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Editorial
Training of Blood Transfusion Teams*

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(Received May 12, 1980)

1. Training of the staff of Blood Transfusion Centres (BTC-s) and postgraduate teaching of clinicians represent a core problem in establishing an efficient National Blood Transfusion Service (NBTS.) 2. The teaching programme should be adapted to the basic function of the BTS. 3. The training programme should give the staff theoretical basis and technical skill to ensure that the BTC can provide the region with whole blood, blood cell concentrates and plasma fractions of guaranteed biological activity. 4. It should demonstrate in practice the importance of a close contact between BTC-s and clinical units. 5. The lack of experts in developing countries is one of the most important limiting factor in the development of their NBTS. Since haematology and immunology overlap and intersect with blood transfusion, the best solution is to train physicians in blood transfusion, haematology and immunology in a combined specialist's training scheme. This type of training renders specialists who are not merely heads of "bottling factories", but who in addition to their high level work in blood transfusion will be well versed in two other specialities both indispensable in modern clinical diagnostics and therapy and in the fight against population diseases.

*

Training of the staff of blood transfusion centres and postgraduate teaching of clinicians represent a core problem in establishing an efficient National Blood Transfusion Service. A regular house-staff and professional staff continuing education programme is indispensable even in the most advanced Blood Transfusion Centres (BTC). This is also of paramount importance in developing countries where the shortage of trained personnel represents the main limiting factor in the development of their NBTS-s.

The aims, trends and scopes of the training programmes have to be adapted 1) to the peculiar aspect of transfusion therapy: to the interaction between the donor and the recipient, and 2) to the basic functions of the BTS. This latter comprises:

- collection and distribution of blood,
- application of laboratory tests,
- advice on clinical usage,
- training and teaching, and
- research and development.

* Abridged version of a paper presented at the ISBT Symposium on Blood Transfusion and Immunohaematology held at Brasilia (Brazil), February 11–15, 1980.

The first three tasks are universally accepted but the two latter ones have not been included into the basic tasks of many Centres. In contrast, in my opinion training and teaching as well as an active research programme are essential features of an efficient blood transfusion service. Fundamental research is to contribute information to the fields of science related to blood and is a *sine qua non* for the development of new techniques in processing blood and blood derivatives and for the scientific evaluation of the results of routine tests.

The first basic task of a BTS is aimed at producing adequate amounts of safe and effective blood components and plasma products. A regional centre has to provide whole blood, blood components and plasma fractions of guaranteed biological activity for the whole area to be supplied.

The staff has to be aware of the fact that the quality of the final product depends upon the quality and the handling of the source material throughout processing. As a consequence, the productive, diagnostic and safety aspects of a BTS are not separated but integrated elements.

What is the optimum basic training for the director of a BTS? I hold the opinion that the director should be preferably a physician, trained in the laboratory and clinical aspects of haematology, immunology and blood transfusion. Advances in these fields of biomedicine have progressed so rapidly that most physicians and surgeons find it difficult to keep up with the changes in basic concepts which underlie the everyday practice of blood transfusion. However, they will only consult the blood bank physician if he is known to have a good scientific reputation and to be well versed in clinical problems.

In setting up a National Blood Programme the director of the BTC has to make calculations of the amount of blood and blood derivatives needed. He has to organize the blood collection and distribution. The optimum size of a donor panel comprises seven donors per one emergency bed and the recruitment of around 2 to 5% of the population as regular donors. In Hungary 5.8% of the population are regular voluntary blood donors. It is also indispensable to have a panel of donors with rare blood group and a panel of donors with high levels of specific antibodies. When planning the size of the blood donor panel the need for different components has to be approximated.

In planning the size of the plasmapheresis and platelet-paheresis panels, the need for plasma for specific plasma proteins and for platelet concentrates has to be calculated. Granulocyte donors are recruited most frequently from family members of the recipient.

In many countries, especially in developing countries, there is a deficiency in whole blood and blood derivatives. But even in countries with highly developed BTS, the amount of blood and plasma collected, has to be increased year by year. New methods have arisen which consume blood voraciously. Let me list some of these fairly new fields of indication. Coronary bypass surgery alone may raise by 26% the need for whole blood. Emergency direct coronary arterial revascularization, infarctectomy, aneurysmectomy, repair of ruptured ventricular septum, liver surgery, surgery of cerebral ischaemia, as well as major surgery in traumatology

and orthopaedics (like hemipelvectomy and implantation of joint prostheses) all consume enormous amounts of blood. Extremely high amounts of platelets are needed for patients on prolonged extracorporeal oxygenation by a membrane oxygenator and for patients on "artificial liver". Great amounts of platelets and granulocytes are needed for patients on aggressive chemotherapy and after bone marrow transplantation.

When new operative procedures (like coronary bypass surgery) are being initiated in a region, due consideration has to be given to immediate and projected blood requirements, and the same is true for all other newly arising fields of indication. The BTC will only be able to meet the quantitatively and qualitatively increasing demands if the director or an expert of the staff will fully understand the basic principles and the hazards involved in the newly applied diagnostic or therapeutic procedures. And that is why continuous postgraduate training is indispensable.

There are three ways of providing more blood products:

– to bleed more donors;

– to have a more widespread use of plasmapheresis and cell-apheresis; and, last but not least,

– to achieve optimum use of the collected fresh blood.

There are essentially two main methods for recruiting blood donors: to encourage paid donations or to build up a completely unpaid donor panel. Hungary relied on paid donors from 1949 until 1959, when we had switched over to unpaid voluntary blood donation. From this time on, the number of voluntary donors increased tenfold in our country. We are also able to recruit voluntary plasmapheresis donors who donate plasma in the blood centres. In some countries like the Netherlands this is achieved by mobile plasmapheresis equipment.

To achieve all voluntary, unpaid blood donation needs much work including propaganda, organization and recruitment. Consequently, a training programme has to include principles and practice of donor propaganda and recruitment as well as motivation of blood donation. You can never give a magic guideline in this subject, you have to find the best arguments which will differ from time to time, and from place to place.

A training programme has to include methods of organization of donor sessions, the preparation and use of mobile equipment, the number of staff participating at a session and the best ways of transportation of the mobile team.

Detailed instructions should be given on incidents and accidents, hazards and contraindications of blood donation.

Thorough practical training has to be given in techniques of collection of blood and specific components as well as in techniques of giving blood transfusions. The participants of a postgraduate training course have to learn the theoretical basis and the practice of the newly arising techniques in blood and plasma donation, cell separation, preservation and plasma fractionation.

We teach our staff not only the use of plasmapheresis and cell-pheresis to obtain high amounts of plasma or cell concentrates from donors, but they have

to be acquainted in addition with the therapeutic use of intensive plasma- and cell-apheresis.

Much has to be learned about contaminated and outdated blood and about the preventive measures to avoid these hazards.

Due time is spent to teach minimum requirements for equipment, reagents, anticoagulants, sterilization procedures, and for the control tests evaluating the quality, efficiency and viability of the final products. A basic principle to be respected throughout the training is that they should never compromise between quality and quantity. The effectiveness of our educational programme is being controlled by frequent monitoring of the preparations produced by the trainees and by proficiency tests of their laboratory work.

Lectures are given on new aspects of structure, function, kinetics and viability of blood cells, which in fact define their optimum storage conditions. Participants have to learn to estimate cell survival. They have to be acquainted with the structure, function and the fractionation of plasma proteins.

Basic immunology, cellular and humoral factors, new concepts on interactions between immunocompetent cells and humoral factors are important subjects in our postgraduate training.

Blood group serology represents of course the most important subject of the training. ABO, Lewis, Ii, Rh system and other RBC antigens have to be learnt in theory and practice. as well as RBC allo- and autoantibodies and red cell incompatibility *in vitro* and *in vivo*.

All participants of the course have to be acquainted with the leucocyte, platelet and serum protein antigens and their respective antibodies as well as with the complement system. They have to learn how to prepare test sera, Coombs sera and cell panels for blood group serology. Blood groups and problems of parentage and identity are being reviewed briefly. Practical training enables the trainees to achieve good command of the application and evaluation of laboratory tests and gain some knowledge on the principles of automation.

Quality control represents again a core subject of the training course.

We also find it important to teach the theory and practice of epidemiology and of the prevention of transmissible diseases, as well as the epidemiology of noninfectious diseases prevailing in the region to be supplied with blood and derivatives.

Lectures on recent concepts of haemostasis and practical training of the methods applied in diagnostics and treatment of haemorrhagic diseases and thrombosis are included in the training programme. Possibility is given during the training course to become acquainted with the methods of the production and use of plasma fractions with haemostatic activity.

Detailed lectures are given on indications of blood transfusion with special attention to component therapy, use and misuse of blood. Theoretical basis and practical aspects of the treatment of hypovolaemic shock and hazards of massive transfusion are being discussed. Quantity and quality of blood components needed for extracorporeal circulation and haemodialysis are also presented. To achieve

optimum use of blood, detailed information has to be given on the use of plasma expanders and electrolyte solutions.

Blood centres often have to face the difficulties in finding compatible blood for patients with autoimmune diseases and this is why it is important to become acquainted with the pathomechanism, diagnostics and treatment of autoimmune diseases with special emphasis on haemotherapy and intensive plasmapheresis applied with success in the treatment of this heterogeneous group of diseases.

Neonates and infants are not small adults. Consequently, the programme has to include the special aspects of haemotherapy in infancy and childhood: the pathophysiology, diagnostics, treatment and prevention of haemolytic disease of the newborn, the technique of exchange transfusion and the treatment of the haemorrhagic disease of the newborn.

Everybody who is involved in the production of blood and derivatives, as well as those involved in the application of haemotherapy have to be fully aware of all hazards and complications of blood transfusion, their diagnostics, treatment, laboratory investigation, their prevention and also the legal aspects involved in blood transfusion.

Last but not least, management, financing, cost/benefit ratios and developmental work in the NBTS are included into the training programme.

I have summarized the most important subjects taught at our postgraduate courses, including the six months' training course organized by WHO in our National Institute of Haematology and Blood Transfusion for experts in blood transfusion from fifteen developing countries. It gave us real satisfaction to hear that most of our students became heads of BTC in their homeland.

The fact that the directors of BTC-s in Hungary can achieve some basic clinical practice and training in haematology and immunology enables them to give high-level postgraduate courses to clinicians in their region. This is the key to building up a close liaison with the clinical units. As a result clinicians consult our experts and we can fight efficiently against the misuse of blood. The results are much better than those which can be achieved by autocratic measures. The efficiency of the postgraduate teaching programme is well reflected in the improved use of red cell concentrates instead of whole blood in the region. According to our experience the new level is being sustained for approximately six to twelve months. Consequently, the battle against the misuse of whole blood cannot be won by a single postgraduate course or with a symposium on the optimum use of blood. Postgraduate training courses have to be repeated regularly and should always include the most recent results to attract the attention of the participants.

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Effect of Platelet Homogenate on Platelet Production of Bone-Marrow-Transplanted Mice*

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(Received May 24, 1979)

Haemopoiesis of mice was reconstituted with 1×10^6 bone marrow cells after 800 r total body irradiation. Then their megakaryocytopoiesis was inhibited by platelet homogenates given at different points of time on three subsequent days. The effect was measured by the peripheral platelet counts on the 14th day after transplantation when the platelet count of irradiated and marrow-transplanted CFLP animals was 410×10^9/l, and that of CBA mice was 185×10^9/l.

Platelet homogenates prepared from $1–2.5 \times 10^8$ platelets daily given on days 1, 2 and 3 did not significantly reduce the peripheral platelet count estimated on the 14th day, while homogenates given on days 8, 9 and 10 reduced it markedly. Homogenates prepared from $0.7–1.0 \times 10^6$ white blood cells did not influence the 14th day platelet count. Thus, platelet homogenates exert their inhibitory effect at a late stage of platelet production, i.e. not by an inhibition of thrombopoietin or a thrombopoietin effect.

*

It has been shown by several authors [1, 2, 3, 4, 6, 10, 12, 13, 14] that megakaryocyte-platelet production can be suppressed in recipient animals by the transfusion of fresh, intact platelets. This, however, occurs only if a very high number of platelets is given.

Krizsa et al. [11] observed that damaged platelets, or more precisely, the rate of their disintegration plays a role in the suppression, and the inhibition after the transfusion of intact platelets might be connected with some substances released by the disintegrating platelets, because inhibition occurs only when the number of disintegrating platelets reaches a certain high level.

In the present study it was attempted to establish the phase of megakaryocytopoiesis primarily affected by platelet homogenates.

Material and Methods

Two to three-month-old male mice of the CFLP and CBA strains were used. The CBA strain is genetically homogeneous (syngeneic), the CFLP strain is inhomogeneous.

* Supported by the Scientific Research Council, Hungarian Ministry of Health (4-20-0304-01-2/CsK).

The animals kept in plastic boxes were given 800 r total body irradiation by a THX-250 (Medicor, Budapest) apparatus (5 min, 28 sec irradiation time, 230 kV, 15 mA, 1 mm copper filter, 40 cm focus distance, 104 r/min air dose).

A few hours later, the animals were subjected to bone marrow transplantation. For transplantation, suspension of bone marrow blown out of both femurs of 4–5 untreated mice of the same strain was used. The bone marrow was adequately diluted with physiological saline to a final concentration of 1×10^6 cells in 0.2 ml.

For preparation of the platelet suspension, blood was drawn from the inferior vena cava of ether-anaesthetized mice and placed into a siliconized container with 5% EDTA-Na_2 pH 6.7 solution.

After centrifugation of the whole blood at 600–800 *g* for 10 min, the supernatant containing the platelets was centrifuged at 2000–2500 *g* for 20 min, then after washing twice, centrifugation was repeated at the same speed and for the same length of time. The residue was resuspended in physiological saline. From the platelet suspension thus prepared, a platelet homogenate was obtained after freezing and thawing three times. Freezing was done in a deep freezer at -18 °C for 45 min, thawing at room temperature. On each occasion each mouse was given intravenously 0.2 ml of platelet homogenate prepared from $1\text{-}2.5 \times 10^8$ platelets.

For the preparation of a white blood cell suspension, the blood drawn as described above, was centrifuged at 600–800 *g* for 10 min, then the buffy coat was suspended in physiological saline for 20 min and centrifuged at 2000 *g*. The white blood cells were washed twice, occasional red blood cells were removed by ammonium oxalate, and the platelets by ADP so that it contained practically no red blood cells or platelets. The white blood cell homogenate was obtained from this by freezing and thawing three times. Each mouse was given on a single occasion homogenate made from $0.7\text{–}1.0 \times 10^6$ white blood cells.

Schedule of treatment

The irradiated and transplanted animals were divided into three groups. The platelet homogenate was given to the first group on days 1, 2, 3 and to the second group on days 8, 9, 10 following transplantation. The control animals received only irradiation and bone marrow cells.

Four separate series of experiments were made with the CFLP strain. Each group of CFLP mice contained the combined results of 40 animals. Another series was done with CBA mice. Each group contained 10 animals. Within each group the animals belonging to the same group as well as the animals of the corresponding control group received irradiation simultaneously.

For the assessment of megakaryocytopoiesis in the bone-marrow-transplanted animals, the peripheral platelet count was determined according to Fischer and Germer [7] on the 14th postirradiation day [9] when the bone marrow was in the phase of regeneration.

Administration of the white blood cell homogenate was performed similarly as the platelet homogenate, i.e. one group received the homogenate on the first three days, the other on the 8th, 9th and 10th days after transplantation. In each of these groups the results of 10 CFLP mice are summarized.

Calculation of the significance was done by Student's *t* test.

Results

Following 800 r irradiation, the platelet count of CFLP mice decreased till days 8 to 10. After this it remained at the low value of 22×10^9/l (S.E. ± 14.2) for a longer time.

If the irradiated animals received 1×10^6 bone marrow cells, their platelet count decreased only to 74×10^9/l, then it increased according to the rate of repopulation and on day 14 it reached 410×10^9/l (S.E. ± 26.3). The mean platelet count of the group treated with homogenates prepared from $1.0–2.5 \times 10^8$ platelets on the first three days was 456×10^9/l on day 14, while after treatment with an identical homogenate on days 8, 9 and 10 it amounted to 273×10^9/l at the same time.

The platelet count of CBA mice after 800 r irradiation and bone marrow transplantation was 185×10^9/l on day 14. The effect of syngeneic platelet homogenate did not differ from that in the CFLP strain (Table 1). The result in the animals treated on days 8, 9 and 10, were significantly different from the control ($p < 0.05$).

Homogenates from $0.7–1.0 \times 10^6$ white blood cells used similarly did not influence thrombocytopoiesis.

Table 1

Platelet counts ($\times 10^9$/l) of bone marrow reconstituted mice on the 14th day after transplantation

Strain	Control	Homogenates given	
		on days 1, 2 and 3	on days 8, 9 and 10
CFLP	410±26.3* (40)**	456±28.4 (40)	273±23.5 (40)
CBA	185±5.32 (10)	184±12.04 (10)	132±11.48 (10)

* Standard error
** Number of mice

Discussion

According to Ebbe and Stohlman [5], maturation of the megakaryocytic series in the rat takes 43 to 75 hours. Several hours are presumably needed for the differentiation of stem cells into recognizable megakaryocyte precursors.

Our assumption was that if the platelet suspension exerts its inhibitory effect in the early stage of maturation of the platelet suspension on the first three days, whereas if it acts at a later stage or inhibits the release, then the suspension given on days 8, 9 and 10 would produce inhibition. The inhibitory effect of such amounts of platelet suspension was shown earlier [11].

The fact that the platelet count in the group which received platelet suspension on the first three days was higher than that of the control group, seems to indicate that the homogenate may constitute an aspecific stimulus for CFU-S cells.

In accordance with the assumption of Goldberg et al. [8] our findings seem to prove that the inhibition develops at a later stage of platelet production.

Thrombopoietin, according to present knowledge, exerts its effect in the early stage of megakaryocytopoiesis. It seems therefore that the suppressive effect of the platelet homogenate develops not through thrombopoietin, but by some other mechanism.

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Иммунологические, цитохимические и цитологические исследования лимфоцитов при неопластических и гипопластических состояниях кровотворения

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(Поступило 8, 3, 1979)

Провели исследование содержания Т- и В-лимфоцитов, цитохимии лимфоцитов и микроструктуры их нуклеол у людей при остром и хроническом лимфолейкозе (ОЛЛ, ХЛЛ) и гипопластической анемии (ГА) и у мышей с хронической бензольной интоксикацией (ХБИ). При ОЛЛ нашли отсутствие Т- и и В-маркеров в стадии рецидива и выраженные цитохимические изменения и изменения нуклеол лимфоцитов во все стадии процесса, включая полную ремиссию. При ХЛЛ подтвердили наличие 2 вариантов иммуноморфологического течения — В-клеточного и смешанного В—Т. Изменения при ГА и ХБИ оказались аналогичными, что позволяет считать ХБИ адекватной моделью некоторых форм ГА людей. Изменения в структуре нуклеол и цитохимических показателей касались как Т-, так и В-лимфоцитов.

