations might somewhat extend the validity of *in vitro* tests.

In the present work, vesicle release from red cells was studied as a function of storage time. Morphology and some other properties of stored erythrocytes were also examined before and after rejuvenation. Furthermore, the failure of rejuvenation to restore normal morphology will be discussed in connection with the membrane loss caused by storage.

Materials and Methods

Blood was collected in acidic citrate dextrose (ACD). Equal volumes from the same donation were stored in separate glass bottles and examined in series. For studies of vesicle release white blood cells and thrombocytes were removed from the blood on the day of collection, under sterile conditions at 4 °C.

Vesicle release. Released vesicles were isolated from the blood plasma after different storage periods. Cells were sedimented by centrifugation of blood ($2500 \times g$ for ten minutes at 4 °C) and about 30% from the middle layer of supernatant plasma was recollected. This part of the plasma was diluted by an equal volume of phosphate buffered saline (PBS) and centrifuged at $55 \times 10^3 \times g$ for ten minutes at 4 °C. Sediments of vesicles were resuspended in buffer for protein and enzyme activity measurements and packed pellets were used for transmission electron microscopy (TEM). Total *protein* content of vesicle suspensions was determined by the method of Lowry et al. [10]. Haemoglobin was calculated on the basis of its iron content measured by an atomic absorption spectrophotometer (Zeis AAS 1).

Acetylcholinesterase (EC. 3.1.1.7) activity was measured according to Ellmann et al. [11].

Packed red cell volume (PCV) was determined by the microhaematocrit method before and after storage. The amount of protein and enzyme activity released from one ml of initial (prestorage) PCV were calculated. For transmission *electron microscopy* tightly packed vesicle sediments were embedded in Durcupan ACM (Fluka) resin after conventional glutaraldehyde-osmiumtetroxide fixation and after ruthenium red [12] staining.

Erythrocytes

Rejuvenation. Adenosine solution (60 mmol/l in PBS) was added to an equal volume of stored blood and the mixture was incubated at 37 °C for 180 minutes.

Morphology. Cells were fixed in a 0.1 mol/l Sörensen's phosphate buffered 1.25% glutaraldehyde solution (pH 7.2 – 7.4; 370 mOsm/l). For scanning electron microscopy (SEM) the fixed cells were washed in buffer several times. They were then sedimented on poly-L-lysine hydrobromide (Sigma) pretreated glass coverslips. The cell-covered coverslips were postfixed in 2% aqueous OsO_4 solution, then dehydrated in graded ethanol and air-dried from ether. Dried preparations were coated with gold-palladium alloy by vacuum evaporation. SE micrographs were taken of randomly chosen fields with a JSM 35 C microscope. Morphological indexes [13] were calculated from data obtained of 400 – 800 cells. (For multiplying factors see Fig. 5.). For TEM ultrathin sections of embedded cells were prepared by conventional methods.

ATP level. Aliquots of blood samples were haemolysed in ice-cold hypotonic Tris-borate buffer (40 mmol/l, pH 9.2) and the haemolysates were boiled for ten minutes. Samples were stored at −20 °C until the day of measurement. ATP was measured by the firefly bioluminescence method adapted to a scintillation counter (Packard, Tri-Carb) [14]. Soluble extracts of dried firefly lanterns (Serva) were reconstituted with water. Appropriate volumes of extracts and haemolysates were mixed in scintillation vials, lowered in the detection chamber and exactly after 30 seconds of mixing the counting was started and three subsequent 10 seconds integral counts were recorded. For calibration graded dilutions of haemolysates from fresh red cells were made with haemolysates of depleted erythrocytes. Final dilutions of samples were adjusted on the basis of the haematocrit to con-

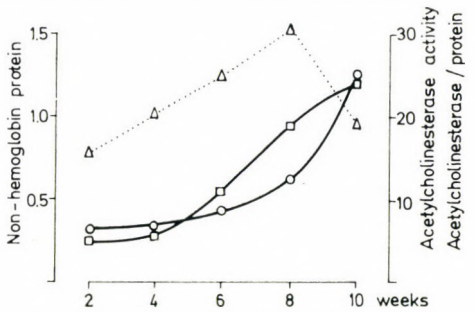

Fig. 1. Vesicle release from red blood cells as a function of time of storage. Non-haemoglobin protein: 0 — mg protein per ml of prestorage packed red cells (PCV); vesicle acetylcholinesterase activity: □ — μmol acetylthiocholine per mg protein × 10 min × ml PCV; acetylcholinesterase activity to protein ratio: △ — μmol acetylthiocholine per mg protein × 10 min. The increase in protein content and acetylcholinesterase activity of vesicles, recoverable from plasma, reflects the progressive accumulation of cell fragments in the liquid preservative medium

tain equal quantities of red cell material. ATP levels were expressed as the percent of ATP content of fresh erythrocytes per unit volume of packed cells.

Cell volume. Cell size distribution curves were established on the basis of electronically measured volumes. The electronic parameters of a counter apparatus (Medicor, Picoscale PS-4) were modified to increase the accuracy of volume signal formation and interfaced with a multichannel analyser (KFKI ICA 70; 16 bit × 4 K). The approximate cell volume corresponding to the channel number was evaluated by calibration using the microhaematocrit and cell count. Distributions of 90° light scatter signal heights of the same samples were examined with a light activated cell sorter (FACS II) at the 488 nm wavelength of incident laser beam. Mean, peak position and peak count/integral count parameters of the distribution curves were computed.

Osmotic fragility studies were done every week up to the seventh week of storage on a separate series of blood. Red cells were washed and resuspended in isotonic PBS at 4 °C. Aliquots of suspensions were lysed in graded concentrations of PBS at 4 °C for ten minutes. Prior to centrifugation lysis was stopped by adding equal volumes of 550 mOsm/kg PBS containing 5 mmol/l $MgCl_2$. Haemoglobin was determined by the haemoglobincyanide method. Osmolarity was estimated with a freezing point osmometer (Knauer, Halbmikro).

Fragility curves were constructed according to the method of Detraglia et al. [15].

Results

Released vesicles. Increasing amounts of vesicles could be recovered per unit volume of packed cells from blood during storage as showed the protein content and the acetylcholinesterase activity of isolated vesicle fractions. Enzyme activity per mg non-haemoglobin protein increased until the eight week of storage (Fig. 1). About 60% of total vesicle protein was haemoglobin. The buds on the spicules of spheroechinocytes, which could be seen by TEM (Figs 2a–b) and SEM (Figs 2c–d), might have been related to the segregation of vesicles. Examination by TEM of isolated pellets revealed that the vesicle fractions consisted of small bodies with round or elongated profile about 100 × 250 nm in diameter (Fig. 3a). Elongated forms were sometimes narrowed at their middle part. The characteristic unit membrane profile of the boundary layer and the ruthenium red staining of cell coat showed the presence of cell membrane components, the internal electron density indicated haemoglobin content inside the membrane envelope of the vesicles (Fig. 3b). Only negligible amounts of contamination by fragments of "ghosts" and some entrapped finely granulated material were found. No changes in the morphological aspects of vesicle pellets related to storage time could be detected.

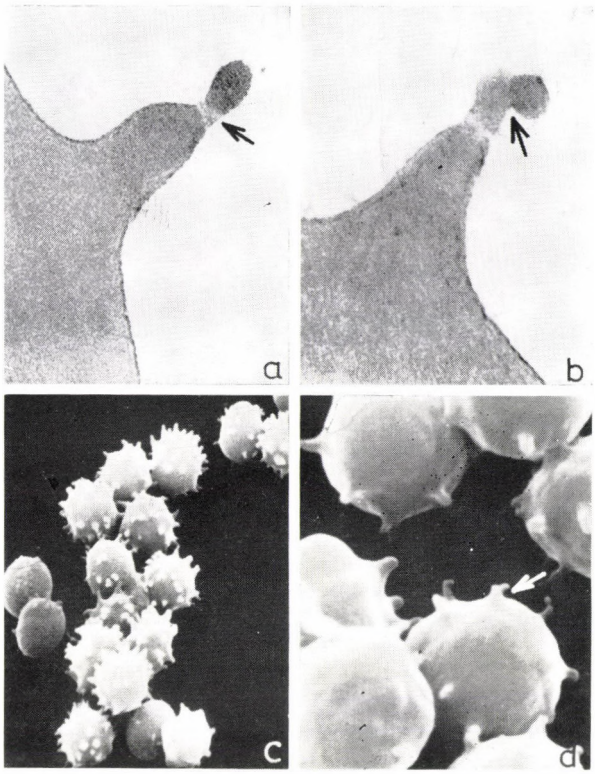

Fig. 2. Transmission (a, b; × 50 000) and scanning (c, d; × 3000 and 12 000) electron micrographs of stored RBC-s. Vesicles (microspherules) are released from the spicules of spheroechinocytes. (Note narrowings on the spicules: marked by arrows)

Changes in properties of red blood cells during storage and effects of rejuvenation in vitro

Morphology. In accordance with previous studies [16, 17] discocyte-echinocyte (D – E) transformation of stored cells resulted in a morphologically heterogeneous population (Fig. 4a). Morphological index (Im) values indicated, however, that shape changes progressed with a certain regularity in the function of storage time (Fig. 5). Low numbers of echinocytes were observed after three hours of incubation in rejuvenated samples in the whole course of experiments and different lengths of previous storage periods did not markedly increase the proportion of residual spiculated cells (Fig. 5). This indicated that most of the stored cells maintained the ability to change the echinocytic shape, even if long storage had preceded the rejuvenation. Morphological indexes, calculated on rejuvenated samples in the usual way (multiplying factor = 1 for all non-spiculated forms) could be used to characterize this decrenation

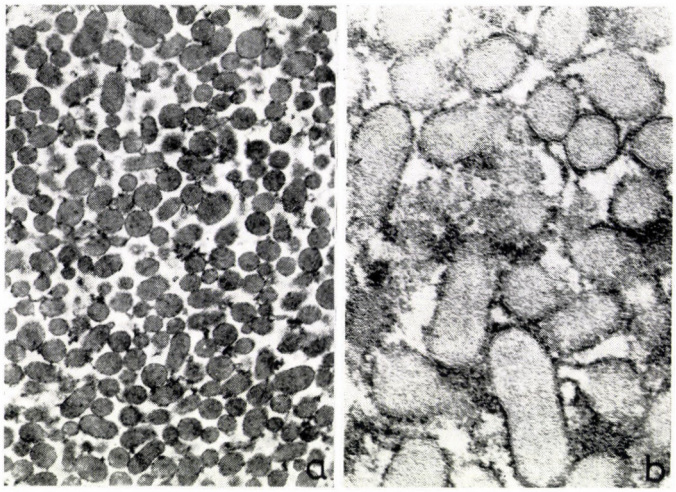

Fig. 3. Transmission electron micrographs of vesicles isolated from the plasma of stored blood. a. Conventional preparation: glutaraldehyde-osmium fixation; uranyl acetate — lead citrate staining; ultrathin sectioning. Small bodies with round or elongated profiles can be seen. Intravesicular electron opacity indicates haemoglobin content of vesicles. × 20 000. b. Preparation as above, but completed with ruthenium red staining. Note unit membrane profiles and staining of the cell coat. × 100 000

capability of stored red cell populations (Im_{r1}, – "level of reversion") It must however, be emphasized that the time of storage was reflected in the morphology of stored-rejuvenated samples. The most striking feature was that a fraction of rejuvenated cells transformed into spherostomatocytes and the number of these forms increased with the increase of storage periods. This indicated that these cells have lost some membrane material and while they could change their shape during rejuvenation they were not able to re-establish the original ratio of surface area to volume (S/V) and to reverse completely into normal discocytes (Fig. 4b–c). Comparison of the morphology of the same samples before and after rejuvenation suggested, that predominantly the spheroechinocytes II of non-rejuvenated samples had become spherostomatocytes during incubation, in other terms, these forms were not reversible if the criteria of reversibility were more strict. Morphological index, calculated with multiplying factor = 0 for spherostomatocytes indicated the possible degree of restoration of the normal morphology.

(Im_{rq}, – "quality of reversion". This term was used to emphasize that some qualitative aspects of cell morphology were taken into consideration in evaluating reversibility.)