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В последние годы при рассмотрении вопросов патогенеза, диагностики и терапии многих гематологических состояний все большее значение придается изучению Т- и В-лимфоцитов. Так, показано, что химический и вирусный канцерогенез сопровождаются определенными изменениями этих клеточных систем, что, по-видимому, связано с появлением опухолево-специфических антигенов [3, 5, 47]. Существенные изменения Т- и В-клеточных систем обнаружены и при гипопластических состояниях кровотворения [24, 32]. Однако имеющихся сведений еще явно недостаточно, особенно мало работ, в которых данные о Т- и В-лимфоцитах сопоставлялись бы с результатами цитохимического исследования лимфоцитов, изучения нуклеолярного их аппарата и т. д. Некоторые ограничения для таких исследований связаны с тем, что в условиях клиники возможно изучение преимущественно клеток периферической крови, соотношение Т- и В-лимфоцитов в которой не полностью отражает состояние этих клеток в лимфоидных органах [14, 23, 25, 39]. Исходя из сказанного, в данной работе наряду с изучением Т- и В-лимфоцитов людей с неопластическими и гипопластическими состояниями параллельно исследовалась цитохимия лимфоцитов и микроструктура нуклеол этих клеток и полученные данные сопоставлялись с состоянием лимфоцитов крови и лимфоидных органов животных с экспериментальной бензольной интоксикацией (БИ). БИ считается адекватной моделью гипопластического состояния [10, 15, 22, 24], кроме того известно, что БИ в клинике нередко сопровож-

дается неопластической трансформацией гемопоэза [19, 37, 44], тогда как данные эксперимента по бензольным лейкозам противоречивы [1, 11, 20, 29, 45, 30].

Материалы и Методы

Исследование проведено у 20 больных острым лимфобластным лейкозом (ОЛЛ), 34 больных хроническим лимфатическим лейкозом (ХЛЛ), 16 больных гипопластической анемией (ГА), а также у 25 мышей СВА с хронической БИ (ХБИ), которую вызывали подкожным введением бензола по 2 мг/кг через день в течение 16 недель. Для сравнения соответствующие показатели изучали у 72 здоровых людей в возрасте 18—36 лет и у 25 нормальных мышей СВА. Клеточный тип лейкоза определяли по клиническим, морфологическим и цитохимическим (гликоген, кислая фосфатаза, неспецифическая эстераза, липиды, пероксидаза) критериям. Среди 20 больных ОЛЛ под влиянием лечения полную ремиссию (менее 5% бластов в костном мозгу) наблюдали у 4, неполную (менее 25% бластов) — у 6, первый рецидив — у 6, второй — у 4 больных. Больных с ХЛЛ обследовали до и после курса лечения (преднизолон, лейкеран), а больных ГА — в период развернутой картины заболевания.

Для идентификации Т- и В-клеток лимфоциты периферической крови выделяли на градиенте плотности фикол-верографин [7]. Исследования с трипановым синим показали, что 90—95% клеток составляли жизнеспособные лимфоциты. Концентрация лимфоидных клеток определялась с помощью смеси 0,1% водных растворов эозина и трипанового синего. Т-клетки у людей определяли в реакции прямого розеткообразования [21] и в цитотоксической реакции (микрометод, 42) с использованием антитимической сыворотки [26], а у мышей — в цитотоксическом тесте [42] с использованием антитимической сыворотки [38]. Субпопуляцию В-лимфоцитов, несущих рецепторы к С3, выявляли по [6]. В случае животных эти исследования проводили не только в крови, но и в лимфоидных органах — тимусе, селезенке, паховых, подчелюстных, мезентериальных и подмышечных лимфоузлах. Для этого готовили суспензии указанных тканей путем извлечения их в среду 199 с 20% бычьей сывороткой и 10% раствора гидролизата лактоальбумина, содержащего 0,005 М 2Н гидроксиэтилпиперазин-N′-2-этансульфониловую кислоту (НЕРЕЗ) и антибиотики (100 ед/мл) с последующим троекратным отмыванием при 200 g 5 мин при 4°С.

Цитохимические исследования проводились в мазках периферической крови, при этом у больных ОЛЛ и ГА в ряде случаев использовали лейкоконцентраты. Интенсивность реакции оценивали по проценту клеток, обладающих активностью и полуколичественным методом (2) с вычислением среднего показателя реакции (СПР).

Микроструктуру нуклеол выявляли по [40].

Результаты

1. *Исследование Т- и В-лимфоцитов*

У больных ОЛЛ в период полной ремиссии соотношение Т- и В-лимфоцитов оказалось близким к норме, при этом имелось соответствие между числом Т-клеток, выявляемых в цитотоксическом тесте, и Т-клеток, дающих Т-розетки (табл. 1). В период неполной ремиссии процент Т-клеток, реагирующих с антитимической сывороткой, не изменялся, но более 25% этих клеток не образовывали Т-розеток. Возможно, это обстоятельство связано с наличием при неполной ремиссии неоплазированных Т-клеток, потерявших способность давать Т-розетки [26]. Содержание В-лимфоцитов при этом было снижено. В период рецидива в изученных нами случаях ОЛЛ лимфоидные клетки были лишены Т- и В-маркеров.

Изучение Т- и В-лимфоцитов при ХЛЛ указывает на то, что большинство случаев его можно было классифицировать как болезнь В-клеточной пролиферации (табл. 1). Действительно, наблюдалось резкое снижение от-

Таблица 1

Иммуноморфологические особенности лимфоидных клеток при остром лимфобластном и хроническом лимфатическом лейкозах

Пациенты	n	Т-лимфоциты (цитотоксический тест, %)	Т-розетки	В-розетки
ОЛЛ, полная ремиссия	4	71,2±2,6	64,1±3,1	8,0±3,2
ОЛЛ, неполная ремиссия	5	69,4±2,2	42,2±3,2*	3,4±3,1*
ОЛЛ, рецидив	5	0	0	0
ХЛЛ, до лечения	15	11,3±1,2*	7,8±2,6*	42,0±3,4*
ХЛЛ, после леч.	10	21,1±2,3*⁰	8,1±2,6*	31,0±2,1*⁰
Здоровые	22	69,1±1,9	67,2±4,3	8,7±2,1

Примечание: *) изменения достоверны по отношению к здоровым лицам, ⁰) достоверность по сравнению с ХЛЛ до лечения ($p \leq 0{,}001$). $\overline{X} + S_{\overline{x}}$

носительного числа Т-клеток, однако их абсолютное число в сравнении с нормой не снижалось. В 3 случаях ХЛЛ абсолютное число Т-клеток повышалось, что в сочетании с увеличенным (абсолютным и относительным) количеством В-клеток позволяет отнести их к сочетанной В—Т-пролиферации. Лечение привело к уменьшению субпопуляции В-лимфоцитов, но возрастание процента Т-клеток наблюдалось только по цитотоксическому тесту.

Больные ГА в зависимости от процента Т-лимфоцитов распределились на 2 подгруппы (табл. 2). У больных 1-й подгруппы (11 пациентов) был отмечен высокий процент этих клеток, и пересчет на абсолютные количества показал, что лимфопения здесь была исключительно В-клеточной. У боль-

Таблица 2

Т- и В-лимфоциты при гипопластической анемии

Группы больных	Лимфоциты (тыс/мкл)	Т-лимфоциты		В-лимфоциты	
		%	тыс/мкл	%	тыс/мкл
Контрольная	1,96±0,14	76,2±4,3	1,32±0,16	7,8±2,3	1,54±0,18
1-я	1,46±0,17	89,6±3,2	1,31±0,15	3,1±0,9	0,45±0,05
p_1	0,01	<0,001	>0,1	0,01	<0,001
2-я	1,29±0,14	61,3±2,1	0,79±0,07	8,1±1,1	1,04±0,13
p_1	0,01	>0,1	<0,001	>0,1	0,05
p_2	>0,1	<0,001	<0,001	0,01	<0,001

Примечание: p_1 — достоверность по отношению к контрольной группе, p_2 — достоверность между подгруппами больных.

ных 2-й подгруппы (5 пациентов) лимфопения носила выраженный В—Т-клеточный характер.

У животных с ХБИ лимфопения в крови была преимущественно Т-клеточной, тогда как процент В-лимфоцитов, несущих рецепторы к С3, не уменьшался (табл. 3). В лимфоидных органах при ХБИ значительно снижалась клеточность, особенно клеточность тимуса (табл. 4) и уменьшалось содержание Т- и В-клеток в селезенке и лимфоузлах.

Таблица 3

Т- и В-лимфоциты в периферической крови при ХБИ у мышей

Группы животных	Лимфоциты (тыс/мкл)	Т-лимфоциты		В-лимфоциты	
		%	тыс/мкл	%	тыс/мкл
Контрольная	7,0±0,4	79±7	5,5±0,7	4,2±0,2	0,30±0,02
с ХБИ	3,4±0,6*	67±5	2,3±0,5*	7,6±0,5*	0,26±0,05

*) $p \leq 0{,}05$.

Таблица 4

Кариоциты и Т- и В-лимфоциты в лимфоидных органах при ХБИ

Группы животных	Селезенка			Лимфоузлы		Тимус
	клетки (млн)	Т-лимфоциты (%)	В-лимфоц. (%)	Т-лимфоциты (%)	В-лимфоциты (%)	(клетки, млн)
Контрольная	110±4	46±2	28±2	76±4	15±1	100±2
Мыши с ХБИ	25±3*	38±1*	3±1*	49±3*	10±1*	8±0,4*

*) $p \leq 0{,}05$.

2. *Цитохимические исследования*

Интенсивность цитохимической реакции на кислую фосфатазу (КФ) и неспецифическую эстеразу (НЭ) при ОЛЛ повышена, особенно при полной ремиссии (табл. 5). Обратная зависимость наблюдалась в отношении сукцинатдегидрогеназы (СДГ) — активность этого фермента при ОЛЛ снижена, особенно в периоде ремиссии. В стадии рецидива повышалась доля гликогенпозитивных лимфоцитов.

Таблица 5

Цитохимия лимфоидных элементов крови при ОЛЛ, ХЛЛ и ГА

Пациенты	КФ		НЭ	
	%	СПР	%	СПР
Здоровые	51,0±2,0	0,67±0,02	34,5±1,1	0,46±0,01
ОЛЛ, полная ремиссия	83,0±1,7*	0,90±0,04*	76,0±1,4*	0,95±0,05*
ОЛЛ, неполная ремиссия	78,3±2,1*	0,84±0,03*	69,1±1,9*	0,83±0,06*
ОЛЛ, рецидив	68,0±2,0*	0,74±0,01*	67,0±2,2*	0,68±0,02*
ХЛЛ, до лечения	32,0±1,8*	0,44±0,05*	68,1±3,4*	0,69±0,08*
ХЛЛ, после лечения	37,0±2,1*	0,44±0,05*	64,1±3,4*	0,69±0,06*
ГА, развернутая картина забол.	64,0±2,0*	0,85±0,08*	38,5±2,1	0,50±0,02

Пациенты	СДГ		Г	
	%	СПР	%	СПР
Здоровые	84,0±2,1	0,98±0,09	13,6±1,2	0,18±0,02
ОЛЛ, полная ремиссия	38,2±2,1*	0,44±0,07*	18,1±2,7	0,24±0,06
ОЛЛ, неполная ремиссия	76,1±1,8*	0,80±0,07	17,4±3,2	0,29±0,08
ОЛЛ, рецидив	79,2±3,6	0,86±0,09	21,0±2,0*	0,26±0,04
ХЛЛ, до лечения	51,2±1,6*	0,69±0,07*	78,1±2,2*	0,94±0,03*
ХЛЛ, после лечения	59,0±1,7*⁰	0,78±0,02*	66,0±1,2*⁰	0,74±0,01*⁰
ГА, развернутя картина забол.	60,0±3,3*	0,70±0,05*	12,0±2,0	0,17±0,01

Примечание: Данны в $X \pm S_x$. *) достоверность различий по отношению к здоровым людям, ^{0}) достоверность различия в группе ХЛЛ до и после лечения.

При ХЛЛ до начала лечения интенсивность реакций на НЭ и гликоген (Г) была повышена за счет резкого повышения доли позитивно реагировавших клеток, что косвенно указывает на возможность появления новой фракции высокоактивных клеток [35]. Активность КФ и СДГ, наоборот, была снижена. Лечение сопровождалось частичной нормализацией цитохимических показателей (табл. 5).

При ГА имелась тенденция к повышению активности КФ, отмечалось снижение СДГ, различия же по НЭ и Г по сравнению с контролем были недос-

товерны (табл. 5). Аналогичными оказались изменения КФ и СДГ лимфоцитов при ХБИ, где наряду с этим повышался процент позитивно реагировавших на НЭ и Г клеток (табл. 6).

Таблица 6

Цитохимия лимфоцитов крови у мышей с ХБИ (СПР)

Группы животных	КФ	НЭ	СДГ	Г
Контрольная	0,10+0,01	0,25+0,03	1,40+0,10	0,05+0,01
Мыши с ХБИ	0,23+0,03*	0,36+0,05*	0,83+0,10*	0,10+0,02

*) $p \leq 0{,}05$.

3. *Изучение нуклеол лимфоцитов*

При ОЛЛ в стадии рецидива увеличивалась доля лимфоцитов с микронуклеолами. Считается, что такие лимфоциты не способны синтезировать РНК и находятся в состоянии дегенерации [40]. Под влиянием лечения доля их снижалась, но при этом возрастало число лимфоцитов с гомогенными нуклеолами, которые находятся в состоянии активированного синтеза РНК [40]. При неполной ремиссии соотношение лимфоцитов с разной микроструктурой нуклеол оказалось близким к норме.

Больные с ХЛЛ по данному показателю распределились на 3 группы вне зависимости от стадии заболевания (табл. 7): 1) с неизмененным соот-

Таблица 7

Структура нуклеол лимфоидных элементов при ОЛЛ, ХЛЛ и ГА

Пациенты		Лимфоциты с		
		гомогенными нуклеолами (%)	микронуклеолами (%)	кольцевидными нуклеолами (%)
ОЛЛ, полная ремиссия		42,0±2,8*	11,4±0,9*	46,6±2,1*
ОЛЛ, неполная ремиссия		3,2±1,9	7,8±2,9	89,0±1,9
ОЛЛ, рецидив		0,9±0,1	19,1±0,4*	80,0±2,2
ХЛЛ	1-я гр. (n = 9)	26,4±2,1*	11,1±0,7	62,5±1,9*
	2-я гр. (n = 12)	2,1±2,3	32,3±3,1*	63,6±2,1*
	3-я гр. (n = 13)	2,8±1,2	8,2±1,3	89,0±4,8
ГА		5,8±1,1	11,6±1,0*	82,6±4,9
Здоровые		2,6±1,1	8,0±1,2	89,4±4,7

*) $p \leq 0{,}01$ по отношению к здоровым людям.

ношением лимфоцитов разных типов (3-я гр.), 2) с резким повышением доли лимфоцитов с микронуклеолами (2-я гр.) и 3) с резким повышением доли лимфоцитов с гомогенными нуклеолами (1-я гр.). Интересно, что 3 больных ХЛЛ, имевших сочетанную В—Т-пролиферацию, относились к группе с повышенным содержанием клеток с микронуклеолами, увеличением НЭ умеренным увеличением КФ.

При ГА происходило увеличение доли лимфоцитов с гомогенными и, в меньшей степени, с микронуклеолами (табл. 7). При ХБИ примерно одинаково возрастали клетки с гомогенными и микронуклеолами (табл. 8).

Таблица 8

Структура нуклеол лимфоцитов у мышей с ХБИ

Группы мышей	Лимфоциты		
	гомогенными нуклеолами (%)	микронуклеолами (%)	кольцевидными нуклеолами (%)
Контрольная	3,8±0,5	3,9±0,2	92,4±9,0
Мыши с ХБИ	10,0±1,0*	11,2±1,4*	78,8±8,6

*) $p < 0{,}001$.

Обсуждение

Изучение содержания Т- и В-лимфоцитов при взятых нами состояниях позволяет сделать ряд выводов и предположений.

Оказалось, что содержание Т- и В-лимфоцитов крови при лейкозах существенно зависело от стадии заболевания и от проводимого лечения. Особенно это проявилось при ОЛЛ, при котором нормализация данного показателя наблюдалась только при полной ремиссии, тогда как неполная ремиссия и особенно рецидив сопровождались резкими изменениями содержания лимфоцитов с Т- и В-маркерами. Отсутствие иммунологических маркеров у лимфобластов при рецидиве ОЛЛ согласуется с данными ряда авторов [4, 8, 9]. Возможно, это является следствием потери клеточных рецепторов при злокачественной трансформации или присутствия некоммитированных стволовых лейкемических клеток. Впрочем не исключено, что изученные нами случаи относятся лишь к одному из иммуноморфологических вариантов ОЛЛ. В литературе имеются указания на существование ОЛЛ с пролиферацией клеток, несущих как Т-, так и Т—В-маркеры [8, 18, 48].

Возможно, что неполное соответствие между клиническим течением лейкозов и соотношением Т- и В-лимфоцитов в крови связано отчасти с тем, что указанное соотношение не точно отражает соотношение Т- и В-клеток в лимфоидных органах. Такое предположение подтвердилось при сравнении динамики этих клеток в крови и лимфоидных органах в экспериментальной

части работы (табл. 3 и 4). Действительно, лимфопения в крови при ХБИ носила преимущественно Т-клеточный характер, тогда как изучение Т- и В-лимфоцитов в лимфоидных органах обнаружило депрессию обеих этих систем иммунитета.

Интересные выводы вытекают и из сопоставления содержания Т- и В-клеток при ГА и ХБИ, а также при лейкозах и ХБИ.