The *ATP level* of RBC-s decreased progressively during storage. Incubation of stored cells in adenosine containing media at 37 °C for 3 h resulted in a marked increase of intracellular ATP level. Although lower values were measured in

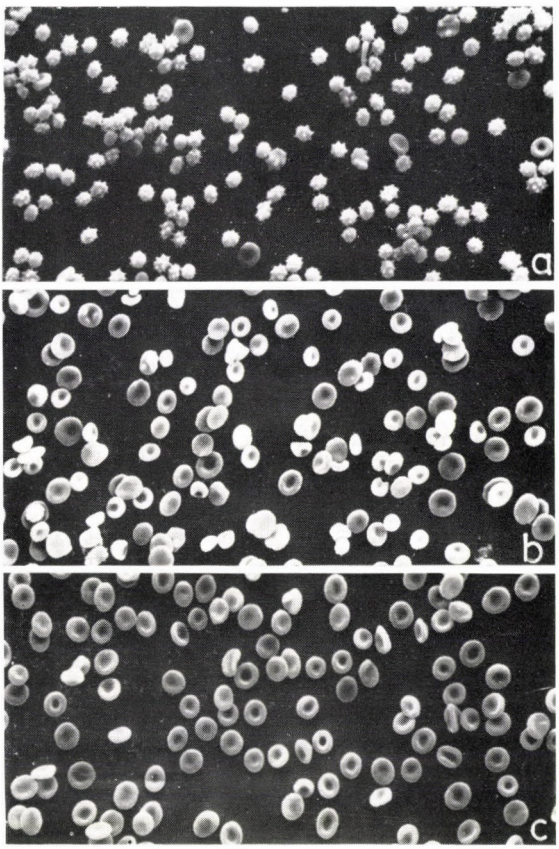

Fig. 4. Scanning electron micrographs of erythrocytes. a. Cells stored for 7 weeks. The population of stored red cells is morphologically heterogeneous: different intermediate forms of discocyte-echinocyte transformation can be observed. b. Cells stored for 7 weeks and rejuvenated. Spiculated forms almost disappeared. Spherostomatocytosis is obvious when compared with the morphology of a fresh sample (c)

rejuvenated RBC-s than in fresh ones, neither the duration of previous storage periods nor the prerejuvenation values were reflected in the ATP level of rejuvenated cells (Fig. 5).

Cell volume. Electronic particle size measurements indicated that during storage the mean cell volume (MCV) increased as a function of time. The changes in the ratio peak count per integral count showed a parallel increase of inhomogeneity in the volume distribution of cell populations. Rejuvenation resulted in a normalization of these parameters (with a slight tendency to overshoot) independently of the time of storage. The 90° light scatter signal distribution curves of the same samples showed that both the mean of the relative signal heights and the inhomogeneity of signal distribution increased during storage. Rejuvenation

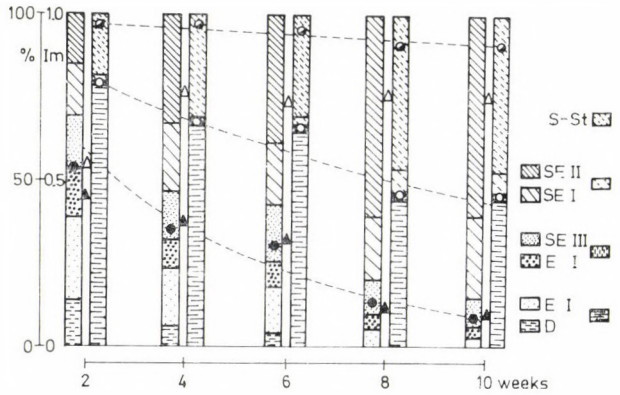

Fig. 5. Proportion of different shapes of erythrocytes in percents as a function of time of storage. Left columns represent non-rejuvenated and right columns rejuvenated samples (D — discocyte; E — echinocyte; SE — spheroechinocyte; S-St — "microspherocyte, spherostomatocyte). Morphological indexes (marks in the columns; tendency of changes showed by broken lines): ● — Im (multiplying factors: D and EI — 1.0; EII—III — 0.5; SEI-II — 0.0), ◑ — Im_{rI} (factors as for Im but S-St shapes designated by 1.0), ○ — Im_{rq} (percentage of S-St forms multiplied by 0.0, otherwise as above). ATP levels (marks between the columns), expressed as percent of ATP content of fresh red cells. ▲ — stored; △ — rejuvenated

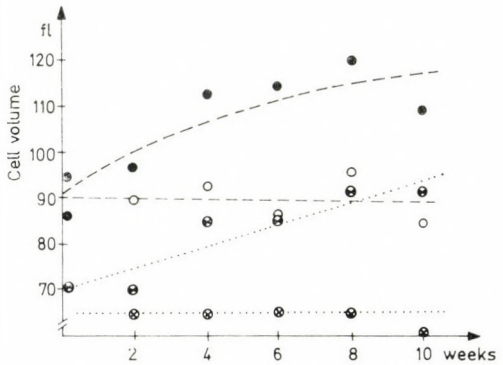

Fig. 6. Relative changes of signal height distribution obtained by electronic particle sizing. (Stored: ● — mean; ⊖ — peak position. Rejuvenated: ○ — mean; ⊗ — peak position)

changed the light scattering of stored cells toward normal, but it could not completely restore the prestorage parameters. The residual abnormalities in the light scattering of rejuvenated cell populations reflected the time of storage (Figs 6–8).

Osmotic fragility. The "median corpuscular fragility" (C_{50}: concentration of PBS in mOsm/kg, when 50% of erythrocytes were haemolysed) increased slightly after three weeks of storage. Rejuvenation lowered C_{50} below its prestor-

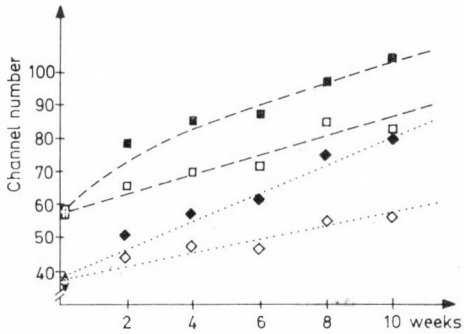

Fig. 7. Relative changes of signal height distribution measured by 90° light scattering method. (Stored: ■ — mean; ◆ — peak position. Rejuvenated: □ — mean; ◇ — peak position)

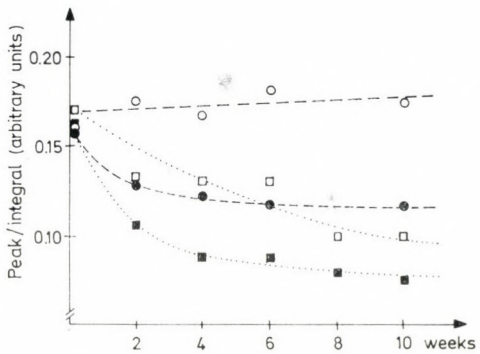

Fig. 8. Changes of the uniformity of cell volume signals expressed as the peak count per integral count ratio of signal height distribution. (Electronic particle sizing: ● — stored; ○ — rejuvenated. 90° light scattering: ■ — stored; □ — rejuvenated)

age value, thus it showed a marked overshoot effect on restoration of the mean osmotic fragility. However, C_{50} of rejuvenated cells increased also after three weeks, so during storage, the time-course of C_{50} changes were alike in both cases.

Although small changes were only observed in C_{50} values, other parameters of osmotic fragility curves (slope and σ) showed clearly that the osmotic behaviour of red cell populations had changed markedly during storage because of the pronounced increase of inhomogeneity in osmotic fragility. Rejuvenation modified the parameters of fragility curves, but not to the prestorage values, and the increase of inhomogeneity of cells during storage was reflected in the fragility curves also after rejuvenation (Figs 9, A and B).

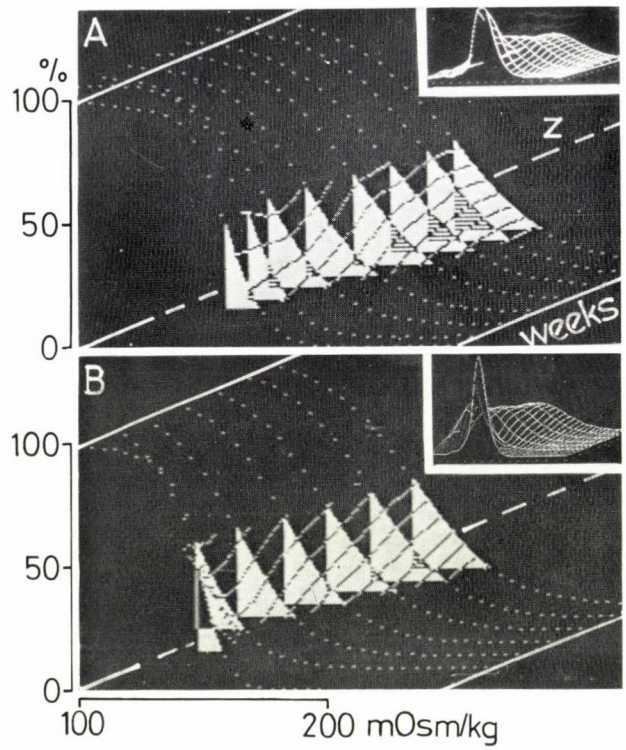

Fig. 9. Osmotic fragility curves during storage. Percent of haemolysis measured in different concentrations (in mOsm/kg) of PBS plotted versus time of storage. Fragility curves after different storage periods (marked on Z axis) on non-rejuvenated (A) and on rejuvenated (B) washed red cells. (Inserts: Fragility distribution curves derived from fragility curves). Triangle like profiles represent the areas limited by the C_{50} to $C_{50} + \sigma$ distances on the fragility curves and by the intersecting projections of this section to the X (concentration) and Y (haemolysis) axis, respectively. Note the pronounced increase of inhomogeneity in osmotic behaviour of the red cell population during storage (slope and σ of curves). Rejuvenation had modified these parameters, but could not restore the prestorage values. Small changes were found in the position of C_{50} ("median corpuscular fragility")

Discussion

Injuries of the *cell membrane* are of particular importance to the reversibility of storage changes owing to the fact that some loss of the cell membrane material could be demonstrated in erythrocytes preserved under blood bank conditions [18, 19]. The mechanism of this damage might have some similarities to the processes observed in other *in vitro* systems [20–26]. Bessis and Mandon [23] demonstrated morphologically a release of membrane fragments and myelin figures in heparinized blood stored at room temperature. A specific release of spectrin-free vesicles from erythrocytes during ATP-depletion at 37 °C was ob-

served by Lutz et al [24] and in subsequent studies these vesicles were compared with the fragments isolated from stored blood [25, 26]. Weed and Reed [20] reviewing the mechanisms of haemolytic disorders concluded that *fragmentation of the red cell membrane* may lead to a shortening of the life span of erythrocytes by decreasing the ratio surface area to volume, which in turn may reduce the cell deformability, a determinant of *in vivo* survival of RBC-s.

Our results support the previous observations that a progressive *membrane loss* of erythrocytes occurs during liquid preservation of blood at 4 °C. This may be *related to the discocyte-echinocyte transformation* of stored cells, because the microspherules or vesicles are *released from the spicules of sphero-echinocytes* in the form of membrane-bounded red cell fragments. Examinations by TEM of isolated fragments on ultrathin sections showed the presence of cell coat components and the right-side-out orientation of the membrane. The progressive fragmentation of the cells was reflected in the *accumulation of released vesicles* in the anticoagulant medium as a function of the time of storage. Fragmentation should limit the *in vitro* reversibility of changes caused by storage, because it can hardly be supposed that rejuvenation of erythrocytes could induce some recovery of the lost membrane material. Some reintegration of lipid components cannot be excluded, either when cells are incubated in the medium in which they have previously been stored, but this is very improbable as far as the membrane proteins are concerned.

Weed et al. [16] called attention to the significance of the morphological heterogeneity of erythrocytes when reversibility is examined in relation to the membrane loss. During storage an increasing fraction of more or less membrane-deficient cells appears, and after rejuvenation the *membrane-deficient cells transform into sphero-stomatocytes* because of their inability to restore the surface area to volume ratio of the normal discocyte. The percentage of cells, which are able to restore the normal discocyte shape, decreases during storage, and this may be considered as the revealing feature of *impaired morphological reversibility*, regardless of the maintenance of the decrenation capabilities. Thus, on a morphological basis we propose Im_{rq} for the semiquantitative approximation of the real D-E-reversibility of stored red cells.

The increase in the ratio of the acetylcholinesterase activity to vesicle protein suggests, that the release of vesicles from older cells precedes the fragmentation of younger erythrocytes. On the base of the acetylcholinesterase activity to density (age) relationships of red cells [27], this is consistent with the previous observations showing that D-E transformation occurs preferentially in the denser cell fractions [17]. As a consequence, the release of vesicles increases the initial *inhomogeneity* of stored red cell populations.