При сопоставлении данных о ГА и ХБИ обращает внимание большая степень лимфопении у больных ГА 2-й подгруппы, что позволяет предположить и большую степень гипоплазии лимфопоэза у этих больных. В этом отношении следует отметить сходство в структуре лимфоидной популяции у больных 2-й подгруппы и у животных с ХБИ, также характеризовавшейся значительной лимфопенией и выраженной гипоплазией лимфоидных органов. Можно предположить, что на начальном этапе гипоплазии, как это наблюдалось у больных 1-й подгруппы, происходит снижение содержания В-лимфоцитов (в селезенке и других лимфоидных органах), в дальнейшем же по мере прогрессирования процесса присоединяется депрессия Т-клеточной системы подобно тому, как это происходило у больных 2-й подгруппы и у мышей с ХБИ. В литературе имеются данные о нарушениях лимфоидной ткани в части случаев ГА [12].

Полученные данные могут иметь и иную интерпретации. Известно что ГА при тимоме сопровождается В-клеточной лимфопенией, что, возможно, связано с депрессивным влиянием гипертрофированного тимуса на костномозговое кроветворение [34]. Развитие ХБИ в наших опытах сопровождалось, наоборот, резкой гипоплазией тимуса и уменьшением количества стволовых гемопоэтических клеток [16, 17] и, возможно, как следствие этого происходило поражение прежде всего Т-клеточной системы иммунитета. По-видимому, эти данные указывают на неоднородность ГА по механизму их развития. Кроме того, они свидетельствуют о том, что ХБИ можно рассматривать как модель тех форм клинической ГА, которые сопровождаются выраженными нарушениями клеточного иммунитета. Подтверждением этого является и почти полная идентичность цитохимических изменений лимфоцитов и данных о состоянии их нуклеол при ГА и ХБИ (табл. 5—8).

В литературе имеется целый ряд данных о том, что у лиц с ХБИ могут развиваться лейкозы, в том числе и лейкозы, исходящие из лимфоидной ткани [13, 19, 37, 44]. Однако в экспериментальных работах, особенно в работах последних лет [29, 45] получены отрицательные результаты. Нам также не удалось наблюдать развитие лейкоза у новорожденных мышей, у которых вызывалась БИ в первые 2 недели жизни с последующим наблюдением в течение 1,5 лет, не было отмечено лейкоза и у взрослых мышей в ходе 12 мес. опыта с ХБИ [17]. Однако у подопытных животных было отмечено целый ряд изменений — такие, как лимфопения, поражение тимуса, нарушение выработки лимфопоэтина [15] — которые принято рассматривать как предлейкозные [33, 49]. В настоящей работе изменения содержания Т- и В-лимфоцитов при ОЛЛ и ХБИ резко различались между собой, тогда как эти

показатели при ХЛЛ и ХБИ имели некоторое сходство (табл. 1 и 2). Интересно, что в некоторых работах допускают значение иммунологических нарушений в патогенезе бензольных лейкозов у человека [41].

Исходя из полученных данных, мы пытались также выявить корреляции между состоянием клеточного иммунитета при ОЛЛ, ХЛЛ, ГА и ХБИ и цитохимическими показателями лимфоцитов. В литературе имеются указания на возможность цитологической и цитохимической идентификации Т- и В-лимфоцитоз [31, 36, 43, 46]. Нам однако такой зависимости выявить не удалось. Так, отсутствие Т- и В-маркеров при рецидиве ОЛЛ сопровождалось не снижением, а как раз повышением интенсивности цитохимических реакций на КФ, НЭ и Г, и только активность СДГ была снижена. Наоборот, нормализация соотношений Т- и В-лимфоцитов крови при неполной и особенно полной ремиссии ОЛЛ сопровождалась выраженными отклонениями цитохимических показателей от нормы. Не удалось выявить соответствия между состоянием клеточного иммунитета и цитохимическими показателями лимфоцитов и при ХЛЛ, ГА и ХБИ. Таким образом, сама по себе принадлежность данной клетки к Т- или В-популяции не определяет ее цитохимическую активность по изучавшимся параметрам, последняя отражает лишь функциональное состояние лимфоцитов.

К аналогичному выводу мы пришли и в отношении возможной связи содержания Т- и В-лимфоцитов и соотношения лимфоцитов с разными типами нуклеол. Действительно, например, при ОЛЛ нормализация соотношения Т- и В-клеток под влиянием лечения сопровождалась не нормализацией соотношения лимфоцитов с разными типами нуклеол, а наоборот, резким возрастанием доли лимфоцитов с гомогенными нуклеолами, т. е. при этом увеличивалось содержание активированных лимфоцитов. При ХЛЛ, как уже отмечалось, выявилось 3 типа изменений соотношения лимфоцитов по структуре нуклеол (табл. 7), но какой-либо связи этого показателя с содержанием Т- и В-лимфоцитов обнаружено не было ни до лечения, ни под его влиянием. Таким образом, и данный показатель — микроструктура нуклеол — не указывает на принадлежность лимфоцита к Т- или В-системам.

Это предположение подтвердилось особенно при изучении цитохимии и нуклеол лимфоцитов при ГА и ХБИ. Такие изменения, как повышение активности КФ при одновременном снижении активности СДГ и повышение доли лимфоцитов с гомогенными нуклеолами принято рассматривать как показатель развития повышенной чувствительности замедленного типа и аутоиммунных расстройств [27, 28], т. е. изменений, характеризующихся сдвигами функционального состояния лимфоцитов.

В целом приведенные данные показывают, что цитохимическая активность лимфоцитов, микроструктура их нуклеол и особенно содержание Т- и В-лимфоцитов являются ценными показателями при изучении патогенеза, клиники и контроля результатов лечения целого ряда гематологических состояний.

Immunological, Cytochemical and Cytological Study of Lymphocytes in Neoplastic and Hypoplastic States of Haemopoiesis

The T and B lymphocytes, their semiquantitative cytochemistry and the nuclei were investigated in human acute and chronic leukaemia (ALL, CLL) and hypoplastic anaemia (HA), as well as in mice with chronic benzene poisoning (CBI). In the acute phase of ALL T and B markers were absent. In all phases, including remission, cytochemical changes and changes in the structure of nucleoli were present. In CLL, two variants of immunological alterations were found, a B cell variant and a mixed B-T cell variant. Immunological and cytochemical changes in HA and CBI were identical and this allowed to conclude that CBI is an adequate model of certain variants of human HA. The immunological and cytochemical changes in CLL, HA and CBI affected both T and B cells.

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Nonsecretory Multiple Myeloma

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A patient is described with myeloma without paraproteinaemia or Bence-Jones proteinuria. In May-Grünwald-Giemsa-stained bone marrow films 32% of the cells were plasma cells containing monoclonal IgG.

Introduction

In patients with multiple myeloma, paraprotein is present in the serum or Bence-Jones protein in the urine in over 98% of the cases [13]. The rare cases where no homogeneous immunoglobulin or light chain production can be detected in the serum or urine by electrophoretic and immunologic techniques, have been termed nonsecretory myeloma [3, 4, 5, 6, 8, 9, 12, 14, 19, 20]. Osserman and Takatsuki [16] studied 262 cases of myeloma and only 3 patients did not display any serum or urine protein abnormality. Similarly, Di Guglielmo [5] found 3 nonsecretory cases among 200 and Hobbs [7] 6 nonsecretory cases among 500 typical cases of myelomatosis.

Report of a Case

A 70-year-old woman, who in 1973 had been diagnosed to have multiple myeloma, and who had been treated with melphalan, cyclophosphamide, prednisone and radiation therapy, was admitted with complaints of generalized bone tenderness in 1978.

X-ray examination showed widespread osteolytic lesions. A search for cancer metastases was negative. The bone marrow was heavily infiltrated with plasma cells typical of multiple myeloma. Therefore, the diagnosis of plasmocytoma was corroborated and further investigations were performed. A bone marrow aspirate was processed for immunofluorescence studies. As recommended by Arend and Adamson [4], the heparinized aspirate was washed five times to remove nonspecifically absorbed serum proteins, and to haemolyze the erythrocytes. The samples of bone marrow were smeared on glass and fixed in acetone. Immunofluorescent studies were carried out using commercial monospecific FITC-labelled immune

sera (Meloy , USA) against heavy chains of IgG, IgM, IgA, IgD, IgE. Additionally the preparations were treated with anti-IgG Fab fragment (Organon Teknika, Holland), and the indirect immunofluorescence test was performed. In the second stage of the reaction FITC-labelled goat globulin directed against rabbit globulin was used (Hyland, USA). For demonstrating light chains, appropriate anti-kappa and lambda sera were used (Behringwerke, FRG). The presence of complement receptors was also studied, using specific immune sera against B_1C/B_{1A} (Behringwerke, FRG). In these cases indirect immunofluorescence tests were performed and the preparation after washing in PBS were treated with FITC conjugated goat anti-rabbit globulin (Hyland, USA). The specificity of immunofluorescent staining was confirmed by appropriate controls. The plasma cells showed bright cytoplasmic fluorescence after treatment with anti-IgG and anti-IgG Fab fragment sera (Fig. 1), and a weak fluorescence for kappa light chains. No fluorescence was seen in plasma cells treated with other non-specific immune sera. Paper electrophoresis revealed no abnormal serum protein and no abnormal serum protein was found on immunoelectrophoresis using antisera against total human serum and specific for all immunoglobulin classes and kappa, lambda light chains. No immunoglobulins were detected in the concentrated urine. Additional laboratory findings were as follows. Red cell count, 4.1 T/l; haemoglobin, 8.55 mmol/l; reticulocytes, 0.014/l; leucocytes, 4.7 *G*/l; bands 0.05, neutrophils 0.72, lymphocytes 0.25, platelets 130 *G*/l; total serum protein, 65 g/l; albumin, 0.59 kg/kg; globulins $alpha_1$, 0.144 kg/kg, $alpha_2$, 0.104 kg/kg, $beta_1$, 0.089 kg/kg, $beta_2$, 0.059 kg/kg, gamma, 0.112 kg/kg. Immunoglobulins: IgA 1.25 g/l, IgM 0.56 g/l, IgG 10.5 g/l; IgD and IgE were not detectable. Sedimentation rate was 2 mm/h, glucose 4.5 mmol/l, non-protein nitrogen 3.33 mmol/l, serum calcium 2.25 mmol/l, inorganic phosphorus 0.965 mmol/l, alkaline phosphatase 10 u.K.A. Bence-Jones in urine was not

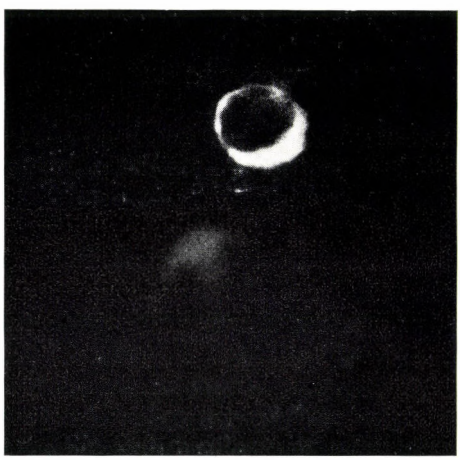

Fig. 1. Cytoplasmic immunofluorescence of bone marrow plasma cell treated with anti-IgG serum

detectable, nor was cryoglobulinaemia observed. HL-A phenotype A2: Dw 22, 16: E rosettes 38%, EAC rosettes 18%, heavy chain surface immunoglobulins on peripheral lymphocytes were as follows: IgG 20%, IgA 5%, IgM 5%. There was only one type of light chain (kappa) detectable on these cells. Skin tests: PPD 5 U, induration 5 mm, SK/SD 40 *u* 8 mm, DNCB challenge dose 100 μg 8 mm. Multidrug combination therapy (vincristine, prednisone, melphalan) [2] was introduced and repeated every month with a satisfactory effect (improvement of physical status, relief of bone pains, weight gain, diminution in size and number of osteolytic lesions).

Discussion

Our patient had clinical multiple myeloma and hypogammaglobulinaemia without paraproteinaemia and Bence-Jones proteinuria. Hobbs [7] considered this form of myelomatosis the most dedifferentiated one, with the worst prognosis. On the other hand, Kim et al. [10] found the survival of 5 nonsecretory myeloma patients to be better than the average for myelomatosis. This was true also for our patient, who is alive and well five years after the diagnosis. This survival is above the mean for the myeloma patients reported by Osserman et al. in 1972 [16].

Immunofluorescence studies of the bone marrow plasma cell infiltrates showed a strong staining for IgG and a very faint one for kappa light chains in the cytoplasm. This faint staining may have been due to differences in the intracellular concentration of heavy and light chains, or due to a difference in the relative potency of the antisera used in the immunofluorescence test [20].

On the basis of the laboratory findings, the case was classified as a typical nonsecretory myeloma. According to some authors [3, 6], the absence of protein may be due to an alteration or block of transport and excretion or an impairment in the genetic mechanisms involved in this kind of neoplasm. Osserman and Takatsuki [16] postulate that in these cases protein synthesis occurs but there is no secretion owing to unfavourable environmental conditions. According to other authors [20] the presence of the heavy and light chains in the plasma cells does not necessarily mean that they are present as intact immunoglobulin molecules which can be released [20]. Interpretation of these data is difficult because the complete pathway of secretion is not clearly known [11, 13]. It has been reported that in myeloma patients a considerable proportion of the B lymphocytes bears membrane-bound immunoglobulin with the same individual antigenic specificity as the monoclonal myeloma protein found in the patient's serum and in cytoplasm of the pathological plasma cells. We also found monoclonal IgG on the surface of circulating B lymphocytes, which could support the hypothesis that these cells are part of the malignant process and may represent early stages of the neoplastic proliferation leading to the formation of malignant plasma cells [13]. In our case the number of T lymphocytes was decreased, in agreement with other observations [12, 13], but the importance of this finding is obscure.

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Function and Role of Peritoneal Macrophages in Experimental Leukaemia

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The specific and nonspecific function of peritoneal macrophages of leukaemic mice has been studied as well as the role of these cells in the induction of lymphocyte cytotoxicity against leukaemic cells *in vitro*. It was found that the phagocytic and digestive function of the peritoneal macrophages remains normal, but the macrophage-mediated cytotoxicity against leukaemic cells is rare and weak in both allogeneic and syngeneic forms of grafted leukaemia. Utilizing the system of generation of cytotoxic lymphocytes in mixed lymphocyte tumour cell cultures *in vitro*, it was observed that the addition of specifically activated syngeneic macrophages lowers the rate of generated cytotoxicity. The mechanism of macrophage-induced suppression is discussed.

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In recent years interest has greatly increased in the role of macrophages in immune reactions, and particularly in the immune response to malignant neoplastic disease. It has been shown that the course of the malignant process displays an inverse relationship to the quantity of macrophages infiltrating the tumour [31] and that activated macrophages exert a tumouricidal effect *in vivo* [20, 21, 22] as well as *in vitro* [23, 27, 45]. The action of these cells is of a specific nature [1, 7, 8, 9, 18] determined by the "macrophage-arming factor" released by specifically sensitized lymphocytes. Other authors advocate the view on the nonspecific effect of macrophages [10, 11, 14, 15, 16, 39] while others [32] assume that lymphocytes stimulated in a variety of ways release mediators capable of transforming normal macrophages in tumouricidal ones.

Our own studies revealed that treatment with allogeneic peritoneal macrophages from healthy animals decrease lethality of experimental leukaemia [35] and the treated leukaemic animals display an increased specific lymphocyte-mediated cytotoxicity [34], as well as an increased immunological reactivity to other antigens [6].

The present paper reports on studies of the nonspecific and specific function of macrophages of leukaemic animals, the phagocytic activity and macrophage-mediated cytotoxicity, as well as the effect of normal and specifically activated macrophages on the induction of specific cytotoxicity *in vitro*.

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Material and Methods

Animals

In the experiments albino mice were used with allogeneic NKLy-leukaemia, C_3H mice with syngeneic NKLy-leukaemia, and DBA_2 mice with syngeneic L_{1210} leukaemia.

Peritoneal macrophages

A suspension of peritoneal macrophages was obtained from the peritoneal cavity of mice by means of washing with Hank's solution. Purification was achieved by adherence on glass by the method of Hoffman [7].

Phagocytosis

The phagocytic and digestive function of peritoneal macrophages was tested *in vivo* against chicken erythrocytes by the method of Donaldson [5] the degree of digestion being determined after the changes in the nucleus of phagocyted erythrocytes stained after Giemsa-Romanovsky. Phagocytic activity *in vitro* was assessed also against chicken erythrocytes in the presence of specific antiserum obtained from rabbits by the radiometric method [28]. For the radiometric assay of phagocytosis, chicken erythrocytes were labelled with ^{51}Cr and the phagocytic activity was determined by the formula,

$$\text{Per cent phagocytic activity} = \frac{A}{C} \times 100$$

where A = ^{51}Cr impulses per minute, released during destruction of phagocyting macrophages, following threefold freezing and thawing in distilled water;

C = ^{51}Cr impulses per minute, total incorporation in the erythrocytes.

Serum, lymphocyte and macrophage-dependent cytotoxicity

Serum cytotoxicity was measured according to Pierce et al. [29]. Lymphocyte-dependent cytotoxicity was determined according to Brunner et al. [3] with spleen cells employed as effector cells. In testing the macrophage-dependent cytotoxicity [3], purified peritoneal macrophages were used as effector cells and leukaemic blast cells, labelled with ^{51}Cr (100 μCi per 10^8 leukaemic cells) as target cells.

Specific cytotoxic activity (SCA) was assessed by the formula,

$$\text{Per cent SCA} = \frac{A - B}{C - B} \times 100$$

where A = ^{51}Cr impulses per minute, released by the leukaemic cells following the effect of the tested serum or effector cells (lymphocytes, macrophages);

B = ^{51}Cr impulses per minute, released by the leukaemic cells after the effect of normal serum or effector cells;

C = ^{51}Cr impulses per minute, total incorporation in the target cells, released by lysis following a threefold freezing and thawing.

Generation of cytotoxic lymphocytes in vitro

Generation of cytotoxic lymphocytes against leukaemic cells was achieved with mixed allogeneic and syngeneic lymphocyte-tumour cell or lymphocyte-macrophage-tumour cell cultures according to Sörensen and Hawkee [38]. The ratio of effector and stimulating cells was 1 : 1 according to the requirements formulated by Cerottini et al. [4] for generation of a primary immune response *in vitro*. In the mixed macrophage-lymphocyte-tumour cell cultures, the ratio of macrophages to lymphocytes was 1 : 10. Specific cytotoxic activity (SCA) was calculated by the formula,

$$\text{Per cent SCA} = \frac{A - B}{C - B} \times 100$$

where A = ^{51}Cr impulses per minute, released from labelled leukaemic cells after the effect of lymphocytes from the tested cultures;

B = ^{51}Cr impulses per minute, released in spontaneous lysis of labelled leukaemic cells during the incubation period;

C = ^{51}Cr impulses per minute, total incorporation in the target cells, released by a threefold freezing and thawing.

Results

Phagocytic and digestive function of peritoneal macrophages in leukaemic animals

Peritoneal macrophages in mice with allogeneic grafted NKLy leukaemia displayed an increased phagocytosis of chicken erythrocytes *in vivo* and *in vitro* as compared with the same cells in healthy animals (Table 1). The percentage of

Table 1
Phagocytic and digestive function of peritoneal macrophages *in vivo* and *in vitro* against chicken erythrocytes in allogeneic NKLy leukaemia

Animals	Day following grafting	Phagocytosis *in vivo*				Phagocytosis *in vitro*	
		No. of mice	Phagocyting macrophages, per cent	Phagocytic index	Digestive activity	No. of mice	Phagocytic activity, per cent
Healthy		9	39.6± 12.7	0.9± 0.6	99,7± 0,9	13	21.4± 7.0
Leukaemic	7th	10	70.9± 25.4	3.2± 2.0	99.7± 0.9	22	31.8± 10.8
Leukaemic	14th	10	75.9± 18.5	3.3± 1.6	100		
P			0.001	0.001	0.05		0.05

phagocyting macrophages in leukaemic mice was 70.9 ± 25.4 and the phagocytic index amounted to 3.2 ± 2 in experiments *in vivo* whereas in healthy animals these indices amount to 39.6 ± 12.7 and 0.9 ± 0.6, respectively. A similar pattern was

obtained *in vitro* in the presence of immune antiserum, as shown by a radiometric method of assay.

The values for the phagocytic and digestive function of peritoneal macrophages from DBA_2 mice with syngeneic L_{1210} leukaemia were not significantly different from the values of healthy mice.

Serum, lymphocyte and macrophage-dependent cytotoxicity

Macrophage-dependent cytotoxicity was tested in parallel with the lymphocyte-dependent and serum-dependent cytotoxicity, in order to obtain comparative data on the significance of the different effector systems and particularly on that of macrophages in the elimination of grafted leukaemic cells. In Fig. 1 the results

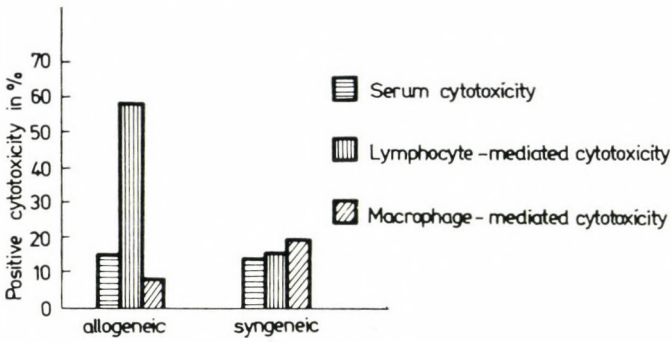

Fig. 1. Rate of serum, lymphocyte and macrophage-mediated cytotoxicity in allogeneic and syngeneic leukaemia

are presented concerning the three types of cytotoxic reaction in allogeneic and syngeneic leukaemia.

Macrophage-dependent cytotoxicity was detected in 2 out of 24 animals with allogeneic leukaemia and in 6 out of 31 animals with syngeneic leukaemia. Specific cytotoxic activity was not increased. In a few cases a weak cytotoxic reaction against leukaemic cells occurred in the serum for both allogeneic and syngeneic leukaemia. In lymphocyte-dependent cytotoxicity, differences occurred according to the type of leukaemia. While in allogeneic leukaemia lymphocyte-dependent cytotoxicity was detected in 57% of the animals, in syngeneic leukaemia it was found in 15%.

Generation of cytotoxic lymphocytes in vitro

Results are presented in Figs 2 and 3. Figure 2 illustrates the generated cytotoxicity in the absence of macrophages; it is seen that the highest cytotoxic activity and the highest specific cytotoxic activity was recorded in cultures of lymphocytes from immunized healthy animals and allogeneic leukaemic cells. Cytotoxic lymphocytes were recorded in 89% of these cultures with a mean specific

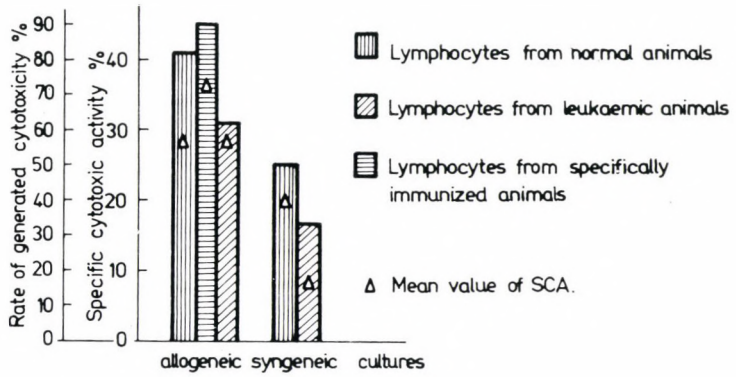

Fig. 2. Rate and degree of generated cytotoxicity against allogeneic and syngeneic leukaemic cells in mixed cultures

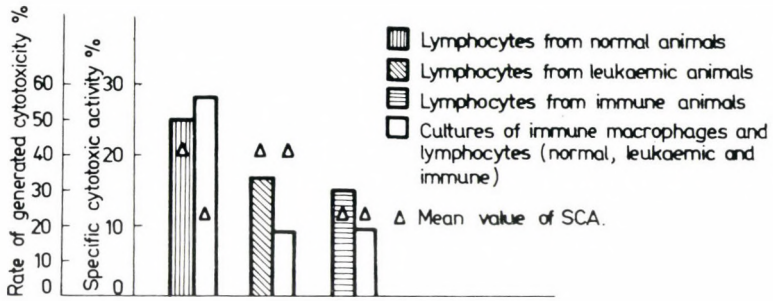

Fig. 3. Rate and degree of generated cytotoxicity in syngeneic lymphocyte-macrophage-tumour cell cultures

cytotoxic activity of 37%. In lymphocytes from healthy, non-immunized animals sensitized *in vitro* with allogeneic leukaemic cells these values were 81% and SCA 28.8%, respectively. A significantly lower rate of generated cytotoxicity (50%, $p < 0.05$) was observed in syngeneic interrelations between effector and stimulating cells. In lymphocytes from leukaemic animals, generation of cytotoxicity was less common in allogeneic and syngeneic cultures (61% and 31%, respectively) than in the corresponding cultures of normal lymphocytes ($p < 0.05$).

On the basis of these control data, the effect was evaluated of normal and specifically activated peritoneal macrophages added as additional effector cells to the lymphocyte tumour cultures. On adding allogeneic macrophages from healthy, non-immunized animals to cultures of normal and leukaemic cells, no substantial deviations were observed from the results obtained with control cultures. The rate of generated cytotoxicity amounted to 80.8%, and SCA to 25%. Similar results were obtained in the presence in these cultures of specifically activated macrophages with cytotoxicity of 82% and an SCA of 24.8%. The highest generated cytotoxicity, one of 90%, was observed in cultures with effector cells

(lymphocytes and macrophages) from specifically immunized mice whereas the SCA value was 21%, lower than the 37.0% in the controls. Similar deviations were recorded on adding activated macrophages to allogeneic culture of leukaemic lymphocytes and leukaemic blast cells. In this case the rate of generated cytotoxicity was 57.0% (*vs.* 61.0% in the controls, and SCA was 24.5% *vs.* 33.4% in the controls).

Significant differences were detected in syngeneic cultures in which the rate or degree of generated cytotoxicity decreased on the addition of peritoneal macrophages from syngeneic, specifically immunized animals in a ratio of 1 : 10 lymphocytes (Fig. 3). Thus, in the presence of activated macrophages the generated cytotoxicity amounted to 18% of the cultures of lymphocytes from leukaemic animals, and to 10% of those with lymphocytes from specifically immunized mice, as compared with 33.0% and 30.0%, respectively, in the controls. Specific cytotoxic activity of sensitized normal lymphocytes was lowered from 20.6% to 13.0%.

Discussion

In the present study, which aimed at analysing the participation of macrophages independently of, or in combination with, lymphocytes in the immune defence against leukaemia, we thought it justified to evaluate their functional condition since leukaemia is a disease of the haemopoietic system. Investigating the fundamental function of macrophages in phagocytosis and digestion of cellular matter, we found that it was unchanged in syngeneic leukaemia and was increased in the allogeneic form of grafted leukaemia. The observed stimulation in allogeneic leukaemia is considered a non-specific activation of the macrophages under the effect of normal tissue antigens of the grafted leukaemia cells. These data, namely the normal phagocytic and digestive function of macrophages in syngeneic leukaemia, as well as their capacity to react to additional stimuli with activation in allogeneic grafted leukaemia, allowed to assume that these cells have not been injured by the neoplastic process.

The specific effect of macrophages on leukaemic cells was followed on the basis of their direct cytotoxic effect on these cells, similarly to that reported for lymphocytes. The tumouricidal effect of macrophages exerted *in vitro* has been established in a number of studies in recent years. It has been reported that activated peritoneal and alveolar macrophages are capable of destroying malignant cells in contact with them, their action being manifested during incubation and being of a non-specific nature [10, 11, 18, 19]. Data are scarce concerning the direct specific effect of these cells, which was the reason why we set ourselves to follow-up particularly that specific function of the macrophages comparing it in rate and degree with the direct cytotoxic effect of lymphocytes. The results indicate that in syngeneic leukaemia lymphocyte-dependent and macrophage-dependent cytotoxicity is equally weak, no statistical difference having been observed in the cytotoxic effect of both types of effector cell. The lack of a cytotoxic effect of the macrophages

against syngeneic tumour cells has been reported also by Fidler [11]. In allogeneic grafted leukaemia, however, we found a lymphocyte-dependent cytotoxicity whereas the rate of the macrophage-dependent cytotoxicity remained low. These data give us grounds to assume that the macrophages act upon leukaemic cells through a mechanism different from that of the lymphocyte action. A more extensive possibility for the study of the interrelations between lymphocytes, macrophages and leukaemic cells in the induction of the specific immune response is offered by the system of generation of cytotoxic lymphocytes *in vitro*. In these experiments it has been established that the addition of allogeneic normal or activated macrophages does not substantially alter the possibilities of lymphocyte cytotoxicity generation which contradicts the findings of some authors [19, 41] but is most likely associated with genetic differences between the two types of effector cells [37]. Interesting are the results obtained in syngeneic interrelations between the three cellular elements, where adding of specifically activated macrophages to the cultures lowers the rate of generated cytotoxicity or the degree of specific cytotoxic activity. These data show that macrophages exert an immunosuppressive effect and decrease the possibility of generation of cytotoxic lymphocytes *in vitro*. The problem of the immunosuppressive role of macrophages has emerged only recently although similar findings have already been reported [33]. The failure of generation of cytotoxic lymphocytes against allogeneic cells in rats was explained by Ohler et al. [25, 26] with the presence of suppressor macrophages in the spleen cell population of these animals. Wing and Remington [44] established that the nature of interrelationship between macrophages and lymphocytes in terms of activation or suppression of lymphocyte reactivity depended upon the ratio between the two types of cell, the ratio of inhibition being 1 : 1 up to 1 : 10. The same authors have shown that at particular cell ratios an activation is induced by normal as well by activated macrophages whereas a suppression was exerted only by activated cells, as established in our studies on the generation of cytotoxic lymphocytes *in vitro*.

As far as the mechanism of the observed suppression is concerned, there are several hypotheses. Some authors assume that the cells undergoing degradation because of macrophage action release thymidine which competes with the labelled thymidine during its incorporation in the DNA molecule and creates the false impression of a suppressed lymphocyte reactivity. This hypothesis, however, has not found any confirmation in other studies [44] and cannot explain the suppression of the generation of cytotoxic lymphocytes *in vitro*. Brunda and Raffel [2] found that the nature of macrophage action upon immunogenetic cells is conditioned by the duration of their mutual contact. The simultaneous short-term incubation of both types of cell contributes to the enzymatic degradation of cellular antigens to less divergent chains, suitable for the induction of a cell-mediated immune response rather than for a humoural one. A more continuous contact, however, might lead to the annihilation of that activity. A similar process probably takes place in mixed cultures in the presence of activated macrophages similarly as in our experiments where a more complete degradation of the leukaemic cells led to a reduction of the immunogenetic stimulus directly affecting the lymphocytes.

A number of other studies provided evidence for the existence of soluble factors facilitating the activation of lymphocytes as well as of soluble substances with a suppressive activity which are released by the macrophages and are capable of suppressing the blastogenic response of T-lymphocytes as regards antigens and mitogens [40]. According to Stobo [40] this "soluble material" plays most likely an immunoregulatory role and exerts its action either directly upon the effector T-cells or through cooperation with other regulator T-cells.

These data indicate that the mechanism of macrophage action in immune reactions in general and in immune responses to malignant tumours in particular remains unelucidated. It is uncontestable, however, confirmed also by our studies, that these cells play a substantial role in the induction of the immune response to malignant, leukaemic cells, most likely by means of a direct effect upon them and their immunogenetic activity.

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Sarcoidosis Associated with Autoimmune Haemolytic Anaemia and Paroxysmal Nocturnal Haemoglobinuria Red Cell Abnormality

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A 29-year-old male with sarcoidosis autoimmune haemolytic anaemia and paroxysmal nocturnal haemoglobinuria is described. Throughout his illness the chest films showed fibrosis in the right hilar region and he had had several pneumonias in the right lung. He had had massive splenomegaly and a splenectomy was performed. He was treated with prednisone and cyclophosphamide. Because his blood group was initially confused, several incompatible blood transfusions were given. Two types of antibody were detected: an autoantibody with "s" specificity and an alloantibody with Rh "D" specificity.

Other interesting features in this case revealed at autopsy were a viral pneumonia and *Toxoplasma gondii* infection of the brain. As far as we know, this is the first reported patient with this unusual association.

*

Anaemia is uncommon in sarcoidosis [13], and haemolytic anaemia is a very rare complication of sarcoidosis, and to date it has been described in few well-documented examples [19]. Paroxysmal nocturnal haemoglobinuria (PNH) is diagnosed on the basis of a positive acidified serum lysis test. There is a well-documented association between PNH and other haematological disorders such as aplastic anaemia, myeloblastic leukaemia and myelosclerosis [9, 21]. The concomitance of a positive antiglobulin test and positive tests for PNH on the red cells of the same patients have been recorded [1, 7, 12, 14].

As far as we know, the coexistence of sarcoidosis, PNH and autoimmune haemolytic anaemia (AIHA) has not been reported. The aim of this paper is to report on a patient with this unusual association. Other unusual features of the case were the presence of toxoplasmic encephalitis and viral pneumonia.

Report of a Case

A 29-year-old white man had first been seen in January, 1972, on account of pallor, marked weakness and weight loss. This was associated with hepatomegaly (3 cm) and splenomegaly (6 cm). A diagnosis of AIHA was made. The blood group was typed as AB Rh-positive. The patient received several blood transfusions and

was treated with prednisone 40 mg/day, which was slowly reduced to a maintenance dosage.

In December, 1972, as the spleen showed progressive enlargement and haemolysis did not respond to prednisone, a splenectomy was performed. The spleen weighed 1500 g and showed microscopically sinusoidal dilatation accompanied by some foci of haemorrhagic necrosis. A biopsy of the liver showed only an increase of lymphoid cells in the sinusoidal spaces and a non-specific inflammatory reaction.

From January, 1973, to January, 1974, he was treated with prednisone as an out-patient. In January, 1974, despite continued therapy with prednisone, haemolysis persisted and he was referred to the Institute of Haematology and Immunology for diagnostic studies.

At admission, the patient was pale and jaundiced. The liver was enlarged to 3 cm below the costal margin, the pulse rate was 92/min and the blood pressure, 130/80 mmHg. There were no palpable lymph nodes. The respiratory system and the nervous system were clinically normal. The optic fundi were normal. Nocturnal urines were darker than during the day with frank haemoglobinuria.

Haematologic findings were: Hb 4 g/dl; reticulocytes 23%, platelets 207×10^9/l, WBC 15×10^9/l with 2% myelocytes, 2% band cells, 81% neutrophils, 1% monocytes and 14% lymphocytes. There were 30 normoblasts per 100 leukocytes. MCV 90 Fl, MCH 27 pg, MCH 30 g/dl. ESR was 90 mm/hr. The peripheral blood film showed hypochromia, anisocytosis, poikilocytosis, macrocytosis, polychromatophilia, basophilic stippling, Cabot's ring, Howell-Jolly bodies, and spherocytosis. The bone marrow aspirate was hypercellular with gross erythroid hyperplasia. Prussian blue reaction demonstrated iron in the bone marrow stores.

The Ham and Crosby tests were strongly positive and so was the sucrose haemolysis test. Haemosiderinuria was positive after Perl's staining. Serum iron was 109 μg/100 ml, TIBC 351 μg/100 ml, serum calcium 9 mg/100 ml.

Total serum bilirubin was 3.1 mg/100 ml, unconjugated bilirubin 2.9 mg/100 ml, haemoglobin electrophoresis revealed a normal pattern. The Hb F was 0.7%. Total serum proteins were 6.9 g/100 ml with 3.1 g/100 ml albumin and 3.8 g/100 ml globulin. Serum protein electrophoresis was within normal limits, erythrocyte acetylcholinesterase 0.05 μ (normal, 0.5 to 1 μ). The leukocyte alkaline phosphatase score was elevated to 127. Other routine biochemical tests were normal. The direct and indirect Coombs tests were strongly positive. The blood group was AB Rh-negative with a genotype cde/cde Mn ss, HLA typing was A2, A11: B5, B7.

Chest X-ray revealed extensive fibrosis in the right hilar region. An intravenous cholangiography showed gallstones. No significant abnormality was seen in the upper gastrointestinal tract, a barium enema study and a bone survey. ECG was normal.

Transfusion of saline-washed red cells was administered and an exchange transfusion was performed. The haemoglobin increased to 12 g/dl and the patient was discharged after 42 days on 40 mg/day prednisone. The evolution of the disease is shown in Fig. 1.