The decrease of the ATP level was comparable with the progression of D-E transformation of cells during storage, while after rejuvenation, neither the percentage of residual spiculated forms nor ATP levels reflected the time of the previous storage. The correlation between shape and ATP content of RBC-s has been studied in various experimental conditions, and it was shown that ATP has

an important role in maintenance of the discoid form [13, 28]. On the other hand, there are evidences that the shape is not strictly correlated with the ATP level of erythrocytes [29–31], indicating that ATP exerts its effect on shape transformation indirectly, and several mechanisms are supposed to play a role in the regulation of red cell shape on the level of the molecular organization of the membrane [32]. In previous experiments we compared the time-course of the ATP level with the shape changes during rejuvenation of stored RBC-s, and we could also observe that ATP/morphology relationships might be modified considerably by the conditions of incubation (e. g. composition of medium, temperature) [33]. Thus, ATP and morphology must be tested separately when the effects of different rejuvenation conditions are examined. The ATP level, however, like other mean cellular parameters of rejuvenated cells, must be considered under reserve when evaluating the reversibility of storage changes.

Rejuvenation-induced changes in morphology, ATP level, cell volume and median corpuscular fragility of stored cells may indicate that the enzyme systems and the regulatory mechanisms (e. g. controlling shape and volume) can be activated by rejuvenation, regardless of the longer than usual periods of storage. This seems natural in view of the remarkable self-repairing abilities of the red cell membrane, for experimental data show that shape changes [34], ion-transport mechanisms [35], etc., can be examined even in resealed ghosts and/or in vesicles prepared from the erythrocyte membrane [36]. The changes of cell volume and volume distribution and their reversibility during storage observed by electronic sizing might seem somewhat embarrassing in the light of the considerable loss of membrane components. It may, however, be estimated from the cell to vesicle size relationship that the release of vesicles leaves the initial bulk mass of the erythrocyte proteins almost unaltered due to the much higher surface to volume ratio of the fragments compared with whole cells. On the other hand, the cell volume is determined by the osmotically active intracellular components and by the permeability of the membrane when erythrocytes are in equilibrium with the suspending medium.

In stored blood the active sodium-potassium transport across the erythrocyte membrane is almost stopped and, despite of the small passive cation permeability of the membrane at 4 °C, the intra- and extracellular concentrations tend to come into equilibrium, which results in an increase in red cell sodium and in a loss of intracellular potassium. The inability of cells for the active maintenance of the concentration gradients between the intra- and extracellular cations and the decrease of the intracellular pH, promote an intracellular accumulation of osmotically active anionic intermediates of cell metabolism (e. g. lactate and phosphate). These events may explain the progressive increase in MCV during storage. The increase in inhomogeneity of volume distribution may reflect that these alterations, like morphological changes, disturb some cells more than others.

On the other hand studies of the sodium-potassium transport of stored RBC-s showed that the active sodium-potassium transport capacity of cells, which has markedly decreased during storage, could be restored to (or above) normal

by rejuvenation [37]. This indicates that the main mechanism by which red cells control their intracellular cation concentration and membrane permeability can be reactivated. The restoration of intracellular ATP and 2,3-DPG levels and pH, creates also favorable conditions for the elimination of the surplus amounts of the osmotically active intracellular substances. Thus, when rejuvenated, stored red cells regain the ability to maintain the steady state ion concentration gradients and this results in a decrease of the cell volume in isoosmotic media, in spite of the different membrane loss, which they had suffered during previous storage.

With electronic particle sizing a close relationship of pulse height to particle volume can be obtained under appropriate conditions. Light scatter signals, however, provide more complex information about size and structure (e. g. shape, structure on the surface and in the internal space, refractive index, etc.) of the particles under study. Therefore, the residual abnormalities found in the light scatter signal distribution curves of rejuvenated samples may reflect the morphological inhomogeneities of the cell populations, which are related rather to the reduced surface to volume (thickness to diameter) ratio of the spherostomatocytes present, than to the cell volume *per se*.

The osmotic fragility of erythrocytes depends on the relationship of the cell volume to the area of non-distensible membrane, thus it should reflect the changes of S/V ratio caused by membrane loss. The osmotic fragility of stored red cells has been studied by numerous investigators, but its relationship to the adequacy of preservation procedures could not be established. More recently, however, Beutler et al. [38] using the sequential haemolysis technique to measure osmotic fragility of reinfused ^{51}Cr-labelled stored cells, demonstrated that a fragile fraction of erythrocytes was removed from the circulation within 24 hours after transfusion.

Haemolysis is known to be influenced, in addition to the S/V ratio, by a number of diverse factors (e. g. temperature, ionic strength, pH, integrity of membrane skeletal components, etc.). In the present experiments it seems that the median corpuscular fragility (C_{50}) reflected predominantly the average of changes in the composition of the intracellular milieu and in membrane permeability, as discussed above in relation to changes of the cell volume. Osmotic fragility measurements were carried out on red cells, washed and suspended in PBS, thus the small increase of the median fragility during storage may be attributed to the washing out of intracellular lactate [38].

The overshoot effects of rejuvenation on the C_{50} values and on the volume of cells may be interrelated phenomena, which presumably are due to the potassium deficiency of stored cells. Rejuvenation reactivates the sodium-potassium transport and permeability of stored cells, but the net uptake of potassium during this incubation remains insufficient to restore the potassium content. The steady state transmembrane ion concentration gradients might be restored at the expense of intracellular water content. More important changes were found in other parameters of the fragility curves. As osmotic fragility curves represent the cu-

mulative frequency distribution of erythrocyte fragility, the analysis of fragility curves can provide information about the distribution parameters of cell populations. The irreversible increase in inhomogeneity of cell populations may be related to the formation of fragile subpopulations due to the preferential fragmentation of spheroechinocytes. Our results suggest that the distribution parameters of cell populations rather than mean or median values might be used in evaluating the reversibility of storage changes *in vitro*.

Abbreviations

ACD	acidic citrate dextrose
ATP	adenosine 5'-triphosphate
2,3-DPG	2,3-diphosphoglycerate
MCV	mean cell volume
PBS	phosphate buffered saline
PCV	packed red cell volume
RBC	red blood cell
SEM	scanning electron microscope/y
TEM	transmission electron microscope/y

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Comparison of the Rheological Behavior of Hemocytometry Controls and Fresh Patient EDTA-Anticoagulated Blood Specimens

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For rheological reasons high viscosity specimens are more sensitive to deficiencies, if any, in the aspiration systems of whole blood flow cytometers and consequently are more likely to give erroneous values. For this reason the rheological behavior of hemocytometry controls was compared with that of patient blood specimens. It is shown that the behavior of controls and patient specimens of comparable hematocrit is similar at shear rates that are probably occurring during aspiration (exceeding approximately 40 s^{-1}). The hematocrit is shown to be a major determinant of the viscosity of both controls and patient specimens. The results suggest that from the rheological point of view the fundamental IFCC premise is met, "that errors detected by means of control specimens exactly mirror errors occurring with patients' specimens", provided their hematocrits are taken into account.

Keywords: EDTA-anticoagulated blood; flow hemocytometry control materials; quality control; rheology; viscosity.

Introduction

Apart from the obvious requirement of stability of values of hemocytometric control materials, Bull [1] states two further desiderata for an ideal control material: "It should be inexpensive and convenient (to encourage frequent use) [2, 3] and it should behave like fresh whole blood (so that it can be processed in the same way as blood samples) and thus provide control for the entire analytical process". Likewise, Groner [4] wrote that for quality control materials "it is desirable that these materials share all relevant characteristics with the specimen (blood sample)".

This requirement implies a set of specific physical features of each material. The rheological properties are one of these features [5]. For a variety of reasons vacuum leaks can occur in the blood aspiration system of Coulter [6] and other counters; this might go undetected if the viscosities of control materials would be much lower than those of patient EDTA-blood specimens. This might result in sluggish and/or incomplete patient blood flow and concomitant erratic values

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for the patient's blood. Our own laboratory experience showed the crucial importance of the viscosity of blood: due to an insidious vacuum leak in the Coulter suction system our "abnormal high" viscosity control (hematocrit (Ht) 63%) invariably gave too low values. (Ht is the most important single determinant of blood viscosity [7]). Rabinovitch [8] showed that the Coulter quality control material "4C" cannot be used on an Ortho ELT-8 instrument "for the simple reason that its viscosity results in variable aspiration volumes".

A thorough search of the recent literature shows no data whatsoever on the viscosity of blood control materials [1, 4, 5, 9–11].

The objectives of this paper are

(1) to gain preliminary insight in the viscosity characteristics (rheology) of 4 commercial and 4 laboratory-made [3] control materials;

(2) to compare these characteristics with those of 4 routine patient EDTA-blood specimens with comparable Ht values;

(3) to investigate whether the use of plasma instead of our artificial preservation medium containing albumin [2] had any beneficial effect on the rheological behavior of laboratory-made control materials.

Materials and Methods

Viscosity measurement technique

The viscosity measurements in this study were carried out with a Contraves Low Shear 30 viscometer (Contraves AG, CH-8052 Zurich, Switzerland) [7, 12–14], using a cup width of 0.5 mm. The (modified) technique, as described in full detail by Goslinga et al. [14], had previously been shown to be highly reproducible due to automation and rigorous standardization. Coefficients of variation of approximately 3% at the lowest (0.016 s^{-1}) and ca. 0.4% at the highest (118 s^{-1}) shear rates were obtained. In short, salient features include:

1. refinement of zero-point setting by applying remote control, thus obviating unsatisfactory vibrations [13, 14];

2. guarantee of accurate measurements by a calibration program with a calibrated standard oil;

3. consistent treatment of the specimens before the actual measurements by rotating them in a water bath of constant temperature;

4. invariable start of measurements, after thorough mixing, at the lowest shear rate, proceeding towards higher shear rates to avoid hysteresis variability [7, 14];

5. routine-plotting of viscosity-versus-time curves (not shown) to facilitate the recognition of artifacts such as an air bubble, a small hair, a non-centered bob, clots or fibrin strands in the sample or an inhomogeneous blood sample, thus controlling the quality of the measurement technique;

6. automatic standardization of the time factor by computer-controled (HP 9815S) measurements. These measurements are spread over the entire range of

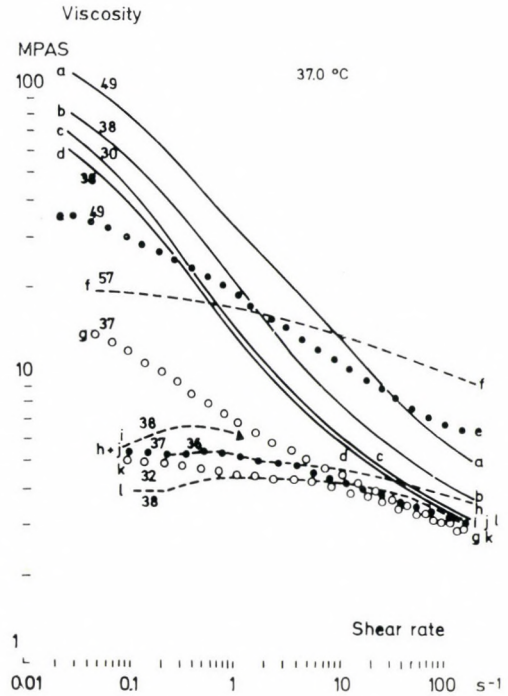

Fig. 1. Composite display of rheograms, showing log. viscosities versus log. shear rates of various groups of specimens. All measurements were performed at 37.0 ± 0.1 °C. Figures accompanying the curves designate hematocrits (in %) as measured in a Coulter Counter Model S-Plus II. Full curves (a – d): fresh EDTA-anticoagulated blood specimens; Dashed curves (f, h, i, l): commercial controls, viz: Hyland's "Eight-parameter Abnormal High" control (f), Coulter's "4C-Plus II Normal" control (h), Ortho's "Normal Hematology" control (i) and Hyland's "Eight-parameter Normal" control (l); Circled curves (e, g, j, k): laboratory-made controls viz. open circles (g and k) plasma as the suspending medium and full circles (e and j) artificial suspending medium containing albumin. For comments see the text

shear rates (0.016 to 118 s^{-1}); at every shear rate a specific length of time is required between the beginning of rotation and the moment of measurement, starting with 110 seconds at a rate of 0.016 s^{-1} and decreasing to 10 seconds at a shear rate of 118 s^{-1}. Thus, measurements on one sample could be made within five minutes, minimizing the effect of settling out of the red blood cells (rbc) during the measurement [13, 14].