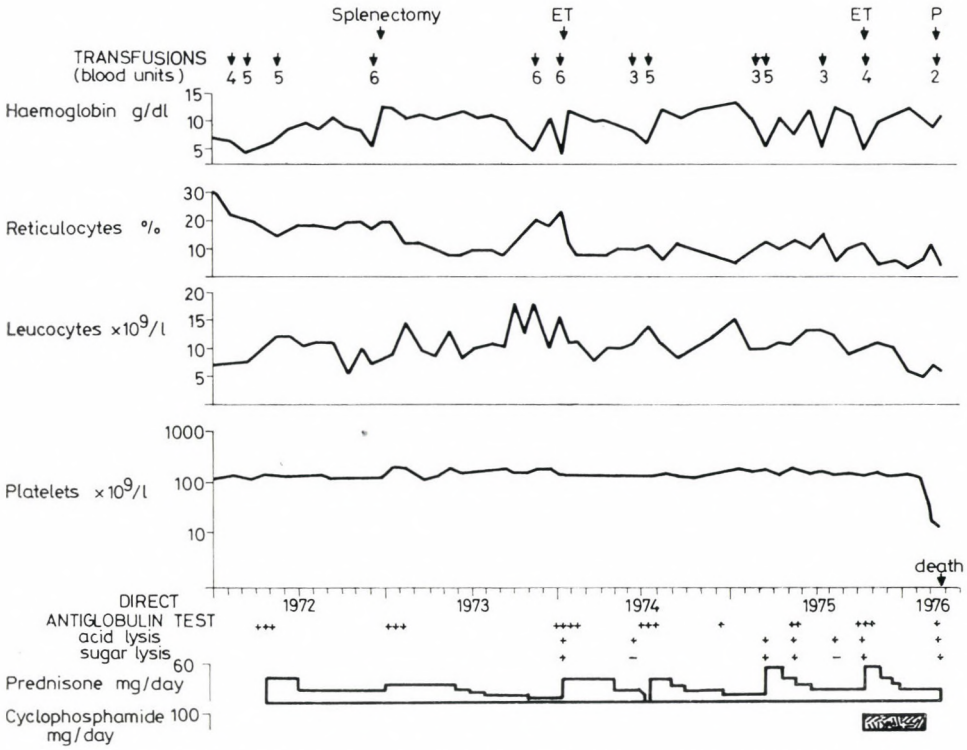

Fig. 1. Haematological evolution of the patient. ET = exchange transfusion, P = plasmapheresis

From June, 1974 to September, 1975, the patient was readmitted three times with fever and pneumonia of the right lung which was responsive to broad spectrum antibiotic therapy.

In September, 1975, he was again admitted on account of fever and pains in both knees and the right elbow. He had atrophy of the muscles of the right shoulder with alate scapula. Deep tendon reflexes and sensory examination were normal. A muscle biopsy of the right deltoid showed atrophic muscle cells without other changes.

Chest X-ray showed persistence of the right parahilar fibrotic process and the bones generalized osteoporosis. Rheumatoid factor, LE phenomenon and antinuclear factor were negative. The antistreptolysin 0 titre was 50 todd units. The Coombs tests remained strongly positive and the acidified serum lysis tests were positive. The direct Coombs test was also carried out on the cells remaining unlysed at the end of the Ham test and it was found to be positive. Haemoglobin that had been between 10 and 11 g/dl, fell to 4 g/dl and the reticulocyte count rose slightly. An exchange transfusion was performed and cyclophosphamide 100 mg/

day was added to the therapy. This was followed by a remarkable improvement with disappearance of the arthralgia. He was discharged on December 31, 1975.

On his final admission on February 8, 1976, he had anorexia, fever, sweats and progressive shortness of breath. Repeated cultures of urine, sputum, blood and bone marrow were negative. Chest X-rays showed only the fibrotic lesion on the right lung. The direct and indirect Coombs tests, the acidified serum lysis tests, and the sucrose haemolysis test, were again positive. Serum calcium was 13.7 mg/100 ml, serum phosphate 3.9 mg/100 ml, urinary calcium 792 mg/24 hr (normal, 30 to 150 mg/24 hr). A skeletal survey showed generalized osteoporosis.

One month after admission the patient still had intermittent pyrexia which was unresponsive to antibiotic therapy and showed continuing clinical deterioration with progressive enlargement of the liver, palpable 7 cm below the costal margin and marked jaundice. The total serum bilirubin increased to 28.5 mg/100 ml and the conjugated bilirubin to 23.5 mg/100 ml. The haematological findings were: Hb 6.6 g/dl, reticulocytes 12%, platelets 28×10^9/1 mm^3, WBC 6.7×10^9/1 with 3% myelocytes, 3% metamyelocytes, 3% band cells, 33% neutrophils, 4% monocytes and 54% lymphocytes.

The coagulation study was normal except for the bleeding time, clot retraction and prothrombin consumption test, which were interpreted as caused by the thrombocytopenia. Tests for intravascular coagulation were negative. Total serum protein level was 5 g/100 ml with 1.7 g/100 ml albumin and 3.3 g/100 ml globulin. The blood urea and the blood ammonia (normal, 75 to 196 µg/100 ml) increased progressively to 120 mg/100 ml and 380 µg/100 ml, respectively. Australia antigen was repeatedly negative.

The patient had flapping tremor of the fingers and hands. Plasmapheresis of 3 litres of plasma was performed over a 3-day period. Initially, the plasmapheresis was followed by a rapid clinical improvement. There was a fall in blood urea to 62 mg/100 ml and in the blood ammonia to 178 µg/100 ml, but the serum total bilirubin persisted at 26.5 mg/100 ml with 20.5 mg/100 ml of conjugated bilirubin.

Subsequently, the patient became progressively dyspnoeic, his clinical condition deteriorated rapidly, pneumonia, cardiac failure, renal failure and coma occurred and he died on March 27.

Immunological studies

Anti-red cell antibodies. Eluates were obtained from the patient's red cells in January, 1974. The eluates contained antibody activity with "s" specificity. Two types of serum antibody were found: an auto-antibody with "s" specificity present in a titre of 1 : 32 and an allo-antibody with Rh "D" specificity present in a titre of 1 : 512. Both antibodies were of the IgG type.

Cellular and humoral immunity. Spontaneous rosette formation with sheep RBC was 11% (normal, 55 to 75%) and rosette assay for cells bearing complement receptors (EAC), 50% (normal, 10 to 33%). Blast transformation of peripheral blood lymphocytes stimulated with phytohaemagglutinin (PHA) was 5% (normal,

40 to 72%). All cutaneous tests performed (PPD, brucellin, toxoplasmin, *Candida albicans*, streptokinase) were negative. Serum protein immunoelectrophoresis revealed a slight increase in IgG and IgM with normal IgA. Complement titration (C'H50) was 27 μ (normal, 28 $\pm$ 4 μ).

Autopsy

Gross findings

The tissues were jaundiced. The mediastinal lymph nodes were slightly enlarged. The right lung showed pneumonic changes. The liver weighed 3100 g. The heart was enlarged. The external surface of the brain was symmetrical. Serial sections revealed an area of greyish discolouration and softening in the left basal ganglion. The wall of the lateral ventricle was slightly elevated. In the right internal capsule there was a smaller area of softening.

Microscopic findings

Necrosis was observed in the brain corresponding to the areas of softening. Multiple toxoplasma pseudocysts were seen (Fig. 2). A viral pneumonia with fibrinous exudate was found in the right lung. Eosinophilic intranuclear inclusions were present in the alveolar cells (Fig. 3). No toxoplasma, bacteria or fungi were seen. Both lungs, especially the right one presented characteristic sarcoid non-

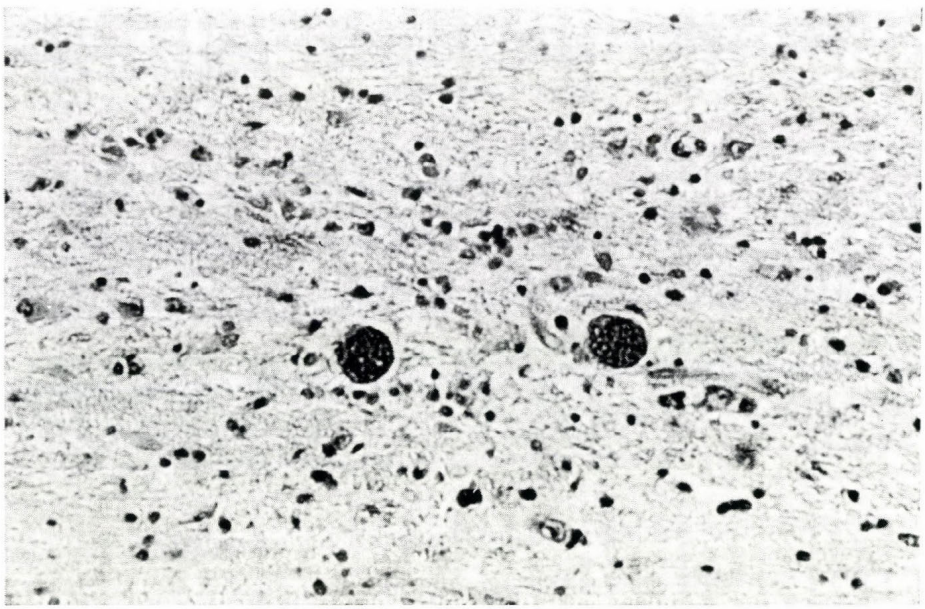

Fig. 2. Two toxoplasma pseudocysts are seen in the brain. Haematoxylin-eosin, × 100

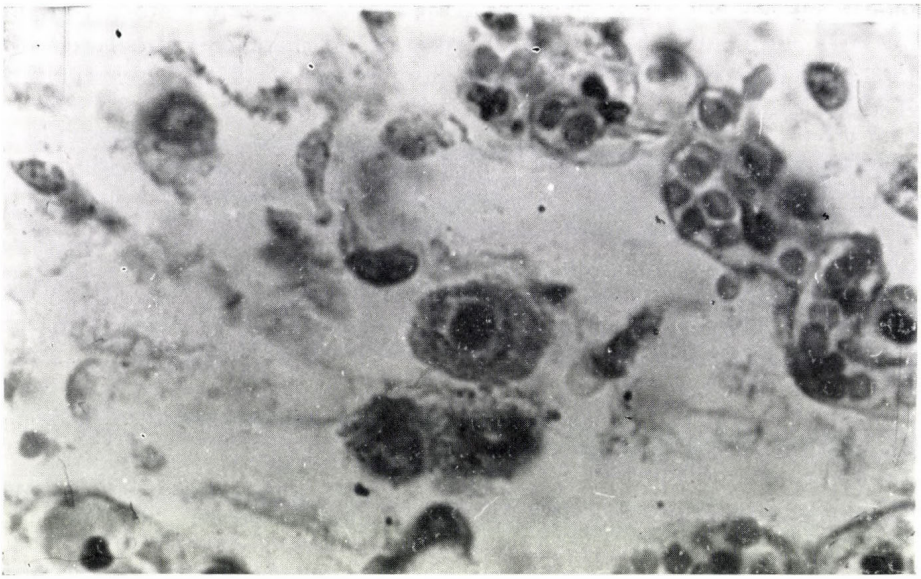

Fig. 3. Eosinophilic intranuclear inclusion in a desquamated alveolar lining cell. Haematoxylin-eosin, ×400

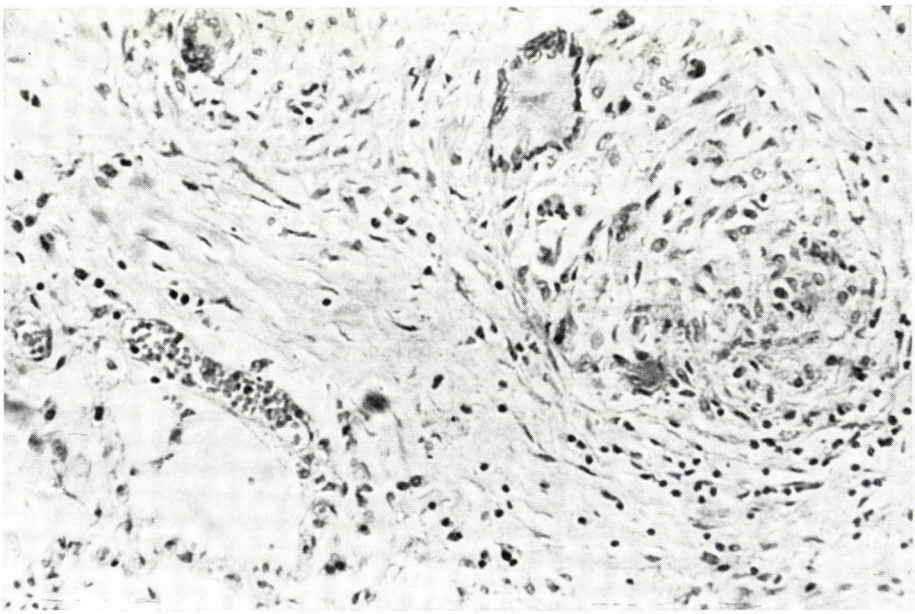

Fig. 4. A sarcoid "non-caseating" granuloma seen in the lung. Haematoxylin-eosin, ×100

caseating granulomas. Langhans giant cells and asteroid inclusions were found (Fig. 4). Some granulomas showed collagenous fibrosis particularly in the right hilar region where the coalescence of granulomas produced extensive fibrosis Ziehl-Neelsen preparations did not contain acid-alcohol-fast bacilli. Mediastinal lymph nodes had lost the basic architectural pattern. Fibrosis and sarcoid granulomas were present. The normal architecture of the liver lobules was preserved. There was marked congestion of the sinusoids. A focal inflammatory infiltration of mononuclear lymphomonocytoid cells involved the portal areas with scattered necrosis of liver cells. No cytoplasmic inclusion bodies were observed. There was fibrosis and haemosiderosis. In the kidneys fibrosis, haemosiderosis and nephrocalcinosis with acholaemic nephrosis were present.

Comments

Sarcoidosis was diagnosed on the basis of the typical histological findings and the exclusion of other categories of granulomatous disease. Great diagnostic problems were encountered in the patient who initially presented with splenomegaly and haemolytic anaemia with a poor response to corticosteroids and splenectomy. The persistence of fever and the fibrosis in the right lung throughout the course of his illness were strongly suggestive of sarcoidosis present since the beginning of the disease. During the course of the disease some other findings common in sarcoidosis were observed: Deficiency of the cell-mediated immune response, arthralgias, hypercalcaemia and hypercalciuria. The correct diagnosis could, however, be made only when the autopsy revealed characteristic sarcoid lesions in the lungs and mediastinal lymph nodes. Although sarcoidosis is usually a multisystemic disease, it is well recognized that it may occur with solitary organs or tissue involvement. This is especially true for pulmonary sarcoidosis [16, 20].

The association of haemolytic anaemia with sarcoidosis is of such infrequent occurrence that it is difficult to state whether the two diseases are related or coexist by chance. Some workers suggest that splenic involvement is not the prime factor in the genesis of haemolytic anaemia in sarcoidosis, so that in some patients an autoimmune reaction must receive consideration and is reasonable to suppose that reticuloendothelial tissue elsewhere in the body may have an aetiological role [19].

In the patient two different antibodies were formed, both incomplete antibodies of the type IgG. One was an auto-antibody with "s" specificity, the other was an allo-antibody with Rh "D" specificity. The latter seems to be related to the incompatible transfusions received at the beginning of his illness on account of an error in the Rhesus blood group characterization.

False positive results may be obtained with red blood cells coated with auto-antibodies, agglutinating spontaneously in albumin when they are tested with the usual commercial albumin-reacting anti-0 serum. For such cases, Rh-typing in albumin is invalid and Rh-typing must be performed either with anti-Rh

saline agglutinin reagents or by carefully controlled adsorption and elution studies [15].

The most surprising feature in this case were the positive tests for PNH. In performing Ham's test, a control with heat-inactivated acidified serum was always included and this was consistently negative. This excluded the possibility that the microspherocytes present in the sample of red cell tested might produce false positive acidified serum tests [7, 14]. An association between PNH and other haematological disorders including AIHA is rare but well documented [1, 7, 9, 17, 21].

A patient who developed a positive acidified serum test during the course of a lymphoproliferative malignancy with a concomitant AIHA has been described [18]. In this case, however, the positive acidified serum test was not characteristic of PNH because it was negative with the patient's own serum, as in congenital dyserythropoietic anaemia [4]. The positive acidified serum test and sucrose lysis test in our case support the development of an abnormal clone of erythroid cells. An increased sensitivity of PNH erythrocytes to lysis by iso- and autoantibodies has been described [7]. This factor might probably lead to an increase of the severity of haemolysis in AIHA, making worse the course and prognosis of the disease.

The possible role of an immune phenomenon in the pathogenesis of sarcoidosis is suggested by the increased incidence of hypersensitivity syndromes in this disease [8]. The association of sarcoidosis and AIHA has an important theoretical significance. It is well-recognized that some patients with sarcoidosis have a deficient immune apparatus and limitations in the ability to initiate delayed hypersensitivity, as in our patient. It has been suggested that an inability to destroy modified immunocompetent cells because of an aberrant immune homeostasis mechanism may result in multiple autoantibody formation. Patients with sarcoidosis would appear to be particularly vulnerable to this sequence of events [13]. An increased antibody response to mismatched blood is also seen in sarcoidosis [11].

An "aplastic" crisis can complicate the evolution of AIHA and refractory anaemia secondary to AIHA has been reported, related probably to a mutation or clonal degeneration of the stem cell [3]. The association between PNH and AIHA may be linked pathogenetically on the basis of a somatic mutation at stem cell level [14]. The same hypothesis could explain the findings in our case.

The chance of sarcoidosis, AIHA and PNH occurring as unrelated diseases in the same patient would seem very remote. The fact that the three events apparently developed together, suggests a close relation among them. A logical sequence of events could be: Sarcoidosis → AIHA → clonal mutation → PNH red cell abnormality.

Recurrent episodes of pneumonia were observed in the patient; we found a deficient cell-mediated immune response, shown by decreased E rosettes, PHA transformation and abnormal delayed hypersensitivity, but immunoglobulin production was unimpaired. Although an increased susceptibility to infections is

the hallmark of impairment in cellular immunity, in sarcoidosis an increased susceptibility to infections is unusual. It has been stated that in sarcoidosis the incidence of bacterial and viral diseases differed little from that in the general population [18]. The toxoplasmic encephalitis and the viral pneumonia complicating this case, may have been related with the absence of the spleen, the decreased cell-mediated immune response, the frequent blood tranfusions and the therapy with corticosteroids and an immunosuppressant [2, 5, 6]. It is probable that fibrosis as a local factor had a predisposing role in the recurrent episodes of pneumonia appearing always in the right lung.

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Plasmapheresis in Patients with Leukaemia, Multiple Myeloma and Immune Complex Diseases

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Therapeutical partial plasma exchange was performed on 9 patients with acute leukaemia, 14 patients with monoclonal and polyclonal gammopathies and 3 with primarily immune complex disease. The procedure was effective in 21 out of the 27 courses performed on patients with gammopathies and in all cases of the primarily immune complex diseases. In acute leukaemic patients the course of the disease was favourably influenced by plasmapheresis. The results show that partial plasma exchange is an effective adjunct therapy in the treatment of patients with haematological diseases.

Introduction

Plasma exchange has been used during the last years in the treatment of a variety of disorders and some striking results have been obtained [1, 7, 10, 12]. Plasma exchange is still an experimental approach and in spite of some exciting preliminary results neither the exact fields of indication, nor the optimum quantity and time schedules of plasma exchange are well established. The increasing availability of continuous flow and discontinuous cell separator allows to apply regimes of frequently repeated large-volume exchange (4 to 5 l of plasma per session).

The present study summarizes the results of repeated partial plasma exchange (300 to 600 ml plasma per session) in 26 patients with haematological diseases.

Materials and Methods

Patient population. Twenty-six patients were treated with partial plasma exchange (PPE): 9 patients with acute leukaemia, 14 patients with monoclonal and polyclonal gammopathies and 3 with primarily immune complex (IC) disease.

Plasma exchange. The patients with different clinical conditions were subjected to manual PPE procedure (300 to 600 ml plasma per session in 1.5 to 2 hours). The removed plasma was replaced by 5% albumin solution or fresh frozen plasma according to the clinical condition. The time schedule of plasma exchange during the chemotherapeutic treatment of acute leukaemic patients is shown in Figs 1 and 2.

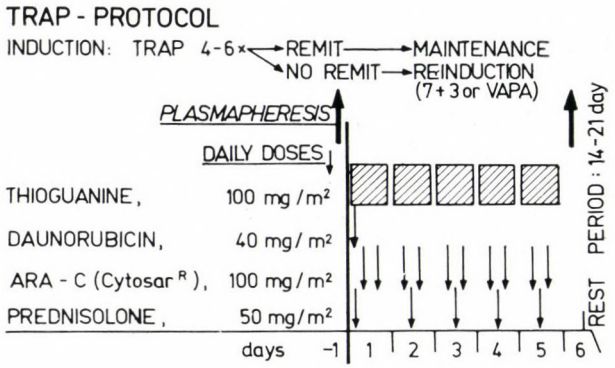

Fig. 1. Time schedule of partial plasma exchange during chemotherapeutic treatment of patients with AML

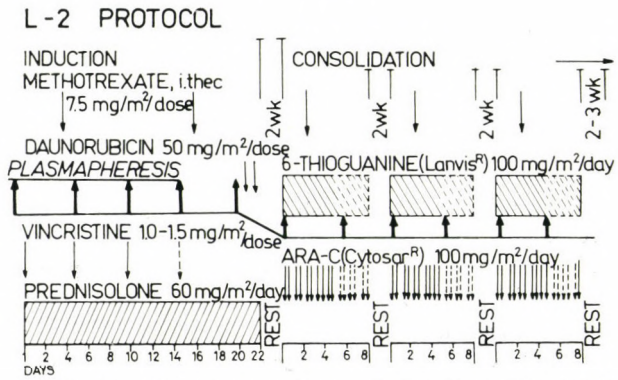

Fig. 2. Time schedule of partial plasma exchange during chemotherapeutic treatment of patients with ALL

The patients with monoclonal gammopathies were treated with M-2 protocol [3], or received intermittent cyclophosphamide treatment (10 mg/kg every four weeks).

The level of circulating immune complexes (CIC-s) was measured by complement consumption test [4], C1q solubility test [8] and C1q-binding assay [13].

Results

Antigen-antibody complexes have been demonstrated in the serum of patients with cancer, including leukaemia [2, 11]. In a previous study we have investigated 75 leukaemic patients for the presence of CIC-s by three different tests [9].

Positive results with at least two tests were found in 64% (Table 1). The role of CIC-s in the genesis of leukaemia is still unknown, but it has been suggested that such complexes could prevent malignant cells from immune destruction [5]. This prompted us to induce plasmapheresis into the treatment of leukaemic patients.

Table 1

Occurrence of circulating immune complexes in acute leukaemia

Type of leukaemia	No. of patients tested	Positive results in at least one serum sample in			
		three tests	two tests	one test	none
AML	27	10	8	9	0
ALL	4	2	2	0	0
CML blastic crisis	11	4	3	4	0
CML	11	0	6	3	2
CLL	22	4	9	7	2
Total	75	20	28	23	4

Among investigated patients 64% gave a positive result in at least two tests.

During the last year, 18 patients with acute leukaemia were admitted to our Department. These patients were randomly distributed into two groups: 9 patients underwent serial plasmapheresis combined with complex chemotherapy and 9 patients were treated by the same chemotherapy protocol without plasmapheresis. The clinical and haematological data which may be of prognostic value are summarized in Table 2. In the main prognostic factor, the type of leukaemia, there was no difference between the two groups. The pheretized group turned out to belong to a slightly better prognostic group according to age distribution, initial white blood cell count and platelet count, and the presence of infection at the time of diagnosis of acute leukaemia. On the other hand, organ infiltration, as a bad prognostic factor, was present in two pheretized patients.

The CIC level was measured by complement consumption and C1q solubility test before and after plasmapheresis. The results showed that this amount of plasma exchange cannot reduce the CIC level (Table 3).

Comparing the number of remissions in the pheretized and control groups, the patients treated by PPE fared better (Table 4). Three complete remissions were obtained in the pheretized group while in the control group none; there were 4 partial remissions in the pheretized group, while in the control 3. The time of observation was too short to evaluate differences in survival, but more patients are alive in the pheretized group. Still, no conclusion can be drawn from these preliminary results because the retrospective evaluation of the randomly selected patients showed that those subjected to PPE had slightly better prognostic factors.

Table 2

Prognostic factors in the plasmapheresis and non-plasmapheresis groups of adult acute leukaemic patients

Prognostic factors	Number of patients	
	Serial plasmapheresis + chemotherapy	Chemotherapy only
Type of leukaemia		
ALL	2	2
AML	7	7
WBC count		
$<50{,}000/mm^3$	7	6
$>50{,}000/mm^3$	2	3
Platelet count		
$<25{,}000/mm^3$	2	2
$25–50{,}000/mm^3$	1	4
$>50{,}000/mm^3$	6	3
Age		
<35 years	5	3
35–60 years	3	4
>60 years	1	2
Infection at diagnosis	3	7
Organ infiltration	2	0

In addition, the results obtained in the control group were worse than those recorded previously in 120 leukaemic patients, where complete remission was obtained in 20% of AML patients and in 58% of ALL patients.

Table 3

Circulating immune complexes in leukaemia before and after plasmapheresis

Test	Before plasmapheresis	After plasmapheresis	p*
Complement consumption	4.32%	4.76%	>0.1
C1q solubility	0.376	0.277	>0.1

* Significance calculated by paired *t*-test

The results obtained in gammopathies are summarized in Table 5. Two patients with light-chain disease were treated in three course by PPE. The indication for PPE was uraemia. In 2 cases the plasmapheresis was effective.

Table 4

Number of remissions in adults with acute leukaemia

	Plasmapheresis + chemotherapy	Chemotherapy only
Complete remission	3/9	0/9
Partial remission	4/9	3/9

Results obtained in 120 leukaemic patients between 1972–1978

	AML	ALL
Complete remission	20%	58%
Partial remission	32%	24%

Ten patients with multiple myeloma and Waldenstrøm macroglobulinaemia were treated in 22 courses of PPE. The indication was hyperviscosity syndrome in each case. Blood viscosity decreased in 17 cases and the clinical signs of hyperviscosity improved in these patients. In 5 cases the amount of exchanged plasma was inadequate. One patient with hyperglobulinaemic thrombocytopathy and one with angioimmunoblastic lymphadenopathy were subjected to PPE because of severe haemorrhagic diathesis. Before starting the plasmapheresis all conventional therapeutic methods failed to influence the severe bleedings. In the patient with hyperglobulinaemic thrombocytopathy, platelets were demonstrated to be coated by IgG, therefore platelet exchange was combined with PPE. This had a dramatic effect, after three days of treatment the haemorrhagic diathesis stopped. In the patient with angioimmunoblastic lymphadenopathy the gastrointestinal, nasopharyngeal bleeding and haematuria stopped after eight plasmaphereses without any other treatment.

Table 5

Effect of plasmapheresis in monoclonal and polyclonal gammopathies

Diagnosis	No. of patients	No. of courses	Indication	Effect	
				improved	none
Light-chain disease	2	3	Uraemia	2	1
Multiple myeloma	6	22	Hyperviscosity	17	5
Waldenstrøm macroglobulinaemia	4				
Hyperglobulinaemic thrombocytopathy	1	2	Haemorrhagic diathesis	2	0
Angioimmunoblastic lymphadenopathy	1				

A 27-year-old boy with fulminant allergic disseminated haemorrhagic skin necrosis was treated during the first four weeks of his illness with steroids and broad-spectrum antibiotics without any effect. As a result of a two-week-course of daily PPE he became afebrile, no new haemorrhagic necroses occurred and he recovered completely (Fig. 3). The post-necrotic wounds healed after skin transplantation. In this patient increased CIC levels were demonstrated by three different tests. The CIC-s decreased dramatically after PPE.

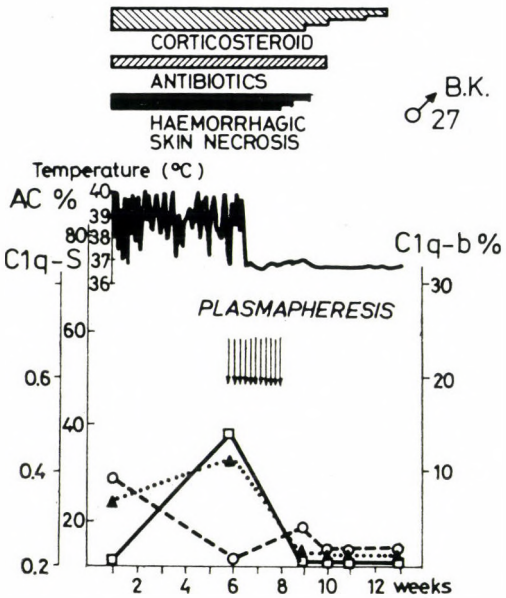

Fig. 3. The CIC level and the course of the disease in a patient with allergic haemorrhagic skin necrosis: —— complement consumption test (AC); - - - - - Clq solubility test (Clq-s); . . . . . Clq binding test (Clq-b)

In a patient with Henoch-Schönlein purpura a two-month course of steroids and immunosuppressive drugs was ineffective, the disseminated haemorrhagic skin manifestations, severe intestinal bleeding and haematuria continued, therefore PPE was introduced. As a consequence, the level of CIC-s decreased, but the haemorrhagic manifestations remained. Combined PPE and immunosuppression with 2.5 mg/kg cyclophosphamide for five months was needed to achieve complete remission (Fig. 4). The same regime was effective in the treatment of another patient with Henoch-Schönlein disease in whom no increase in the CIC level was detected by our assay methods.

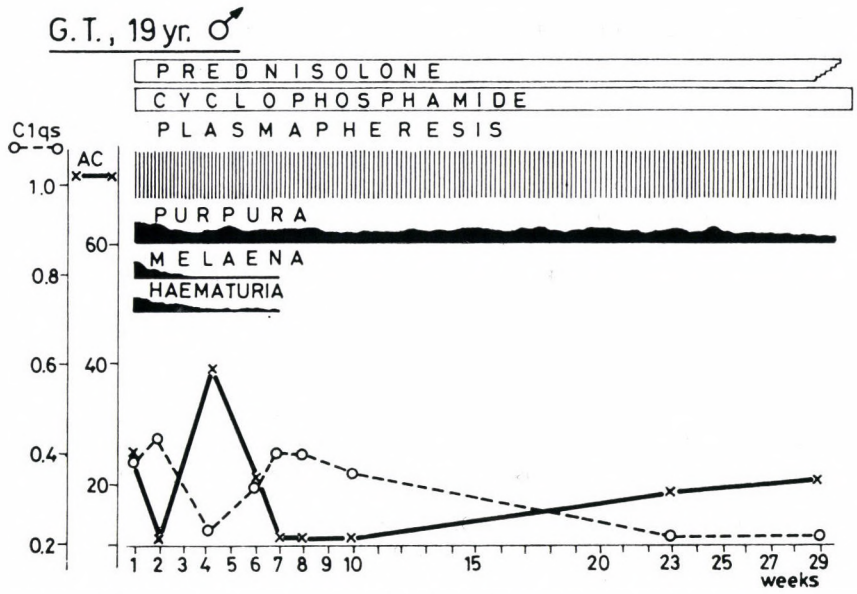

Fig. 4. CIC level and the course of the disease in a patient with Henoch-Schönlein disease treated by partial plasma exchange —— complement consumption test (AC) - - - - C1q solubility test (C1q-s)

Discussion

In this study, manual plasmapheresis was performed and the effect of this less intensive PPE was examined in acute leukaemia, monoclonal and polyclonal gammopathies and IC diseases.

In acute leukaemic patients the course of the disease seemed favourably influenced by plasmapheresis. The number of treated patients was small, so that the slightly different prognostic factors may have also influenced the result. On the other hand, the beneficial effect of PPE could not be correlated with the simultaneous change in the CIC level. The decrease of acute phase reactants – which have been demonstrated to act as non-specific blocking factors of cell-mediated immune reactions in hosts against tumour cell reactions [6] – might have also contributed to the favourable result.

In monoclonal and polyclonal gammopathies, plasmapheresis was effective in most cases. In monoclonal gammopathies the improvement could not be attributed to the PPE alone, because these patients have to be treated by cytostatics at the same time. But in polyclonal gammopathies, PPE without any other specific treatment was effective in the relief of severe clinical symptoms.

Plasmapheresis performed in 3 patients with primarily immune complex diseases was effective in every case, although the clinical improvement was not always reflected in the decrease of CIC-s. In these cases immune complexes of other composition which are not detectable by our assay methods may have been present,

but the beneficial effect of plasmapheresis was due rather to the removal of other biologically active proteins such as immunoglobulins, complement components, acute phase reactants and clotting factors. On the other hand, IC diseases have a changing clinical course and spontaneous recovery may coincide with plasmapheresis in some cases.

In conclusion, even a less intensive plasma exchange is effective in some patients with haematological disorders, especially immune complex diseases and gammopathies. In acute leukaemia these preliminary results pointed to a beneficial effect, but long-term follow-up of a larger patient population is needed to draw definite conclusion.

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Formation of Debris in Stored Human Blood*

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The formation of debris in human blood, stored under standard blood bank conditions, was investigated by measuring debris weight, screen filtration pressure (SFP) and counting the number of aggregates. Formation of microaggregates was most pronounced in blood five to fifteen days old as was the SFP. A tendency of progressive increase was found in debris weight with storage time.

When blood is stored using standard blood bank conditions, degenerative changes occur. Microaggregates of platelets, white blood cells and fibrin are one manifestation of these changes. Aggregates are formed if the blood is stored in acid-citrate-dextrose (ACD) or citrate-phosphate-dextrose (CPD) solutions [1, 2]. In stored packed cells microaggregate formation is even more rapid [3]. Apart from microaggregates the debris consists of disintegrating cells, precipitated fibrin and clots.

Three methods are currently available for estimation of the amount of debris in blood: weight of debris, screen filtration pressure (SFP) and counting the number of aggregates by using a coulter counter. These three methods measure different aspects of debris formation [12, 13].

In earlier reports a progressive increase in SFP and quantity of microaggregates with storage time has been reported [1, 7, 9, 16]. From extensive studies on dog blood as well as human blood this has, however, not been the experience of this laboratory. In dog blood the mechanisms for aggregate formation are probably different from humans [6] and aggregate formation seems to be maximal between the fifth and eighth day of storage. Before and after that time SFP is seldom elevated.

It was the aim of the present investigation to study the formation of debris during storage of human blood by using these three different methods.

* This study was performed at the Surgical Research Laboratory, Department of Surgery, Tulane University, New Orleans, La., USA.

Material and Methods

Blood. Forty-seven units of stored human blood were used in these experiments. The age of the blood was between 2 and 21 days and the blood was stored using normal blood bank conditions in CPD.

Screen filtration pressure (SFP). The technique described by Swank et al. [18] was used. By this method blood was forced at a constant rate against a fine-mesh screen with 20 μm diameter pores and the resistance of flow through the screen was measured as the backward pressure. The mean of triple determinations was calculated.

Coulter counting. The technique described by Solis et al. [14] and by Marshall et al. [8] was used. One millilitre of blood was mixed with 200 ml of Isoton (Coulter Diagnostics, Inc.) containing 0.3 ml Lysing agent (B 3145 – 6 A, Scientific Products, Inc.). The number of aggregates was counted in 0.5 ml of the sample within 15 seconds using a 400 μm aperture detecting tube in 12 different channels from 16.0 to 203.0 μm in diameter. The mean of three different determinations was calculated. The volumes were calculated by multiplying the number of particles counted by the mean geometric volume of particles found by a given channel.

Debris weight. The mesh screen used for SFP measurement was weighed before and after the passage of the amount of blood required for pressure determination (0.68 ml).

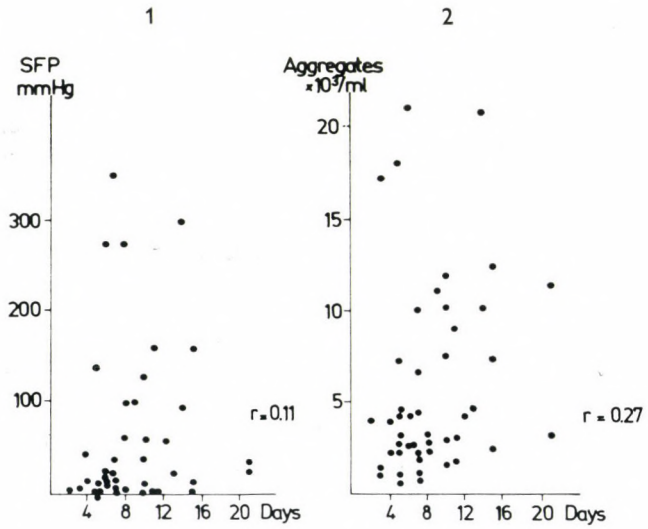

Fig. 1. Relation between screen filtration pressure (SFP) and storage time. Peak values of SFP within days 5–15

Fig. 2. Relation between total number of microaggregates/ml and storage time. Peak values within days 5–15

Results

The results are presented in Figs 1 to 4.