To facilitate comparison of different specimens, the viscosity is represented graphically, versus the shear rate in a double logarithmic coordinate system yielding rheograms (Fig. 1).

For practical reasons the measurements were performed at 37.0 ± 0.1 °C, although some samples were also measured at 20.0 ± 0.1 °C (Fig. 3).

Fresh patient EDTA-anticoagulated blood specimens

Four fresh patient EDTA-anticoagulated blood specimens (Fig. 1: full curves *a*–*d)* of varying hematocrits (Ht's) were measured within seven hours after drawing.

Hemocytometric controls

a. Laboratory-made controls

Two of the measured controls (Fig. 1, curves *e* and *j*) were prepared in an artificial (high-K, low-Na) suspending medium containing 3% albumin, as described before [3, 15].

Although this preservation medium is satisfactory, there are two reasons to study the possibility of using normal transfusion plasma instead:

1. Further simplification of blood control processing: (compatible) plasma can be stored frozen and consequently is ready at hand;

2. Hemorheological considerations.

In a previous paper [15] we have mentioned that substances such as citrate, mannitol and sucrose are impermeable to rbc and consequently exert an oncotic effect. We found that the citrate concentration in the plasma anticoagulant CPD-A was insufficient for fully counterbalancing the impermeable hemoglobin and other osmotically active substances such as lactate [15]. This has recently been confirmed by Hoegman et al. [16]. We invariably found satisfactory Mean Cell Volume (MCV) constancy after addition of 0.55% mannitol (final concentration) to CPD-A plasma. Nevertheless, our impression was that neither MCV nor Red cell Distribution Width (RDW) are constant during periods as long as those found in our control [15]. This needs further investigation. Furthermore, it should be realized that the percentage of mannitol to be added is dependent on the (quite variable) citrate concentration in the citrate-containing anticoagulants [17].

One of the objectives of this paper was to study whether the use of plasma instead of the artificial medium had any beneficial effect on the rheological behavior of laboratory-made control materials (Fig. 1, curves g and k).

b. Commercial controls

One "abnormal high" control viz. Hyland's "Eight-parameter High Abnormal" control, lot No. 3118 H was measured, 30 days before its expiration date (E. D.) (1 March 1984) (Fig. 1, curve f). Its stated Ht was 56.5%. Three "normal" controls were measured: Coulter's "4 C-Plus II Normal" control, lot No. 9378, was measured 7 days before its E. D. (9 January 1984) (Fig. 1, curve h (Ht = 36%); Ortho's Hematology control blood, lot No. 83 LO7/746, was measured 4 days before its E. D. (5 February 1984) (Fig. 1, curve i) (Ht = 38%); Hyland's "Eight-parameter Normal" control, lot No. 398N, was measured 33 days before its E. D. (31 December 1983) (Fig. 1, Curve l) (Ht = 38%).

Results

Figure 1 (full curves a – d) shows the well-known non-Newtonian character of fresh patient EDTA-blood specimens [7]. The figures accompanying the curves designate the Ht of the specimens. Although in normal blood, Ht is the most important single determinant of blood viscosity [7], inspection of curves a – d shows that other (partly unknown) determinants can decisively affect fresh blood viscosity values over the entire shear rate range.

The dashed curves f, h, i and l in Fig. 1 represent the rheograms of the 4 commercial controls examined. They all show a characteristic (nearly –) Newtonian behavior (i. e. independence of viscosities on shear rates). The viscosities of the "Normal" Coulter (h), Ortho (i) and Hyland (l) controls are of the same order of magnitude, and so are their Ht's (36 – 38%). In contrast, curve f, representing Hyland's "Abnormal High" control, roughly shows three to four times higher viscosities than the "Normal" controls. This clearly demonstrates that in commercial controls, too, Ht has a decisive influence on the viscosity.

Figure 1 also shows that the viscosity of the "Normal" controls (h, i, l) approaches those of EDTA-blood specimens with comparable Ht's (b – d) only at shear rates exceeding at least 40 s^{-1} (as estimated from the numerical values, which are not shown). On the other hand, Fig. 1 demonstrates that the viscosity of Hyland's "Abnormal High" control (f) at shear rates exceeding 0.5 – 10 s^{-1} is substantially higher than the viscosity of fresh EDTA-anticoagulated blood specimens with normal Ht (Fig. 1, a – d).

The circled curves e, g, j and k in Fig. 1 represent the rheological behavior of our laboratory-made controls. The full-circled curves *e* and *j* make up the rheograms of cells in our artificial suspending medium, containing approximately 3% albumin; the open-circled curves *g* and *k* represent those of cells in mannitol-containing plasma. These media are called artificial and plasma medium, respectively. Controls *j* (artificial medium) and *k* (plasma medium) approximately coincide with those of the "Normal" commercial controls *h*, *i* and *l*. Consequently, their viscosity is also much lower than that of comparable EDTA-blood specimens with corresponding Ht *b* – *d*, up to shear rates of approximately 40 s^{-1}. At the time of measurements, specimens *j* and *k* were 6 weeks and 11 weeks old, respectively. Specimen *j* (artificial medium) showed minimal hemolysis, whereas specimen *k* (plasma) exhibited substantial hemolysis. In contrast, curves *e* (artificial medium) and *g* (plasma medium) show a non-Newtonian, more or less parallel, behavior. However, they fail to coincide with the fresh EDTA-blood curves. Curve *e* represents the rheogram of the multiparameter hemocytometry control recently described in detail (3). The control was 11 weeks old at the time of the measurement, consequently hemolysis was rather extensive (3). Its viscosity at a shear rate exceeding 30 s^{-1} was comparable to the EDTA-specimen with an equal Ht (curve a). Curve *g* exhibits the rheogram of fresh rbc suspended in plasma and measured 6 days after preparation.

Figure 2 shows the viscosity changes of the laboratory-made fresh red blood cell-plasma control corresponding to curve *g* in Fig. 1, during storage at 4 °C as a function of the shear rate. At low shear rates the viscosities decrease, whereas at higher shear rates (exceeding 0.1 to 1.0 s^{-1}) they increase. These effects are more pronounced with measurements at 20 °C (full curves) than with those at 37 °C (dashed curves).

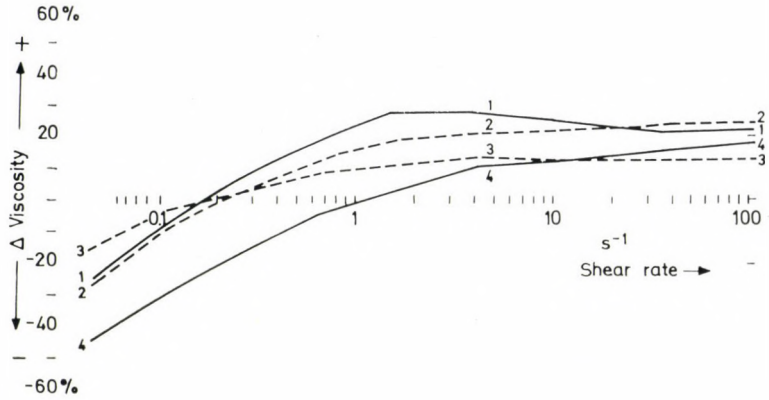

Fig. 2. Viscosity changes of the laboratory-made fresh red blood cell-plasma control (corresponding to curve g in Fig. 1) duringstorage at 4 °C as a function of shear rates. The full curves 1 and 4 represent measurements at 20 °C, the dashed curves 2 and 3 those at 37 °C. Curves 1 and 3 show the changes after 1 month of storage, curves 2 and 4 those after 2 months of storage. At low shear rates viscosities decrease due to decreased aggregation, whereas at higher shear rates (exceeding 0.1 to 1.0 s^{-1}) viscosities increase due to decreased red blood cell deformability. These effects are more pronounced with measurements at 20 °C than with those at 37 °C

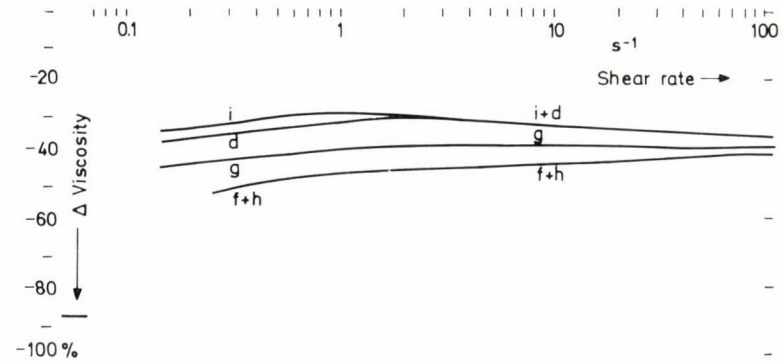

Fig. 3. Viscosity decreases due to temperature elevations from 20.0 ± 0.1 °C to 37.0 ± 0.1 °C. The curves represent specimens identical to those of Fig. 1 with corresponding letters. Curve d: a fresh EDTA-anticoagulated patient sample; curve i: Ortho's "Normal" control; curve g: laboratory-made fresh red blood cell-plasma control. Curves f and h: Hyland's "Abnormal High" and Coulter's "4C Plus II" controls (coinciding). Viscosity changes vary from 30–50% at low (0.3 s^{-1}) to 30–40% at high shear rates (100 s^{-1})

Figure 3 shows that elevating the measuring temperature from 20 °C to 37 °C, a range largely spanning worldwide ambient temperatures, results in decreases of viscosity of approximately 30 – 50% at low (0.3 s^{-1}) and 30 – 40% at high shear rates (100 s^{-1}) for both a fresh EDTA-blood specimen and for several commercial and laboratory-made controls (see legends to Fig. 3).

Discussion

The fresh patient blood specimens (Fig. 1, full curves a – d) exhibit the well-known non-Newtonian behavior [7]. Viscosities increase exponentially at the lower shear rates due to the presence of the plasma proteins albumin, fibrinogen and immunoglobulins; they overcome the zeta potential between red blood cells (rbc) and the rbc aggregate, forming rouleaux, causing a disproportionate increase in viscosity [7, 12, 18]. It seems appropriate to point out that erythrocyte sedimentation (ES), as routinely determined by the ESR(ate)-test, actually is a low-shear system exerted by gravity [7]. At higher shear rates, the rouleaux are dispersed and individual rbc are deformed into ellipsoids with their long axes aligned in the direction of flow [7, 12, 19].

In contrast, the commercial controls (Fig. 1, dashed curves *f*, *h*, *i* and *l)* and two of the four laboratory-made controls (Fig. 1, circled curves *j* and *k)* show a near-Newtonian behavior and their curves approximately coincide (apart from that of specimen *f* owing to its higher Ht). The general rule is that in non-aggregating suspensions much lower viscosities are observed at lower shear rates, whereas at higher shear rates viscosities slightly increase due to decreased rbc-deformability and hence these suspensions tend to behave as (near-)Newtonian fluids [20 – 25]. It might be mentioned that we found very low ESR-values for Coulter's and Hyland's "Normal" controls (Fig. 1, curves *h* and *l)*: 4 mm after 3 h and 12 – 18 mm after 24 h of sedimentation. On the other hand strong influences of the plasma composition on ESR-values and consequently on low shear viscosities are well-known from clinical practice.

Finally the remaining two laboratory-made controls (Fig. 1, circled curves *e* and *g)* showed a non-Newtonian behavior. However they did not coincide with fresh EDTA-blood specimens with comparable Ht.

Unfortunately, we can only partly explain the discordant rheological behavior of our laboratory-made controls. The data show that this discordance was not only due to the nature of the suspending medium: our albumin-containing artificial medium (curves *e* and *j)* versus plasma (curves *g* and *k)*. The non-Newtonian behavior of the controls *e* and *g* at lower shear rates might be related to incomplete discocyte-echinocyte shape transformation and consequently only partly decreased cell-cell interaction.

One of the controls was made from fresh rbc (curve *g)*, the other three controls were 6 – 11 weeks old and consequently partly hemolyzed. Viscosity is known to change during storage. At corresponding shear rates, hemolyzed packed

rbc (packed ghosts) in suspension generally show a viscosity slightly lower than that of intact rbc [23].

Figure 2 shows the viscosity changes of the fresh plasma control g during storage at 4 °C as a function of shear rates. We observed similar typical changes during storage of fresh EDTA-blood: viscosities at very low shear rates decrease due to diminished aggregation and viscosities at high shear rates increase due to diminished deformability of rbc (20 – 25). Figure 2 shows that these storage changes are slightly more pronounced when the viscosity is measured at 20 °C. However, Figure 2 also shows that the changes in this particular control specimen are only at most 20% in the probably relevant shear rate range above 40 s^{-1} (see below), which is negligible in comparison to the Ht-effect (vide infra). Similarly, we found negligible viscosity differences (ca. 10% at 20 °C) after storage of the Coulter control. (Not shown; it should however, be mentioned that we measured the control 6 weeks after its expiration date.)

Figure 3 shows that the temperature effect between 20 °C and 37 °C on the viscosity is similar for both fresh EDTA-blood and the controls including Hyland's "Abnormal High" control and is only slightly dependent on the shear rate. The viscosity changes amount to approximately 30 – 50%. It is important to realize that room temperature changes seem to have an equal effect on the viscosity of both patient and control specimens.

Figure 1 shows similar rheograms for Ortho's control and Coulter's "4C Plus II" control. Assuming that the rheogram of Coulter's control "4C" is similar to that of Coulter's control "4C Plus II", we cannot explain Rabinovitch's statement (8) mentioned in the Introduction, that Coulter's "4C" control cannot be used on an Ortho ELT-8 instrument "for the simple reason that its viscosity results in variable aspiration volumes".

Figure 1 – 3 confirm that the Ht is by far the most important single determinant of viscosity of both fresh blood and the controls (7): Fig. 1 (f) shows increases in the order of 300 – 400% for a commercial control and Fig. 1 (a) some 200% for fresh EDTA-blood due to Ht-differences, whereas Figs 2 and 3 show only 20% increase due to deformability decreases and only 30 – 50% due to temperature changes in the 20° – 37 °C range. Thus, specimens with high Ht will be much more sensitive to deficiencies, if any, in the aspiration systems of flow hemocytometers and consequently non-linearity or even erroneous values might occur. This explains our findings of too low values in an "abnormal high" control, mentioned in the Introduction. It would be even more important if polycythemic specimens were to be analysed.

In the Contraves viscometer, 28.7 revolutions per minute (rpm) and 98.0 rpm correspond to shear rates of 34.6 s^{-1} and 118.2 s^{-1}, respectively. The fundamental question is what the magnitudes of shear rates are that occur during whole blood flow in the aspiration systems of whole blood flow cytometers. It depends on the vacuum applied and the different geometries in the suction system of the particular instrument used. Consequently, shear rates are likely to vary markedly at various locations in a particular instrument and, a fortiori, in different instru-

ments. However, the appropriate shear rate range is very difficult to estimate; it would require extensive empirical model simulation studies [26]. For this reason the viscosities were measured over a wide range of shear rates. Nevertheless, judging from the force with which blood samples are generally sucked up into the hemocytometers, it seems reasonable to speculate that shear rates will be rather high, probably exceeding 40 s^{-1}. As Fig. 1 clearly demonstrates, the magnitudes of occurring shear rates are decisive for the rheological behaviour of fresh EDTA specimens in comparison to that of control specimens: at rates exceeding approximately 40 s^{-1}, their viscosities are numerically comparable. Consequently, the results suggest that from a rheological point of view the fundamental IFCC-premise [27] is met that "errors detected by means of control specimens exactly mirror errors occurring with patients' specimens".

In conclusion, this paper provides insight in the viscosity characteristics of hemocytometry controls in comparison to those of fresh patient EDTA-blood specimens (Fig. 1). Moreover, the results strongly suggest that the use of plasma instead of our artificial suspending medium, containing 3% albumin [3], does not make the rheogram more identical to that of fresh blood and consequently does not seem to have any beneficial effect on the rheological behavior of laboratory-made hemocytometry controls.

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Cytotoxic Activity of Peripheral Mononuclear Cells in Normal Pregnancy

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Killer-cell activity was studied in healthy pregnants in all three trimesters of pregnancy. Results were compared with values obtained three months after delivery, as well as with similar data of non-pregnant women. The effect of pregnant and non-pregnant sera on cytotoxic activity has also been investigated. Killer-cell activity was measured by a capacity test on antibody coated human red blood cells.

According to the results, healthy pregnancy did not influence the maternal killer-cell activity. On the other hand, the various sera had a decreasing effect on the killer-cell activity of both pregnant and non-pregnant women. Attention is called to the aspecific inhibitory effect of sera.

Keywords: maternal killer-cell activity, cytotoxic capacity, effect of sera, ADCC

Introduction

The immunological paradoxon of pregnancy was first described by Medawar in 1953 [1]. Immunologically speaking, the fetus and the placenta are hemiallografts, i. e. antigens foreign to the mother but histocompatible with the father are present in the conceptual tissue. Because of this presence of paternal antigens, one would expect that the fetus, as a victim of the maternal immune system, would be rejected by the organism. Fortunately, this does not happen under normal physiological circumstances, thus in the absence of such immune rejection the pregnancy can be regarded as an allotransplantation.

By knowing more about the immunological connection of the mother and the fetus, we might explain the physiological functions of the immune system during pregnancy, and it may be easier to clarify whether a pathological alteration in pregnancy has an immunological basis. According to present knowledge, the fetus and the placenta are protected from rejection by a combined mechanism [2]. Numerous authors have observed changes in the maternal cellular immune response during pregnancy. Functional investigations of maternal lymphocytes revealed a decreased reaction to mitogens [3–5], while others observed inhibitory effects of pregnant sera on lymphocyte functions [5–7], thus changes of the lym-

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phocyte subpopulations during pregnancy are unequivocal. At the same time, literature on the non-specific immune reaction transmitter O-cells that incorporate the natural killer (NK) and the killer (K)-cells, is rather scarce.

In the present study we have attempted to determine with the antibody dependent cell-mediated cytotoxic (ADCC) assay, whether there were any changes in maternal killer cell activity during pregnancy. Cytotoxic activity was expressed by cytotoxic capacity, which measures the maximal target cell-killing capacity of a lymphocyte unit [8]. Answers were sought to the questions as follows:

1. Is there any change in the cytotoxic capacity of maternal peripheral mononuclear cells (PBMC) during pregnancy?

2. How does the serum of pregnants and non-pregnants affect the cytotoxic capacity of PBMC of pregnant and non-pregnant women?

Materials and Methods

Sera and PBMC of healthy, symptom-free women were tested in the 6–11 (n = 40), 20–22 (n = 40) and 37–40 (n = 24) weeks of pregnancy. In 20 cases examinations were done in all the three trimesters of pregnancy. The controls included sera and PBMC of 20 women who had delivered three months earlier, and those of 42 healthy, non-pregnant women of reproductive age.

Separation of PBMC

PBMC were isolated from heparinized blood by Ficoll-Uromiro gradient centrifugation [9]. The glass-adherent and non-adherent cells were counted. The non-adherent cell concentration was adjusted to working values with RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum.

Cytotoxicity assay

The human 0 Rh(D) +ve erythrocyte (phenotype R_1R_2) ADCC test was carried out according to Urbaniak [10]. The cytotoxic capacity test was applied by the enzyme-like kinetic model of Zeijlemaker et al [11] and Garam et al [8]. We measured the maximum target-cell killing capacity – set onto 1×10^6/ml non-adherent cell number – of PBMC.

Supernatant activity was measured by an NK-350 gamma counter.

Cytotoxic reaction modifying effect of sera

Based on earlier investigations, we have observed that regardless of the PBMC-target cell ratio, the sera inhibited or stimulated the cytotoxic reactions of the PBMC similarly. Consequently, it was necessary to measure the cytotoxic

capacity only at one point, in this case at 10×10^6/ml target cell concentration. Accordingly, 50 ml inactivated serum was added to the test system (inactivation lasted 1 hour at 56 °C). The serum dilution was 25%. We also investigated the effect of serum of pregnant as well as non-pregnant women, and the pooled sera of AB RH-ve donors on the PBMC function of pregnant and non-pregnant women.

Statistics

Results are expressed as mean valued ± S. E. The data were analysed with Student's *t* test.

Results

Table 1 shows the cytotoxic capacity of healthy pregnant women during the first, second and third trimesters, as compared to that of healthy but non-pregnant women. It is obvious from Table 1 that during the various phases of pregnancy the cytotoxic capacity remained stable and showed no change as compared to the cytotoxic capacity of healthy women.

Table 2 shows the cytotoxic capacity of the 20 pregnants examined in all the three trimesters as well as three months after delivery. The data confirmed the finding that cytotoxic capacity is unchanged during a normal pregnancy.

Table 1

Cytotoxic capacity of the PBMC of healthy pregnant and non-pregnant women

	Pregnants			Non-pregnants
	I	II	III	
	trimester			
Cytotoxic capacity	n = 40 1.07 ± 0.09	n = 40 0.98 ± 0.08	n = 24 0.84 ± 0.11	n = 42 0.96 ± 0.07

Human erythrocyte ADCC/mean/± SE/ × 10^6

Table 2

Cytotoxic capacity of the PBMC of 20 healthy pregnants in the three trimesters of pregnancy and three months after delivery

	Pregnants		n = 20	Controls	
	I	II	III	Three months after delivery n = 20	non-pregnants n = 42
	trimester				
Cytotoxic capacity	1.01 ± 0.11	1.02 ± 0.11	0.95 ± 0.13	1.07 ± 0.18	0.96 ± 0.07

Human erythrocyte ADCC/mean/± SE/ × 10^6

Table 3

Effect on cytotoxic capacity of PBMC of serum of pregnant and non-pregnant women

	Pregnants			Non-pregnants
	I	II	III	
	trimester			
Cytotoxic capacity	n = 40 1.07 ± 0.09	n = 40 0.98 ± 0.08	n = 24 0.84 ± 0.11	n = 42 0.96 ± 0.07
Cytotoxic capacity measured with own serum	n = 38 0.53 ± 0.06*	n = 40 0.44 ± 0.11*	n = 22 0.44 ± 0.11**	n = 40 0.53 ± 0.06*
Cytotoxic capacity measured with AB blood group pooled sera	n = 40 0.62 ± 0.0 *	n = 40 0.53 ± 0.08*	n = 24 0.38 ± 0.10***	n = 40 0.55 ± 0.06*

Human erythrocyte ADCC/mean/± SE± $\times 10^6$
*$p < 0.001$; **$p < 0.01$; ***$p < 0.0025$ as compared to basic values

As to the PBMC activity modifying effect of sera, both pregnant and non-pregnant sera significantly decreased the cytotoxic capacity of PBMC of both pregnant and non-pregnant women *(Table 3)*.

Discussion

The specific killer-cell activity of healthy pregnant women was tested by our own method [8]. Considering the enzyme-like nature of cytotoxicity, its activity was determined by a cytotoxicity assay. The women were investigated in the first, second and third trimesters of pregnancy. It was also determined how the serum of pregnant and non-pregnant women affected the cytotoxic capacity. At the same time, K-cell activity was found to be unchanged in normal pregnancy. This supports earlier findings [12–15] that during pregnancy the mother's lymphocyte functions, at least as far as the killer-cells are concerned, remain unchanged.

On the other hand, the sera of pregnants had a marked inhibitory effect on the cytotoxic activity of PBMC, as noted in both pregnant and healthy non-pregnant women, as described by other authors [16–20].

Inhibition of cellular immunity by serum was attributed to several factors. Hormones produced during pregnancy may be one of these, while Stites et al [21] have demonstrated similar effects of cortisol and progesterone. The lowering effect on lymphocyte reactivity of progesterone was also shown by Siiteri et al [22] and Szekeres et al [23]. Other authors emphasized the role of placental oestrogens [24, 25], while some suggested that human chorio-gonadotrophin (HCG) was the key factor [26–28]. Murgita et al [29] stressed the role of alpha-

fetoprotein, while according to Petri et al [19] the so-called soluble mediators produced by lymphocytes were responsible for the inhibition.

Prostaglandins (PGs) may also have a role in the immune response. For example, PGE has a decreasing effect on the cellular immune response [30, 31], but the most important lymphocyte blocking agent is produced by the immune system itself. It is an IgG-type blocking antibody and this substance, in one respect, obstructs sensibilization, while on the other it protects the antigen structured fetal tissues from the direct effect of cytotoxic lymphocytes [32–33].

These studies have shown that the inhibitory effect of non-pregnant, AB blood group pooled serum was similar in cytotoxic capacity to that of PBMC of both pregnant and non-pregnant women. Thus, the effect can be exerted by other than pregnancy specific substances. In tests in vitro, the serum of non-pregnant women also contains materials that are equally effective inhibitors of the cytotoxic activity of killer-cells of both pregnant and non-pregnant women.