No linear correlation was found between SFP, the quantity of microaggregates and storage time (Figs 1 to 3). SFP, volume and number of aggregates seemed to reach their highest value between days 5 and 15. A weak tendency towards a linear correlation was seen between debris weight and storage time (Fig. 4). There were, however, considerable variations in the data.

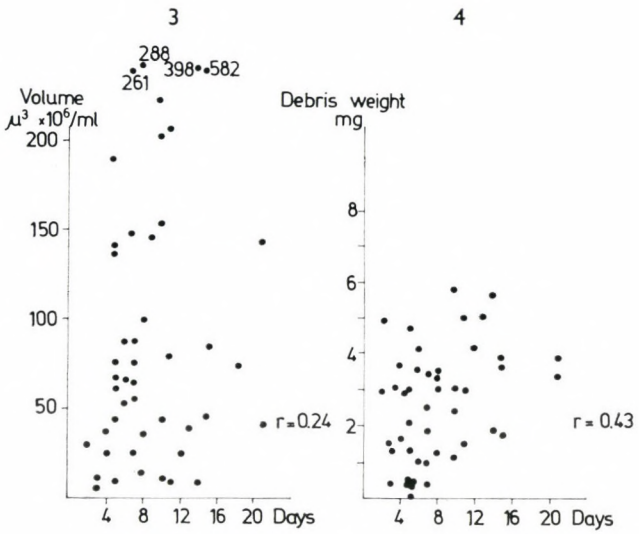

Fig. 3. Relation between volume of microaggregates/ml and storage time. Peak values within days 5–15

Fig. 4. Relation between weight of debris on the filter screen and storage time. A tendency to progressive increase with time (r = 0.43)

Discussion

The present investigation demonstrated a tendency to a progressive increase in debris weight with increasing storage time. The screen filtration pressure (SFP) and the volume or number of microaggregates did not increase progressively but attained a maximum between 5 and 15 days of storage.

The three methods for estimation of debris in blood used in this study measure different aspects of debris formation [8, 12, 13] and the data from the present study seemed to confirm these reports.

Screen filtration pressure is preferentially a measure of the adhesiveness of cells and aggregates [17]. Maximal SFP values were seen between days 5 and 15, when the number of aggregates had reached a peak value. In previous papers no firm correlations were found between these two parameters [4, 5] and the relation

in time of the peak values could just be coincidental [8]. Measurement of screen filtration pressure is probably the method that has been used most extensively to follow microaggregate formation in blood. A simultaneous increase in SFP and debris volume has been described [15]. In the present study a similar relationship was found in that both parameters reached their peaks between 5 and 15 days.

The tendency to a correlation between debris weight and storage time was remarkable. It must, however, be stressed that the correlation was not absolute, due to variations in the data. This variation is part of the methodology used. In a previous study debris weight was correlated to the number of aggregates [13]. The fact that in the present study the quantity of aggregates did not increase with storage time indicated that microaggregates are only part of the debris, other factors being disintegrating cells, precipitated fibrin and clots.

It has earlier been stressed [12, 13] that elevated SFP in the transfused blood is correlated with organ (e.g. pulmonary) dysfunction both in experimental animals [2, 5, 6, 10] and in humans [4, 11, 17]. No such correlation has been observed between the quantity of transfused aggregates and organ failure. For this reason it seems to be of clinical relevance to measure SFP of the transfused blood before transfusion.

The present results demonstrated no linear increase in SFP with progressive storage time. Sometimes such a correlation has been assumed to exist in that all stored blood has been presumed to have high SFP. It is obvious from this study that the assumption is misleading.

From the present investigation it is concluded that formation of debris in stored human blood seemed to occur progressively with storage time as reflected by debris weight. The quantity of aggregates and screen filtration pressure did not, however, increase with time but reached peak values within 5 to 15 days of storage, but the variations in the data were considerable.

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Clinical Relevance of Cytotoxic Antibodies Occurring in Pregnancy

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Cytotoxic antibodies were studied in 809 pregnant women's sera. Two kinds of antibody population: HLA-A, B, C and Ia-like, were detected and a correlation was established between their presence and some clinical conditions, such as preterm delivery, spontaneous abortion, pre-eclampsia, neonatal hyperbilirubinaemia and congenital anomalies. An opposite effect of HLA-A, B, C and Ia-like antibodies was observed as far as the first three parameters are concerned. On the basis of these investigations a protective role of Ia-like antibodies for the newborn can be implied.

*

From the immunological point of view, a similarity exists between organ transplantation and pregnancy, as the fetus can be regarded as a haploidentical allograft tolerated for a limited time by the mother. This is a simplification that would certainly be questionable by gynaecologists. Hormonal immunosuppression, a number of known and even more unknown non-specific humoral factors [9, 11, 30] and the placenta acting as an immunological barrier [9] complicate the issue.

In spite of all the differences, it is not seriously doubted that immunological processes may have some influence on the outcome of gravidity [21, 22, 27]. As for the components of the specific immune response (antibody production, cellular sensitization), their effects are easily detectable both in transplantation and in the feto-maternal relationship [2, 3, 5, 15, 16, 33].

In the present study a brief account is given of the correlation between the presence of cytotoxic antibodies in pregnancy and the incidence of some clinical conditions associated with maternal immunoreactivity.

In organ transplantation, the deteriorating effect of HLA antibodies directed against HLA-A, B and C antigens has been discussed extensively [17, 28]. Yet the poor outcome associated with hyperacute rejection and short graft survival may be due not so much to the antibodies themselves than to the simultaneously occurring cellular immune response [31]. A different cytotoxic antibody population is the B-cell-specific (human Ia-like) antibody, to which no definitive role in transplantation could be attributed [8, 14, 24, 25, 35]. One of our major concerns was to provide evidence for or against the protective role of Ia-like antibodies in pregnancy.

Material and Method

Detection of cytotoxic antibodies in pregnant women's serum was performed by the NIH lymphocytotoxicity microtest with prolonged incubation time at room temperature. 809 sera taken within 24 h after delivery were investigated with target cells separated from patients suffering from chronic lymphocytic leukaemia (B cell type) and from normal donors.

T and B lymphocytes were isolated from heparinized peripheral blood by the E rosette technique followed by Ficoll-Uromiro gradient centrifugation.

Using leukaemic B cells from 20 patients, we selected the sera reacting positively with at least two of the twenty. Positivity was detected by trypan blue exclusion.

In further parallel tests with T and B cells from normal donors the sera reacting with T and B cells alike were separated from those reacting with B cells alone [19]. Thus the sera belonging to the first group contained anti-HLA-A, B, C and probably also Ia-like antibodies [14].

The sera of the latter group were depleted with platelets (using a pool of 110 donors' thrombocyte suspension at +4 and +20 °C for each stage, respectively) to remove residual HLA-A, B, C and different "non-Ia-like" reactivity [29]. In this way three groups were set up, consisting of sera

a) without cytotoxic antibodies,
b) with HLA-A, B, C and probably also Ia-like antibodies,
c) with Ia-like antibodies,

in which several feto-maternal clinical conditions were evaluated statistically. The average incidence of each parameter in the total material served as reference in the χ^2 test.

The following criteria were used:

preterm delivery	– less than 2500 g body-weight
spontaneous abortion	– before the 28th week of gestation
pre-eclampsia	– proteinuria, hypertension and oedema
congenital anomalies	– no restriction
neonatal hyperbilirubinaemia	– when treatment was needed (UV light, penicillamine, exchange transfusion)

Results

The average number of pregnancies was different in each group; the incidence of preterm deliveries and spontaneous abortions per pregnancy is shown in Table 1.

In the presence of Ia-like antibodies a significant ($p < 0.05$) decrease in the frequency of preterm deliveries was observed. HLA-A, B, C antibodies seemed to be responsible for the slight but not significant increase. The incidence of spontaneous abortions was similar though this deviation was not significant in the presence of Ia-like antibodies.

Table 1

Incidence of preterm delivery and spontaneous abortion in the history

Antibody	No. of subjects	Average no. of pregnancies	Preterm delivery			Spontaneous abortion		
			no.	%	per grav.	no.	%	per grav.
Ia-like	92	2.36	9	9.18	3.89*	8	8.16	3.46
HLA and Ia-like	208	2.85	60	28.85	10.12	46	22.11	7.75
Without antibody	509	2.28	94	18.47	8.10	68	13.36	5.86
Total	809	2.49	163	20.15	8.29	122	15.08	6.21

* $p < 0.05$ significant

Table 2

Frequency of preterm delivery and pre-eclampsia in the presence of cytotoxic antibodies

Antibody	No. of subjects	Preterm delivery		Pre-eclampsia	
		no.	%	no.	%
Ia-like	92	4	4.08*	12	12.24
HLA and Ia-like	208	23	11.06	35	16.83
Without antibody	509	50	9.82	81	15.91
Total	809	77	9.52	128	18.80

* $p < 0.1$; not significant

Table 3

Spontaneous abortions occurring in 340 pregnant women

Antibody	No. of members in the groups	Spontaneous abortion	
		no.	%
Ia-like	23	0	0
HLA and Ia-like	73	3	4.1
Without antibody	244	5	2.0
Total	340	8	2.4

The frequency of preterm deliveries, spontaneous abortions and gestoses in the last pregnancy of the members of each group is shown in Tables 2 and 3.

Although significant deviations could not be observed, there was a remarkable decrease in the incidence of preterm deliveries and spontaneous abortions in

the presence of Ia-like antibodies, whereas a non-significant increase was found whenever HLA-A, B, C antibodies were also present in the sera. In the distribution of neonatal hyperbilirubinaemia and congenital anomalies, the opposite effect of HLA-A, B, C and Ia-like antibodies could not be confirmed. Neonatal hyperbilirubinaemia caused by the high-titre anti-A, anti-B or anti-D antibodies was left out of consideration.

Table 4 shows the difference in incidence of these clinical conditions.

Table 4

Distribution of congenital anomalies and neonatal hyperbilirubinaemia in the presence of cytotoxic antibodies

Antibody	No. of members	Congenital anomalies		Neonatal jaundices	
		no.	%	no.	%
Ia-like	92	1	1.08	4	4.35
HLA and Ia-like	208	3	1.44	12	6.77
Without antibody	509	12	2.36	17	3.34
Total	809	16	1.98	33	4.08

As seen from Table 4, if antibody was present, relatively more cases of neonatal jaundice were found. On the other hand, the number of congenital anomalies in the groups investigated did not seem to vary in dependence on the presence of antibodies.

Discussion

The influence of immunological events on fetal development has been confirmed by several authors [5, 20, 32, 34]. The aim of our study was to establish a correlation between the presence of the two sorts of maternal cytotoxic antibody as a result of alloimmunization, and the incidence of some clinical conditions.

Apart from congenital anomalies and hyperbilirubinaemia, a very characteristic distribution of the investigated parameters was observed in the presence of cytotoxic antibodies. The number of congenital anomalies was small. The higher frequency of congenital anomalies in the absence of antibody might be due either to the role of a human HLA-linked T-like complex where homozygosity and consequently the lack of antibody gives rise to the risk [7, 18] or to the fact that some rather severe cases developed in the presence of HLA antibodies might result in spontaneous abortions.

HLA antigens being present on the surface of erythroblasts, HLA antibodies may damage these immature red cells; this is the most probable explanation for the association of HLA antibody and neonatal hyperbilirubinaemia [10].

Ia-like antibodies are not likely to interfere in the same way; their effect might have even been due to HLA antibody contamination. As to the incidence of preterm deliveries, spontaneous abortions and gestoses in each group, HLA and Ia-like antibodies seemed to have a contrasting effect. This observation was not unexpected though the favourable effect of Ia-like antibodies is steadily questioned in transplantation.

Several authors succeeded in obtaining human Ia-like antibodies from placenta eluates [6, 12]. The antibodies were shown to have an inhibitory effect in the MLC and CML tests [12, 13]. The passage of maternal lymphocytes through the placenta being well known [1], the specific inhibition of the maternal cell-mediated immunity may be of considerable importance in the immunological protection of the conceptus. The slightly higher frequency of the examined parameters found in the presence of HLA antibodies can probably be considered to be merely the consequence of maternal sensitization. In the ABO system where the antigens are tissue antigens too, Lauritsen [22] ascribed to an incompatibility the significantly higher frequency of spontaneous abortions with normal karyotypes. The question is open why human Ia-like antibodies unlike HLA antibodies should have such a distinct and peculiar function. After Michie and Andersson [26] had failed to obtain homozygotic Wistar rats after inbreeding for more than 70 generations, it became obvious that strong forces are acting to preserve heterozygosity at certain MHC loci. There is evidence to indicate that, in human relations, the MLC locus is at least one of the loci where heterozygosity is of similar importance. Lauritsen [23] reported that lymphocytes from women were hard to stimulate with those from their male partner in one-way MLC tests in the case of infertile couples. MLC homozygosity may also increase the placentary passage of sensitized maternal lymphocytes through the genetic control of this process [20]. Both observations reveal a definite advantage for heterozygotic individuals. Because of the well-known and close relationship that exists between MLC antigens and human Ia antigens, it is clear that if human Ia-like antibodies should be harmful, a force to the favour of homozygotics would come into existence. This would be a contradiction not easy to explain. It would be interesting to know the biological role bound to these loci that might explain the necessity of heterozygosity preservation. For the time being, however, it is no more than a hypothesis that the immune response genes are localized in this region and because of their dominant mode of inheritance heterozygotic individuals can respond to a larger antigen scale than can homozygotics.

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WORKSHOP

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Introduction

In the framework of the joint protein research program of the Academies of Sciences of Czechoslovakia, the German Democratic Republic, Hungary, Poland and the Soviet Union a constant collaboration has been established between scientists searching for the structure and function of antibodies. The collaboration extends to the study of the steric structure of immunoglobulins and the localization of functionally active groups, as well as to the comparison of the results obtained by different physical methods.

Participants of the above collaboration, following the previous meetings in Czechoslovakia, the German Democratic Republic and the Soviet Union, have met in Visegrád, Hungary in 1980. The following selected lectures and abstracts of papers presented at this meeting give evidence of the fruitful collaboration of this research program.

Reviews presented at the meeting but not submitted in manuscripts were the following:

Lisowski, J.: Cytophilic antibodies.

Füst, G.: Interaction between immune complexes and the complement system.

Fiebig, H., Ambrosius, H.: Use of hybridoma produced monoclonal antibodies for studying Ig structure and function.

Franěk, F.: Primary structure of immunoglobulin polypeptide chains and the relation to gene structure.

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Physico-Chemical Investigations of Immunoglobulin Structure in Solution

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The mechanism of how the antibody molecule mediates the effector function is of great importance to immunology and medicine. Metzger [1] considered three possible models to account for the exposure of functional sites as a consequence of antigen-binding, *viz.*

(a) the allosteric model proposes a conformational change in the Fc subunit as a result of secondary force perturbations following antigen-binding by the Fab subunit;

(b) the distortive model suggests that because of the binding of antigen, the molecule is "stretched" so as to reveal or cover up critical sites;

(c) the associative model considers the reactions of antibody with antigen as a means of bringing together or aggregating antibodies. This polymerization of antibodies can then result in the formation of a new site or the creation of a polyvalent site from several monovalent ones.

The most complicated for investigation and understanding is the allosteric model. As most effector sites are situated in the Fc subunit, the allosteric model must include three moments: *(i)* the change of the variable part conformation; *(ii)* the change of interactions between the variable and constant parts of the Fab subunit; *(iii)* the change of interactions between the Fab and Fc subunits.

Essential non-covalent interactions must exist between the different parts of the immunoglobulin molecule for realization of the allosteric mechanism. This, however, needs a labile structure. The lability is not only a result of antigen-binding but may be induced by changes of the different physico-chemical properties of the environment. In this review we discuss the data revealing some kinds of lability of the immunoglobulin structure and its possible role in the realization of the allosteric mechanism of activation of antibody effector functions.

As early as 1969 [2] we reported the existence of a reversible conformational transition of immunoglobulins induced by the temperature change near the physiological interval of the temperatures of the internal environment of homothermic animals. The different proteolytic fragments of immunoglobulins indicate the transition for Fab subunits and for the variable part of the Bence-Jones protein [3]. Similar data were obtained by other investigators [4, 5, 6]. The changes occurring at a temperature change are accompanied by a decrease of exposure to solvent

perturbation and an increase of rigidity of the microenvironment to the thermal perturbation of a significant part of tyrosine residues [7]. Kitagawa et al. [8] who revealed a change of the Raman spectrum of Bence-Jones protein suggested the formation of stronger hydrogen bonds by tyrosine residues at increased temperature in the transition interval. They showed [8] that it is highly probable that the reversible structure change reported by us [3] is also associated with the conformational change of the cysteine bridge. This change is different from that caused by thermal denaturation, because the negative CD band at 218 nm which reflects the β-structure of the polypeptide backbone is not changed or increased on raising the temperature [3, 6]. In spite of the cooperativity, the transition is not accompanied by an appreciable enthalpy effect [3, 7]. The investigations by the spin-echo method of NMR [9] show that the "thermophysiological" transition is stipulated by the change of interdomain interactions through the intermediate highly mobile state. This transition may explain the anomalous effect of temperature variations on the binding of ε-dinitrophenyl-L-lysine to anti-dinitrophenyl antibodies which yielded a non-linear Van't Hoff plot [10]. Studies of rabbit anti-dinitrophenyl antibodies by differential thermal perturbation spectroscopy [11] show that the binding of the ε-dinitrophenyl-L-lysine shifted the equilibrium to the low temperature state. The chemical relaxation studies of hapten-antibody interactions revealed two conformational states of antibodies and its Fab subunits and the shift of the equilibrium between them [12].

Thus, the variable part of immunoglobulins forming the antigen-binding site exists in some conformational states with different interactions between domains. The equilibrium between the states is shifted without appreciable enthalpy effect.