These results are only the beginning of a series of investigations calling attention to some of the aspecific inhibitory factors of sera in a given immunological process. The isolation of inhibitory factors and the role of killer-cell activity in various pathological pregnancies demands further investigations.

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Granulocyte Enriched Buffy Coat Transfusions in Neutropenic Patients

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Twenty four courses of granulocyte enriched buffy coat transfusions were administered to 22 different infected neutropenic patients. Those patients who received an average of ≥ 13 units per transfusion, which represented a mean of 1.02×10^{10} granulocytes, had a survival rate of only 30% which was not significantly different from the 28.5% found among patients who received an average of ≤ 12 units per transfusion, which represented a mean of $.63 \times 10^{10}$ granulocytes. In addition, no significant difference in survival rate was found between patients who received a course of $\geq$ four transfusions and those who received a course of $\leq$ three transfusions. Finally, no significant difference in survival rate was found between patients with acute leukemia and those with other disorders or between patients with positive cultures and those whose cultures were negative. Given the poor clinical results associated with buffy coat transfusions, it is concluded that every effort should be made to recruit single leukapheresis donors for the support of infected neutropenic patients, rather than use granulocyte enriched buffy coats as they are presently produced.

Keywords: buffy coats, granulocyte transfusions, granulocytopenia, leukopenia

Introduction

The preparation of granulocyte enriched buffy coats [1, 2, 3] and their transfusion to infected neutropenic patients [1, 4, 5, 6] has been reported briefly in the literature. Most of these reports deal with the use of these concentrates in infants [1, 4, 5]. In only one report was an attempt made to assess the efficacy of this therapy in adults [6]. In that uncontrolled retrospective study, the survival of patients receiving granulocyte enriched buffy coats in numerous Greater New York regional hospitals during 1976 was determined. The infusion of a mean of 8.1×10^9 granulocytes to patients on a daily basis was not associated with an improved survival rate when compared to patients who received only a mean of 5.1×10^9 granulocytes daily. Furthermore, the overall survival rate of 44% among patients receiving the higher dose was much less than the rates reported to be associated with the transfusion of granulocytes obtained by leukapheresis [5, 6,

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7]. It was suggested that transfusion of greater numbers of buffy coats, further enriched with granulocytes, might be associated with an increased rate of survival.

In the present study, we have examined the efficacy of the transfusion of buffy coats containing 33% more granulocytes than those used in the study described above. These transfusions were administered to neutropenic patients in a single institution during a four year period.

Methods and selection of patients

During the period cited, all patients admitted to the Westchester County Medical Center who were neutropenic ($< 500/mm^3$) due to myelosuppression and who had evidence of infection (fever [$> 38.3\,^{\circ}C$], localizing signs or positive cultures) for more than 48 hours which had not responded to appropriate antibiotic therapy were considered to be candidates for granulocyte transfusion. Those patients with adequate numbers of ABO compatible family members and friends received leukapheresis concentrates while the others received buffy coats. It is those patients who received buffy coats who are the subject of this report. The buffy coats were prepared from CPD or CPDA-1 whole blood by differential centrifugation in a refrigerated centrifuge, as previously described [2]. Each unit contained a mean of 6.5 ± 3.8 (2SD) $\times 10^8$ ganulocytes, had a mean heamtocrit of 38.8 ± 7.9 (2SD) vol. % and a mean volume of 35.3 ± 4.5 (2SD) ml. They were stored at $1^{\circ} - 6\,^{\circ}C$ for variable periods up to 24 hours prior to transfusion.

A course of buffy coat transfusions was defined as the daily infusion of varying numbers of units which continued until clinical resolution of the infection, recovery of bone marrow function or death of the patient. The number of units infused was frequently limited by the number available. In some cases, the course included occasional days when transfusion was not performed because of non-availability of product. Those days were included in the calculation of the duration of therapy and the average number of units infused per day of therapy. Survival of the recipients was defined as the preservation of life for at least 4 days following the cessation of transfusions and the complete or partial resolution of the acute infectious episode and its immediate sequelae.

Table 1

Patient Population

Group	Units/Tx. (Mean, S. D.)	Male/Female	Age (Mean, S. D.)
$\geq$ 13 Units/Tx.	15.7 (2.2)	7/3	58.8 (14.9)
$\leq$ 12 Units/Tx.	9.8 (2.6)	9/5	56.9 (20.5)

AL = Acute leukemia, ST = Solid tumor,

Twenty two adult patients received a total of 24 courses of transfusions. The total number of transfusions was 109, consisting of a total of 1.446 buffy coat units. Nine courses were administered to patients with acute leukemia and 15 to patients with other disorders (solid tumors, multiple myeloma, and aplastic anemia). Fifteen were to patients with positive cultures, 12 of whom were infected with gram negative organisms. All of these patients were grouped according to the average number of units infused per transfusion (≥ 13 units and ≤ 12 units), (Table 1). The entire patient population as well as these two groups were in turn evaluated according to the number of transfusion days comprising each course (≥ 4 days and ≤ 3 days), and the presence or absence of positive cultures.

The significance of the observed differences in the mean number of units infused per transfusion between the various groups was determined by the Student "t" test. The significance of the observed differences in survival rate between all these groups formed from the 24 cases and from the patients with either acute leukemia or other diagnoses was determined by the chi square method using Yates' correction.

Results

Division of the 24 courses into those who received ≥ 13 units per transfusion and those who received ≤ 12 units per transfusion resulted in the creation of two groups statistically different from each other ($p < .001$). Similar separation of the groups which had been created according to the number of transfusion days comprising each course and the presence or absence of positive cultures into those who received ≥ 13 units per transfusion and those who received ≤ 12 units per transfusion resulted in the creation of sub-groups statistically different from each other ($p < .001 - < .05$).

Each of the twenty four courses, consisted of a mean of 5.9 ± 4.0 SD daily transfusions (range $2 - 17$). The mean of the average number of units administered per transfusion was 12.3 ± 3.8 SD. Survival occurred in only 7 of the 24 cases (29.2%). No significant difference in survival rate was seen between the ten patients who received an average ≥ 13 units per transfusion (mean of the averages $= 15.7 \pm \pm 2.2$ SD), who had a survival rate of 30.0% and the fourteen who received an

of Study Groups

Group	Diagnosis	Pos Culture	Survival
$\geq$ 13 Units/Tx.	3AL, 6ST, 1AA	7/10	3/10
$\leq$ 12 Units/Tx.	6AL, 6ST, 1AA, 1MM	8/14	4/14

AA = Aplastic Anemia, MM = Multiple Myeloma

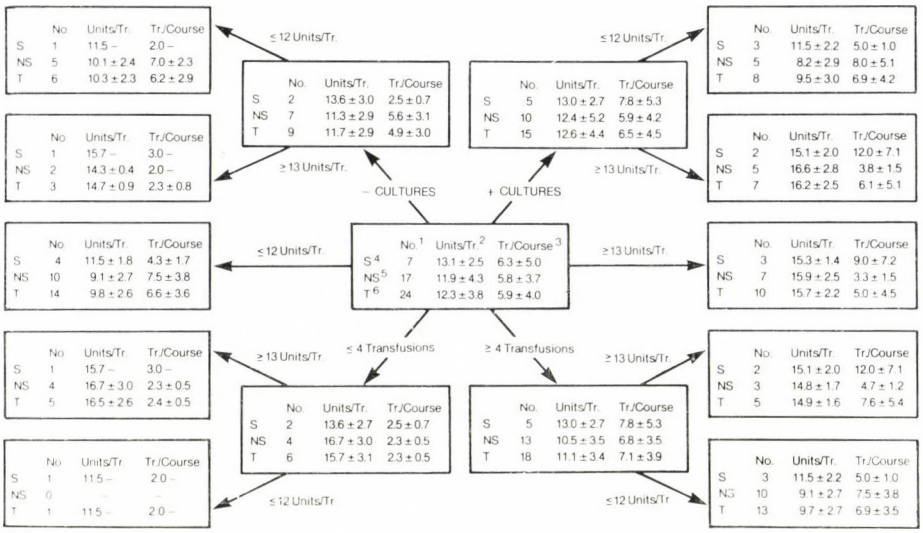

Fig. 1. Survival rates and descriptions of the 24 patient treatments with CPD and CPDA1 buffy coat transfusions according to groups and sets determined by transfusions per course, units per transfusion, and presence or absence of positive cultures (1 = number in group; 2 = units per transfusion: mean and standard deviation; 3 = transfusions per course: mean and standard deviation; 4 = survivors; 5 = nonsurvivors; 6 = total number). No significant difference in survival rate was found between the group of patients who received ≥13 units per transfusion and the group of patients who received ≤12 units per transfusion or between these divided groups formed from the patients who had positive or negative cultures and from those who received ≤4 transfusions per course and ≤3 transfusions per course

average ≤12 units per transfusion (mean of the averages = 9.8 ± 2.6 SD), who had a survival rate of 28.5%. Similarly, those 18 patients treated for ≥4 days (mean = 7.1 ± 3.9 SD) had a survival rate of 27.8% which was not significantly different from the survival rate of 33.3% noted among the remaining six patients who were treated for ≤3 days (mean = 2.3 ± 0.5 SD). Lastly, the 15 patients with positive cultures had a survival rate of 33.3% which was not significantly different from the survival rate of 22.2% found among the nine patients with negative cultures.

Those members of the group infused with ≥13 units per transfusion who received ≥4 transfusions did not have a significantly different survival rate (40%) than those members of the group who received ≤3 transfusions (20%). In addition, among those patients who received ≥13 units per transfusion the survival rate of those with positive cultures (29%) was not significantly different from those with negative cultures (33%).

Separate examination of the 18 patients who received ≥4 transfusions per course showed no difference in survival rate between the patients who received ≥13 units (40%) per transfusion and ≤12 units (23%) per transfusion. Similarly, separate examination of the 15 patients with positive cultures showed no difference

between those who received ≥ 13 units (28%) per transfusion and those who received ≤ 12 units (37%) per transfusion. All of the above results are summarized in Figure 1.

Similar separate divisions of patients with acute leukemia and those with other diagnoses into groups according to the number of units administered per transfusion, number of transfusions per course and culture results were performed. The groups were comprised of relatively small numbers of patients, and none of them had a significantly different survival rate from the others (data not shown).

Discussion

In a previous report, the transfusion of granulocyte enriched buffy coats was described as being commonplace in the Greater New York region for the support of infected neutropenic patients when sufficient leukapheresis donors were not found among the patients' family members or friends [6]. In that report, which analyzed the results of a multi-institutional study in 1976, a course of transfusions consisting of a mean of 8.1×10^9 granulocytes per transfusion was associated with an overall survival of 44.4%. This survival rate was not significantly different from the survival rate of 50.0% found in another group who received a mean of only 5.1×10^9 granulocytes per transfusion. Those buffy coats contained a mean of 4.9×10^8 granulocytes [2]. It was concluded that the buffy coats produced during that period and transfused in the numbers reported were without clinical benefit. It was suggested that this poor survival rate might be improved if the number of granulocytes infused were increased.

In the current study, buffy coats containing a mean of 6.5×10^8 granulocytes per unit were administered to infected neutropenic patients in a single institution. These buffy coats therefore contained an average of 33% more granulocytes than the buffy coats utilized in the previous study. In spite of the infusion of a mean of 10.2×10^9 granulocytes per transfusion (15.7 units), which represented an increase of 26% in dose of granulocytes over the number infused into the higher dose patients in the prior study, such transfusions were associated with a survival rate of only 30%. This survival rate was not significantly greater than the 28% associated with the infusion of a mean of 6.3×10^9 granulocytes per transfusion (9.8 units). As in the previous study, such survival rates compare poorly with rates reported in the literature for the transfusion of leukapheresis products, [7, 8, 9]. Although direct comparison is difficult beacuse the recipients treated were not a strictly comparable group, a survival rate of 69.2% was found among the infected neutropenic patients in this same institution who were transfused with leukapheresis concentrates during the same time period as this present study. The lack of good survival in this study was found in spite of the fact that the quantity of granulocytes infused per transfusion was comparable to the amounts contained in leukapheresis products obtained without steroid stimulation ($7.1 - 13.3 \times 10^9$) and which have been reported to be associated with greater survival [10]. In addition, the mean amounts infused per transfusion of buffy coats was in excess of

the 10^{10} granulocytes cited by Higby as the minimum daily dose necessary for therapeutic effect [11].

The reason for the poor therapeutic effect associated with infusion of these buffy coats during this period is unclear. It is probably not related to the general supportive care afforded these patients, given the much better survival rate cited above for our patients treated with leukapheresis concentrates. It may in part relate to the overall condition of the patients selected to receive buffy coat transfusions, since they were older and many had received repeated courses of chemotherapy and had been intensively transfused. Although the dose used would have been expected to be adequate, the number of granulocytes which functioned after infusion in patients might have been much less than anticipated. It has been shown that incompatible granulocytes infused into immunized patients do not migrate into foci of infection [12, 13] and also that the clinical response of patients with granulocyte specific antibodies against infused granulocytes is poor [14]. Since many of our patients had been heavily transfused, they could have been sensitized to some granulocyte specific antigens. In those cases, it is reasonable to assume that some of the buffy coats consist of granulocytes possessing these antigens. It could therefore be hypothesized that larger numbers of granulocytes might have to be administered in the form of random buffy coat units than would have to be administered in the form of single leukapheresis concentrates negative for those antigens in order to detect clinical benefit. In addition, the conditions of storage might have also been associated with decreased in-vivo function. While some studies have shown that buffy coats stored at $1-6\,^{\circ}C$ for up to 24 hours have good in vitro function [5, 15], other studies have shown that granulocytes have both decreased in vitro function [16] and decreased intravascular recovery, survival, and migration into skin windows with storage at $1-6\,^{\circ}C$ [17].

Since a marked increase in the number of granulocytes contained in the buffy coats separated by simple differential centrifugation [2] has not been consistently possible, an increase in dose would have to be achieved primarily by an increase in the number of buffy coats utilized. The administration of 25 units would entail the infusion of a total of 1.63×10^{10} granulocytes which represents an increase of 39% over the dose utilized in this study and approximates the $1 \times 10^{10}/m^2$ BSA suggested as the appropriate dose for children [4]. The administration of 25 units of buffy coats as they were produced during this study would entail the infusion of an average volume of approximately 900 ml, consisting of plasma and an average of 350 ml of red cells. As has been previously pointed out, a course of such infusions might be associated with hypervolemia and erythrocytosis. In addition, the infusion of this large number of blood products from different donors could also entail an increased risk of posttransfusion hepatitis and broad sensitization to HLA antigens [6].

Alternate methods of buffy coat preparation and storage at $22\,^{\circ}C$ might partially alleviate the above problems. Buffy coats separated from units of whole blood by differential centrifugation on model 2991 IBM blood processor have been reported to contain $1.11 \pm 0.4 \times 10^9$ granulocytes [5]. The use of the rouleaux-

inducing agent, hydroxyethyl starch as an additive to whole blood units, increases the separation of granulocytes into the buffy coat. Single units of whole blood treated in this way have been shown to produce buffy coats containing $2.0 \pm \pm 0.6 \times 10^9$ granulocytes [1].

Because of the lack of demonstrable clinical effectiveness at the doses used and the above potential complications which might be associated with the infusion of large numbers of buffy coats, we continue to discourage the routine use of buffy coats as produced. The transfusion of buffy coat concentrates with increased numbers of granulocytes and stored at 22 °C should be undertaken in an experimental setting. We feel that every effort should be made to recruit leukapheresis donors for the granulocyte support of those infected neutropenic patients who do not respond to antibiotic therapy.

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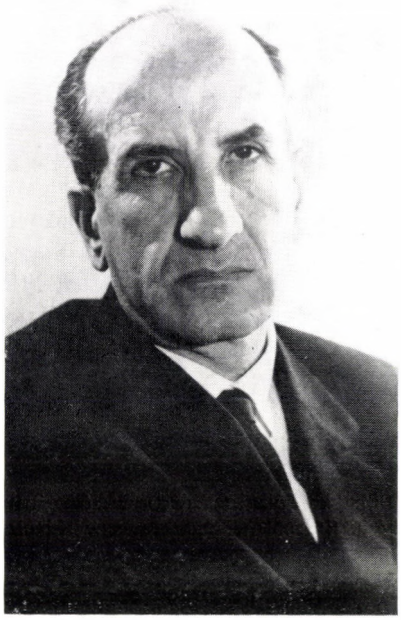
Obituary



Professor ISTVÁN SIMONOVITS, the director-general of the National Blood Transfusion Service of Hungary and a member of the Editorial Board of *Haematologia* passed away on May 12, 1985, aged 78 years.

His adult life, which he himself divided into four stages, was inspired by science and a constant seeking of truth. As a young medical student he worked in the Department of Biochemistry of the Semmelweiss University Medical School in Budapest. His scientific work was characterized throughout by exemplary precision in examination methods, by the elimination of background systematic errors and a critical evaluation of results. Thanks to this, he succeeded in showing as fine a difference as that existing between oxy- and deoxyhaemoglobin molecules, with an instrument which was up-to-date at the time, but which, looking back now, was very simple. It was this discovery that made him world famous. His results were quoted in detail in the first volume of the *Biochemical Yearbook* by Nobel prize winner Hans Fischer; 30 years later, Max Perutz, also a Nobel prize winner in the field of Hb research referred to it as the first proof of the molecular structure change that takes place during blood pigment activity.

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István Simonovits considered his work at the Department of Internal Medicine to be his second incarnation. Even as a practicing doctor he continued his research work. The biggest acknowledgement he received during this period was for a new diuretic method in the treatment of cardiac failure.

During the third stage of his life, he created long enduring values in the organization of the Hungarian health service, of which he was one of the most important leaders for decades. As all his life, this work too was characterized by a commitment to society, by an honest and fighting integrity, a respect for the progressive traditions and social progress of his country, and by faith in the organization of a high-standard health service for all citizens. He laid down the theoretical and scientific basis for the organization of the health service in a socialist country with classical conciseness in the textbook entitled "*Social Health Service and Medical Organization*", which had four editions. This book is still the textbook of graduate and postgraduate teaching in Hungary. He educated generations of doctors with his scientific articles and university lectures. He fought against the bureaucratization of the health service and against the alienation in the doctor-patient relationship. His educational activity was permeated by the firm belief that only someone who had been a practicing physician and who is aware of even the tiniest details of the way the health service works, can be a good organizer of same.

He always depended on the suggestions of the best specialists. His scientific research work and his activity as physician, as well as his work in different fields of the health service made him capable of determining the most important tasks and of concentrating the available human and economic resources for their solution. This characterizes his M. D. thesis which dealt with the causes of infant mortality.

As one of the directors of the Hungarian Ministry of Health, in 1949 he started the organization of the Hungarian National Blood Transfusion Service. From 1964 until his death – in the fourth incarnation of his life – he worked as the director-general of the National Blood Transfusion Service. He played an important role in the decentralization of the Service, in the standardization of methods of the regional centres and in the raising of the level of their work. It was he, who started and organized the unpaid plasma donation in the country. The creation and careful control of the anti-D IgG prevention and the ensuring of the production of the needed product in Hungary was just one of his lasting achievements. The fact that we were the first in the world who could protect all Rh-negative mothers at risk, free of charge and not only after delivery but also after interruption was internationally acknowledged.

This highly appreciated man, beloved by his colleagues and all those who knew him had received a number of Hungarian and international medals and decorations and was one of the pillars of the Hungarian Health Service.

Susan R. Hollán, MD

International Society of Blood Transfusion

Dear Readers:

The members of the International Society of Blood Transfusion (ISBT) Working Party on Terminology for Red Cell Surface Antigens wish to bring their Munich Report, (Vox Sang. 49:171 – 175, 1985) to your attention.

The Working Party was established to promote uniformity and non-duplication in alphabetical nomenclature for red cell surface antigens and to provide a standard numerical terminology. The Report contains listings of ISBT approved designations and guidelines for the designations of "new" antigens. Interested persons may wish to contact local committee members: Australia (S. Young); Canada (M. Crookston, M. Lewis, B. P. L. Moore); Denmark (J. Jørgensen), England (D. J. Anstee, G. W. G. Bird, M. Contreras, G. Daniels, C. M. Ciles, A. Lubenko, P. A. Tippett); Finland (H. Nevanlinna, P. Sistonen); France (Ch. Salmon); Germany (W. Dahr, S. Seidl); Holland (C. P. Engelfriet); Japan (Y. Yasuda); New Zealand (P. Booth, G. Woodfield); Norway (L. Kornstad, R. Nordhagen); U. S. A. (F. H. Allen, Jr., E. Brodheim, P. D. Issitt, W. L. Marsh, P. Morel, J. J. Moulds, R. E. Rosenfield, B. Sabo, R. H. Walker).

Respectfully submitted,

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Abstracts

Reversible and irreversible inhibition of phosphate transport in human erythrocytes by a membrane impermeant carbodiimide. J. D. Craik and R. A. F. Reithmeier (Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada). *J. Biol. Chem. 260*, 2404 (1985).

Phosphate entry into chloride-loaded human erythrocytes is inhibited by treatment of cells with the water-soluble carbodiimide 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide (EAC) in the absence of added nucleophile. EAC does not penetrate the erythrocyte membrane or lead to significant intermolecular cross-linking of membrane proteins. At neutral extracellular pH in chloride-free medium, only about 50% of transport is rapidly and irreversibly inhibited, but at alkaline pH the inhibition is more rapid and complete. EAC inhibition was reversible in the presence of extracellular NaCl. Modification of membrane sulfhydryl groups does not prevent inhibition of phosphate transport by EAC, but an almost complete protection is afforded by 4,4-dinitrostilbene-2,2-disulfonic acid, a reversible competitive inhibitor of anion transport. N-(4-Azido-2-nitrophenyl)-2-aminoethylsulfonate, a reversible noncompetitive inhibitor of anion transport did not protect against EAC inhibition of transport but prevented reversal of inhibition in saline medium. Transport inhibition by [^{3}H]EAC did not lead to specific incorporation of radioactivity into Band 3, the anion transport protein. These results suggest that inhibition of anion transport by EAC is due to modification of a carboxylic acid residue in or near the transport site accessible from the external face of the membrane. The subsequent fate of the modified carboxyl residue appears to be sensitive to the orientation of the anion transport site.

G. Gárdos

The effects of terbium III on the Ca-activated, K channel found in the resealed human erythrocyte membrane. P. G. Wood and H. Mueller (Max-Planck Institut für Biophysik, Frankfurt, FRG). *Eur. J. Biochem. 146*, 65 (1985).

Incorporated terbium, Tb^{3+}, activates the Ca-activated K channel found in the resealed erythrocyte ghost membrane and allows the net efflux of K. As in the case of Ca activation, low levels of external K stimulate the net efflux of K. The action of incorporated Tb^{3+} seems to be analogous to that in incorporated Ca. Externally applied Tb^{3+}, however, inhibits the net efflux of K after either Ca or Tb^{3+} activation. The net-efflux of K can be inhibited by Tb^{3+} even after partial digestion of the channel by low levels of incorporated trypsin. Furthermore, the channel when incorporated into planar bilayers, can be inhibited by Tb^{3+}. Externally applied Tb^{3+} does not seem to inhibit net K efflux indirectly via inhibition of the pathways for the co-transported anions. In addition, because of the low concentrations of Tb^{3+} required for inhibition, it seems unlikely that an alteration of surface potential is responsible for the observed effects.

G. Gárdos

VNU Science Press, Utrecht
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Proton-activated rubidium transport catalyzed by the sodium pump. R. Blostein (Departments of Medicine and Biochemistry, McGill University and the Montreal General Hospital Research Institute, Montreal, Quebec, Canada). *J. Biol. Chem. 260*, 829 (1985).

Although the sodium pump normally exchanges three sodium for two potassium ions, experiments with inside-out red cell membrane vesicles show that the stoichiometry is reduced when the cytoplasmic sodium concentration is decreased to less than 1 mM. The present study was designed to gain insight into the question whether other monovalent cations, particularly protons, can act as sodium congeners in effecting pump-mediated potassium transport (ATP-dependent rubidium efflux from inside-out vesicles). It was shown that at low cytoplasmic sodium concentration, an increase in proton concentration effects a further reduction in sodium: rubidium stoichiometry, to a value less than minimally expected ($1\ Na^+ : 3\ Rb^+$). Furthermore, when vesicles containing $^{86}RbCl$ are incubated in nominally sodium-free medium, ATP-dependent net rubidium efflux (normal influx) occurs when the pH is reduced from approximately 7.0 to 6.2 or less. This efflux is inhibited by strophanthidin and vanadate. These experiments support the notion that the sodium pump can operate as an ATP-dependent proton-activated rubidium (potassium) pump without obligatory countertransport of sodium ions.

G. Gárdos

Reduced transglutaminase-catalyzed cross-linking of exogenous amines to membrane proteins in sickle erythrocytes. S. K. Ballas, N. Mohandas, M. R. Clark, S. H. Embury, E. D. Smith, L. J. Marton and S. B. Shohet (Cardeza Foundation for Hematologic Research, Department of Medicine, Jefferson Medical College, Philadelphia, PA, USA). *Biochim, Biophys. Acta 812*, 234 (1985).

In order to determine the capacity of sickle cells to undergo transglutaminase-catalyzed cross-linking of membrane proteins, human normal and sickle erythrocytes were incubated with [ring-2-^{14}C] histamine in the presence of Ca^{2+} and ionophore A23187. The [^{14}C] histamine incorporation into membrane components was observed in freshly prepared erythrocytes. Incorporation of radioactivity into spectrin and Band 3 membrane components was significantly ($P < 0.001$) less in sickle erythrocytes than in normal cells. Transglutaminase deficiency was excluded by the finding of increased activity of this enzyme in sickle cells from patients with reticulocytosis. The incorporation of [^{3}H]spermine into red cell membranes was also less in sickle erythrocytes than in normal cells under the same conditions of incubation used for [ring-2-^{14}C]histamine. Sickle erythrocytes were more permeable to these amines than normal cells. It is proposed that the γ-glutamyl sites of membrane proteins in sickle erythrocytes are less accessible for transglutaminase-catalyzed cross-linking to histamine and polyamines in vitro. This may be due to prior in vivo activation of this enzyme by the increased calcium in sickle cells and/or shielding secondary to altered membrane organization.

Ilma Szász

The effect of hypercholesterolemia on guinea pig platelets, erythrocytes and megakaryocytes. B. P. Schick and P. K. Schick (Thrombosis Research Center and Department of Medicine, Temple University School of Medicine, Philadelphia, PA, USA). *Biochim. Biophys. Acta 833*, 291 (1985).

This study has examined the effect of diet-induced hypercholesterolemia on guinea pig platelets, erythrocytes, megakaryocytes and plasma. The cholesterol/phospholipid ratios of plasma and erythrocytes began to increase after one day on the diet and increased steadily for two weeks and more slowly thereafter for 30 days. In contrast, the cholesterol/phospholipid ratio of platelets remained constant for 4—5 days, then increased until reaching a maximum of about 0.85 in two weeks. Thus, the time-course for increase of the cholesterol/phospholipid ratio is different for platelets than for erythrocytes and plasma. The increase in the cholesterol/phospholipid ratio of megakaryocytes was small and not dependent on the degree of increase in the plasma choles-

terol/phospholipid ratio. The cholesterol esters of both platelets and megakaryocytes increased with time for two weeks. The increase in megakaryocyte cholesterol esters appeared to precede that of platelets. The protein content of platelets and megakaryocytes and average megakaryocyte size were increased. Normal platelets incubated in plasma from hypercholesterolemic guinea pigs did not accumulate excess cholesterol, but erythrocyte cholesterol increased 45% in 6h under the same conditions. Cholesterol synthesis in megakaryocytes was depressed 50—80% by cholesterol feeding and by in vitro incubation of the cells in hypercholesterolemic plasma. The data suggest that the platelets and erythrocytes may accumulate excess cholesterol by different mechanisms. The effects of cholesterol feeding on megakaryocytes and the lag in accumulation of cholesterol in platelets relative to erythrocytes and plasma suggest that a defect in the megakaryocyte may be a primary determinant of accumulation of cholesterol in platelets.

Ilma Szász

The liver is an organ site for the release of inosine metabolized by non-glycolytic pig red cells. R. B. Zeidler, M. H. Metzler, J. B. Morgan and H. D. Kim (Department of Pharmacology and Department of Surgery, University of Missouri-Columbia, School of Medicine, Columbia, MO, USA). *Biochim. Biophys. Acta 838*, 321 (1985).

The metabolic energy source used by the pig red cell, which is unable to metabolize blood-borne glucose, was examined. Potential physiological substrates include adenosine, inosine, ribose, deoxyribose, dihydroxyacetone and glyceraldehyde, of which inosine was previously implicated. A net ATP synthesis by red cells occurs during in situ perfusion through the adult miniature pig liver. HPLC analysis of the perfusate revealed the presence primarily of inosine and hypoxanthine. Inosine production by the liver was 0.015 μmol/g per min. Moreover, red cells maintain ATP when suspended in a balanced salt medium during a 6 h incubation at 38 °C, in which inosine is continuously infused to give an external concentration of no more than 3 μmol/l, mimicking its plasma level. Inosine consumption under these infusion conditions was 56 nmol/ml cell per h, which is two orders of magnitude lower than when inosine is present in millimolar concentration. The total red cell inosine consumption of 9.63 μmol/h is much less than the total liver inosine production of 212 μmol/h. These findings suggest that the liver is an organ site elaborating inosine, and that maintenance of a 3 μmol/l inosine in plasma is sufficient to meet the energy requirements of the pig red cells.

Ilma Szász

Acyl selectivity in the transfer of molecular species of phosphatidylcholines from human erythrocytes. P. Child, J. J. Myher, F. A. Kuypers, J. A. F. Op den Kamp, A. Kuksis and L. L. M. Van Deenen (Laboratory of Biochemistry, State University of Utrecht, Utrecht, The Netherlands). *Biochim. Biophys. Acta 812*, 321 (1985).

This report describes the molecular species composition of phosphatidylcholines (PC) transferred from human erythrocytes to acceptor vesicles composed of cholesterol and single PC species in the presence of PC-specific transfer protein from bovine liver. The compositions of the PC isolated from the vesicles were determined by capillary GLC as the diacylglycerol trimethylsilyl ethers. The cellular PC species appearing in the acceptor vesicles were enriched in unsaturated species and showed a low content of dipalmitoyl PC compared to untreated erythrocytes. This trend was independent of the composition of the PC used to construct the acceptor vesicles and it was possible to determine that the relative rates of efflux of the palmitoyl-containing phosphatidylcholines decreased in the order: palmitoyl-linoleoyl > palmitoyl-oleoyl > dipalmitoyl and in the stearoyl series, stearoyl-linoleoyl > > stearoyl-oleoyl. No clear trend was distinguished for the influence of chain-length on the efflux, thus preventing an unambiguous assignment of the order of removal of all species from the cell membrane. Results derived for arachidonoyl-containing species were compromised by evidence for oxidation occurring during incubations at 37 °C. To confirm that acyl selectivity was also pos-

sible during transfer in the absence of the transfer protein, the efflux of ^{14}C-labeled soya PC and [^{14}C]dipalmitoyl PC from prelabeled erythrocytes was measured using plasma as the acceptor. As predicted by the chromatographic analyses, ^{14}C-labeled soya PC effused up to 10-times faster than [^{14}C]dipalmitoyl PC from the red cell membrane. Thus, the more rapid transfer of unsaturated PC cannot be explained entirely as a specificity of the transfer protein and is consistent with the hypothesis that intermolecular interactions involving PC molecules within the erythrocyte membrane, become weaker with increasing unsaturation. The results suggest a potential role of PC-specific transfer protein as a probe of the nature of PC interactions within biological membranes.

B. Sarkadi

Limited proteolysis of human erythrocyte Ca^{2+}=ATPase in membrane-bound form. Identification of calmodulin-binding fragments, E. I. Emelyanenko, M. I. Shakhparonov and N. N. Modyanov (Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow, USSR). *Biochem. Biophys. Res. Comm. 126*, 214 (1985).

Water-solube and membrane-bound calmodulin-binding polypeptides formed upon limited proteolysis of erythrocyte ghosts were isolated by mean of affinity chromatography. Immune blotting revealed that all isolated fragments originated from Ca^{2+}-ATPase. Among the fragments obtained those having formed an acylphosphate intermediate were identified. The N-terminal residue of purified intact Ca^{2+}=ATPase was shown to be blocked (probably acylated).

B. Sarkadi

Cyclic AMP inhibits platelet activation independently of its effect on cytosolic free calcium. A. Pannocchia and R. M. Hardisty (Department of Haematology and Oncology, Institute of Child Health, The Hospital for Sick Children, London, UK). *Biochem. Biophys. Res. Comm. 127*, 339 (1985).

Stimulation of platelets with the ionophore A23187, thrombin, ADP or PAF-acether resulted in a rapid increase of cytosolic free Ca^{2+}, as measured with Quin-2, and in aggregation, 5HT secretion and — in the case of the first two agonists — thromboxane generation. PGI_2 and dibutyryl cyclic AMP inhibited all these responses, except in the case of A23187, in response to which the increase in Ca^{2+} was unaffected, although the other responses were inhibited. The inhibition of aggregation and secretion in response to the combination of thrombin and A23187 was indistinguishable from that in response to thrombin alone. It thus appears that cAMP inhibits these responses independently of its effect in lowering cytosolic free Ca^{2+}.

B. Sarkadi

Inositol 1.4.5-trisphosphate releases Ca^{2+} from a Ca^{2+}-transporting membrane vesicle fraction derived from human platelets. F. A. O'Rourke, S. P. Halenda, G. B. Zavoico and M. B. Feinstein (Department of Pharmacology, University of Connecticut Health Center, Farmington, Connecticut). *J. Biol. Chem. 260*, 956 (1985).

Human platelet membrane vesicles that accumulated Ca^{2+} in the presence of ATP were isolated on an isoosmotic KCl-Percoll gradient. ATP-dependent Ca^{2+} uptake was stimulated by oxalate and phosphate to steady-state levels of $>$ 100 nmol/mg protein, and the accumulated Ca^{2+} could be largely released by ionophore A23187. Inositol 1,4,5-trisphosphate, in a dose-dependent manner (0.5—5.0 μM), caused the rapid release ($<$ 5s) of 40—70% of the total A23187-releasable store of accumulated Ca^{2+}. The membrane vesicles that release accumulated Ca^{2+} in response to inositol 1,4,5-trisphosphate were enriched in enzymes characteristically found in smooth endoplasmic reticulum. These results support the hypothesis that inositol 1,4,5-trisphosphate, produced by the hydrolysis of phosphatidylinositol 1,4-bisphosphate in response to stimulation of cell surface receptors, is a second messenger mediating the release of Ca^{2+} from intracellular storage sites.

Ágnes Enyedi

Breakdown and synthesis of polyphosphoinositides in fMetLeuPhe-stimulated neutrophils. S. Cockcroft, M. M. Barrowman and B. D. Gomperts (Department of Experimental Pathology, University College London, London, England). *FEBS Letters 181*, 259 (1985).

The interconversions of the inositol-containing lipids (PI, PI—P and PI—P_2) and their products (DG, inositol phosphates and PA) in human and rabbit neutrophils stimulated with fMetLeuPhe and PMA have been examined. PMA causes only the phosphorylation of PI to PI—P whereas fMetLeuPhe causes phosphorylation of both PI and PI—P yielding PI—P_2 and the hydrolysis of all three lipids. While the predominant reaction is breakdown of PI to PA catalysed by phospholipase D, approx. 2% of PI is converted to polyphosphoinositides and then broken down by the phospholipase C route yielding inositol phosphates and DG. The latter reaction occurs without detectable lag and is a function of receptor occupancy. The amount of inositol trisphosphate thus formed would be sufficient to release Ca^{2+} from intracellular stores.

Ágnes Enyedi

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SPOTLIGHTS (meetings, congresses)

7 – 10 December 1985

Annual meeting of the American Society of Haematology ASH reg. supervisor, C. B. Slack, Inc. 6900 Grove Rd.; Thorofare, NJ 08086, USA

11 – 16 May 1986

19th Congress of the ISBT – 21st Congress of the ISH GPO Box 2609 Sydney, NSW 2001, Australia

21 – 27 August 1986

14th International Cancer Congress

Dissemination of current knowledge of cancer, its prevention, early detection, diagnosis, treatment, rehabilitation and continuing care, with special emphasis on the multidisciplinary approach to the cancer problem.
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Organization of public and professional cancer educational programmes for professionals, allied health personnel and for volunter cancer agency participants. (Open)

For further information, please contact: Prof. S. Eckhardt
Secretary General, 14th International Cancer Congress, National Institute of Oncology, Ráth Gy. u. 7/9, 1525 Budapest, Hungary

INSTRUCTIONS TO CONTRIBUTORS

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