Let us see what is known about interactions between variable and constant parts of the Fab subunit. The X-ray structural data show that these parts are joined through the stretched segments of the polypeptide chain, the so-called "switch region" [13, 14]. The non-covalent interactions between the constant and the variable domains of the same chain are significantly weaker than those between the homological domains of different chains. The angle between the variable and constant parts of the same chain is very different for different immunoglobulins [13, 14]. These data allow to suggest another kind of lability of immunoglobulins: the transition of the variable and constant parts of the Fab subunit relative to each other. The question arises whether the non-covalent interactions between the variable and constant parts are strong enough to allow the cooperativity of the Fab structure. Microcalorimetric studies [15] suggested that the Bence-Jones protein has very weak non-covalent interactions between the variable and constant parts. But in spite of that, reduction of the interchain disulfide bond in the constant region induces the temperature dependent conformational mobility of the variable part of the Bence-Jones protein [5]. Recent microcalorimetric investigations of the Fab fragments [9] indicate the existence of a higher cooperativity of the Fab structure and essential non-covalent interactions between the variable and constant parts. This fact correlates with the higher stability of the Fab subunit to proteolytic digestion in comparison with Bence-Jones proteins. According to the microcalori-

metric data, the hapten-binding induces an increase of the cooperativity of the Fab subunit [9]. The spin-label method of EPR [16] indicates a shift of equilibrium between two conformational states of the Fab subunit to a more compact conformer. Thus, one can distinguish two kinds of conformational lability of the Fab subunit: The conformational lability of the variable part of antibodies stipulated by the ability of this part to exist in some conformational states with different interactions between domains; and the conformational lability of the Fab subunit stipulated by its ability to exist in some conformational states with different interactions between variable and constant parts. The hapten-binding induces not only the shift of the equilibrium between the variable part of conformers, but also changes the interactions between variable and constant parts, resulting in the formation of a more cooperative and compact conformer.

As to the interactions between Fab and Fc subunits of immunoglobulins, in 1965 Noelken et al. [17] suggested a flexible model of IgG structure. According to this model, the Fab and Fc subunits are joined by flexible hinges. At present the structure of the so-called "hinge region" is known for some IgG molecules [18, 19]. The "hinge region" in human, IgG1, IgG2 and IgG4 consists of 15 to 17 amino acid residues. In human IgG3 this region is about four times larger. The region of IgG3 has a strikingly well-ordered secondary and tertiary structure revealed by very simple circular dichroism spectra of an isolated hinge fragment [20]. Thus the main part of the "hinge region" is the rigid rod. The ends of the rod are connected through the relatively flexible segments of the polypeptide chain to the Fab and Fc subunits. One can predict the most rigid structure for the human IgG4. The "hinge region" of this subclass has the amino acid sequence of seven amino acid residues including five prolines and two cysteines which fix this segment to another heavy chain. A slightly more flexible "hinge region" can be expected for the human IgG2 in which two prolines in the N-terminus from the interchain disulfide bonds are substituted by valine and lysine. The next in the level of flexibility can be IgG1 in which five amino acid residues are inserted to the N-terminus from the interchain disulfide bonds. This sequence has no prolines and includes two threonines. A greater flexibility can be predicted for IgG3 because the long "hinge region" rod is joined to the globular subunits through relatively flexible segments of polypeptide chains. The polarization of fluorescence and the spin-method of EPR [22, 23, 24] offer experimental evidence allowing to suggest a significant degree of flexibility between the Fab arms. But some experiments show [25, 26] that the flexibility is limited, and recent studies indicate that there are subtle differences in the internal flexibility of immunoglobulins of different classes and subclasses and of different animals [9, 23]. According to the polarization of fluorescence, the non-precipitating pig antibodies have the most rigid structure, and the rabbit antibodies, the most flexible structure [23]. According to data of two methods (the study-state polarization of fluorescence and the spin-label method of EPR), IgG3 has the most flexible structure among human IgG subclasses. The correlation time for two other subclassess – IgG1 and IgG2 – is significantly higher [9]. The microcalorimetric investigations of normal rabbit IgG and of homogeneous

rabbit anti-polysaccharide antibodies to the pneumococcal polysaccharide fraction (SIII) indicate a significantly different energy of non-covalent interactions between the Fab and Fc subunits [9]. In the antibody these interactions were essential, but in normal IgG they were practically absent. Significant non-covalent interactions were observed between the Fab and Fc subunits of pig anti-dinitrophenyl antibodies [9]. Thus, in any case for the part of immunoglobulin subclasses and for immunoglobulins of some animals, significant non-covalent interactions exist between the Fab and Fc subunits.

The question arises whether the hapten-binding induces a change of interactions between the Fab and Fc subunits. X-ray small-angle scattering data of Pilz et al. [27] indicate a decrease in the radii of gyration of rabbit antipoly-(D-analyl) and anti-azophenyl-lactoside antibodies after their complexing with tetra-D-alanyl-amide and p-azophenyl-β-lactoside haptens, respectively. Neutron small-angle scattering experiments [28] gave similar results for pig anti-dinitrophenyl antibodies. To account for these changes two alternative hypotheses can be proposed. First, it can be contended that the liganded molecule exists in a more compact conformation than the unliganded one. Alternatively, segmental flexibility of the liganded antibody is presumed to be higher than that of the unliganded antibody, thus resulting in a lower average value of the radii of gyration. The polarization fluorescence studies indicated a decrease of flexibility of liganded antibodies, but recent investigations of Käiväiräinen et al. [30] revealed an opposite effect.

The next question is that of the conformational change in the Fc subunit as a result of antigen-binding. Schlessinger et al. [31] investigated the interactions of antibodies with ribonuclease, poly-D-alanine and the "loop" of lysozyme by circular polarization of luminescence. The emission spectrum shows changes accompanying the antigen-binding with the antibody in antigen excess. When the Fab fragments alone were studied, the results observed were not the same as for the total molecule, indicating that some of the changes took place in the Fc region. Microcalorimetric investigations of the binding of monovalent oligosaccharide by homogenous rabbit anti-polysaccharide antibodies allowed to reveal the changes that were absent in the Fab subunits [32].

Recent investigations show that the effect studied may be explained by the induction of conformational changes in the Fc subunit and, most probably, by the increasing interactions between C_H2 and C_H3 domains [9], as this sample of antibodies indicates essential non-covalent interactions between the Fab and Fc subunits. In the precipitating pig anti-dinitrophenyl antibodies the opposite effect was studied. Most probably the changes can be explained by the weakness of the interactions between C_H2 and C_H3 domains [9]. In the same antibodies, differential thermal and solvent perturbation spectroscopy allows to reveal an increase of rigidity of some parts of the Fc subunit, most likely of the C_H2 domains [9]. There are several experiments that do not indicate any conformational change in the Fc subunit as a result of antigen-binding [33, 34, 35].

Practically all physico-chemical investigations of conformational changes of

antibodies induced by hapten- or antigen-binding were performed for IgG. The association between IgG molecules plays, however, an important role in effector functions (two or more molecules of IgG are needed for complement fixation) and at present it is impossible for IgG antibodies to distinguish between associative and allosteric models. More definite results may be obtained for IgM which is already "associated" and fixes the complement more efficiently after binding the univalent antigen [36, 37].

The results discussed above allow to conclude that though it is generally agreed that immunoglobulins are flexible at least at a gross level, recent studies reveal essential differences in internal flexibility (within and between domains) and in energy of non-covalent interactions between domains and subunits of immunoglobulins of different subclasses and of different animals. It cannot be excluded that different mechanisms of activation of the effector function may be significant for various antigens, effector functions and for immunoglobulins of different classes, subclasses and different animals.

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Conformational Flexibility of Antibody Molecules

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The recognition of antigen by antibodies triggers the secondary effector functions leading to the elimination of the antigen bearing material. In the organism antibodies recognizing a practically unlimited range of antigens can be generated, and the recognition is based on highly specific molecular interactions. The secondary effector functions are less diverse and they are limited to a small number of mechanisms initiated by the formation of antigen-antibody complex. These multiple functions are associated with discrete globular domains within the antibody molecule [1]. The molecular mechanism by which the initial antigen recognition on the Fab part of the molecule is transferred to the Fc part is still a matter of controversy [6]. In this debate domain-domain distortions, antigen induced aggregations and allosteric changes are discussed as possible mechanisms. Not too much attention was devoted so far to the intramolecular (intradomain) flexibility and motility of the immunoglobulin molecules in the interpretation of the transition of information between domains.

The three-dimensional structure of the polypeptide chain in a protein is stabilized by weak secondary interactions (hydrogens bonds, hydrophobic interactions) the energy of which is comparable with the kT thermal energy, at room temperature. The idea of the flexible protein structure was introduced by Linderstrøm-Lang and Schellman [5]. A variety of techniques have been employed during the last decade to demonstrate intramolecular motion. Thermal agitation leads to a continuous breakage and reformation of secondary bonds, allowing segmental intramolecular motion, breathing of the protein molecules. To elucidate the mechanism of information transfer between the distant functional domains of immunoglobulins, we studied the effect of site-filling monovalent and bivalent antigens on the conformation, conformational stability and intramolecular mobility of homogeneous rabbit antibodies. Since our purpose was to separate the intramolecular effect induced in one single antibody molecule from the effect due to intermolecular aggregations, experiments were carried out with solutions of monomeric antibody saturated with monovalent and bivalent antigens [8]. Differential sedimentation and small angle X-ray scattering experiments did not reveal significant changes in the average atomic positions (shape and volume) of the immunoglobulin molecule upon antigen binding, while significant changes were

observed in the relaxation spectra of the hydrogen-deuterium exchange (Fig. 1). The hydrogen-deuterium exchange in globular proteins occurs by the conformational fluctuations exposing the buried peptide groups to the solvent. The probability distribution of solvent exposure is summarized in the relaxation spectra [4]. According to our hydrogen-deuterium exchange studies [7] the immunoglobulin molecules are among the most mobile protein molecules of this size. It can be seen from Fig. 1 that the probability of solvent exposure of more than half of the peptide hydrogens was increased ten-fold, reflecting a 4–6 kJ/mol increase in the standard free energy of the reversible conformational fluctuations upon antigen binding. This change was also reflected in the shift of transition temperatures measured by adiabatic scanning microcalorimetry. The changes in conformational mobility and stability extended to all domains of the IgG molecule.

Comparative studies with Fab and Fc fragments of rabbit IgG show that the Fab fragment, which contains the preformed antigen binding site is more rigid compared with the Fc fragment, which contains the secondary binding site for the C1q complement molecules. The difference in conformational mobility is well seen in the relaxation spectra in Fig. 2. The experimentally observed relaxation spectrum of intact IgG cannot be derived by algebraic summation of those of the Fab and Fc

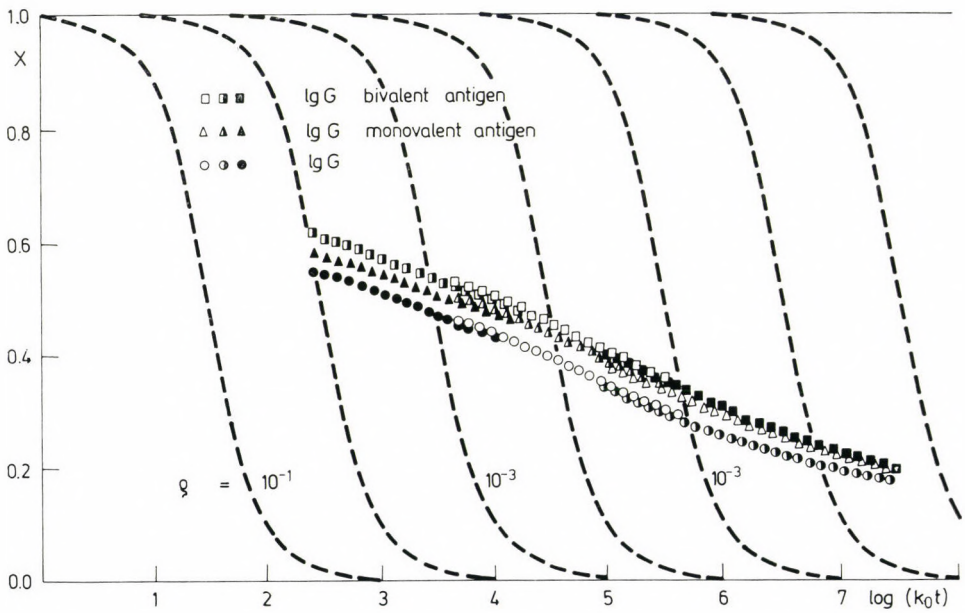

Fig. 1. Hydrogen-deuterium exchange data summarized in the form of relaxation spectra. The measurements were carried out at three pH values: 5.42, 7.25, 8.52. The temperature of the measurements was 298 °K. X is the fraction of unexchanged peptide hydrogens, k_0 is the chemical exchange rate constant of the solvent exposed peptide hydrogens, t is the time elapsed from the start of the exchange. The dashed curves represent hypothetical polypeptides which expose their peptide hydrogens to the solvent with equal, ρ probability (Závodszky et al., 1980)

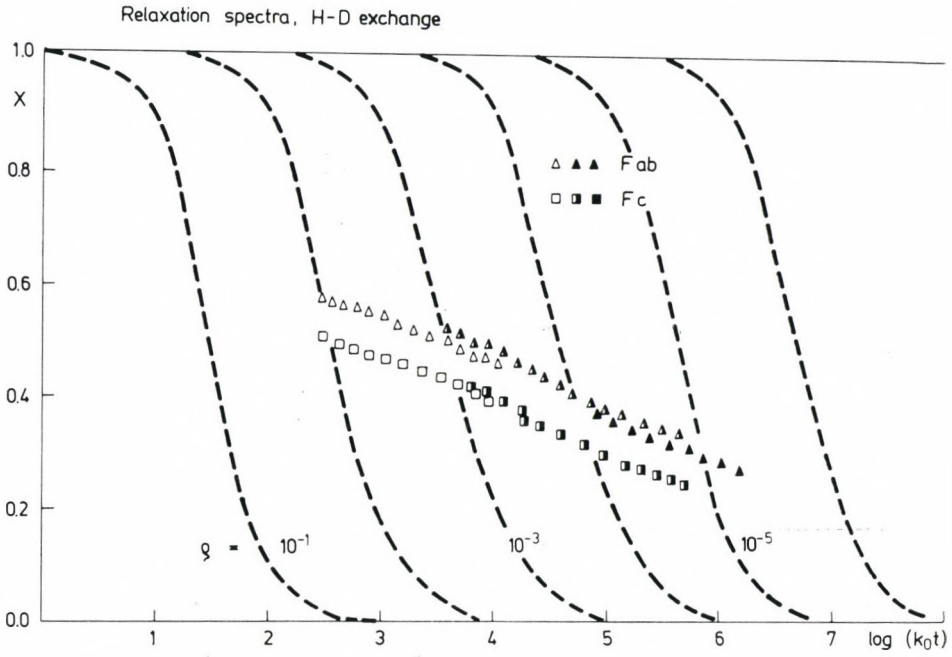

Fig. 2. Relaxation spectra of the Fab and Fc fragments of rabbit immunoglobulin G. (Symbols and experimental conditions as in Fig. 1.)

fragments. This dicrepancy can be explained, if we suppose that the fluctuation patterns of the Fab and Fc fragments in the IgG molecule are coupled. The comparison of the high resolution proton NMR spectra of the Fab and Fc fragments also suggests that intramolecular mobility and flexibility in the Fc fragment is greater than in the Fab. The well-resolved proton NMR spectra of the Fc fragment is interpreted as evidence for substantial segmental flexibility [2]. This mobility was monitored using special NMR pulse sequences which exploit either the multiplet structure (spin coupling) or the differential linewidths of resonances. Several aromatic residues with mobility independent of the rest of the protein could be detected by these techniques. Figure 3 shows the subspectrum at 270 MHz of the Fc fragment obtained from a Carr-Purcell A pulse sequence.

In the system investigated, the fluctuation spectrum of the antibody molecule has been changed upon antigen binding. Since protein conformations are specific structures, stabilized by highly cooperative weak interactions, their fluctuations should be concerted. Concerted conformational fluctuations may serve as a possible mechanism of information transfer over large distances within the macromolecule. "Retuning" of some components of the fluctuation spectrum will be reflected in the entire system. The observed changes in the relaxation spectrum of IgG could explain, in part, the role of the antigen binding as a trigger for com-

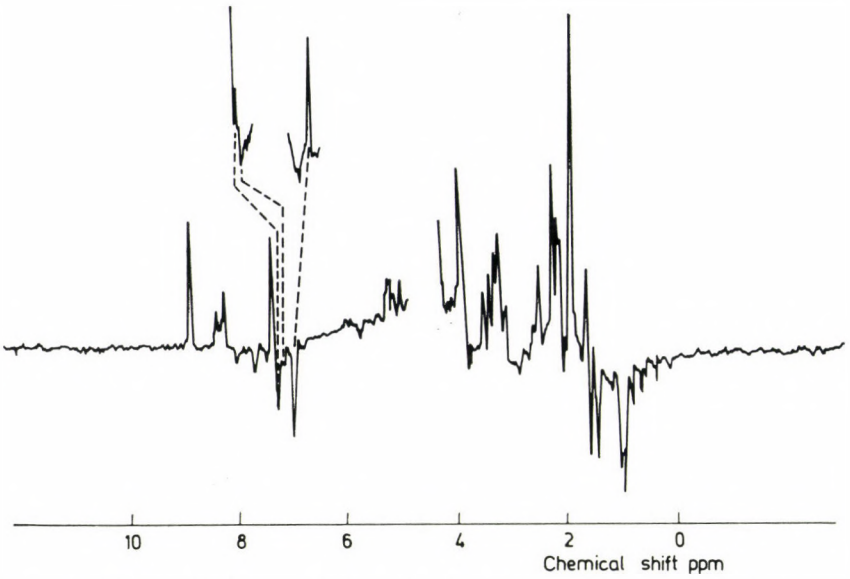

Fig. 3. 270 MHz proton NMR subspectrum, obtained from Carr-Purcell A pulse sequence at 313° K. Irradiation at 6.7 ppm causes decoupling of the doublet at 7.0 ppm as seen by phase inversion, while irradiation at 7.0 ppm has the same effect on the doublet at 6.7 ppm (Boyd et al., 1979)

plement fixation. The observed changes in conformational fluctuations are possibly parts of the process leading to the formation of the complement binding site. The great conformational mobility of the Fc fragment is in good agreement with the view that the complement binding site takes its high-affinity form only upon the effect of antigen binding. Further essential changes are than brought about by the formation of suitable density of complement binding sites in antibody aggregates.

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Correspondence between Structure and Function of Immunoglobulin G Subclasses

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In the well-known model of a highly flexible IgG molecule the Fab and Fc subunits are joined through the flexible hinge region and very weak non-covalent interactions exist between them. The IgG of different subclasses and of different animals has a very different primary structure of the hinge region. As a consequence of such a local change of the primary structure, the IgG subclasses obtain different biological properties (fixation of the complement, binding to one of the several types of cells, agglutination and precipitation of antigen). The most probable explanation of these differences is a different extent of freedom of rotation and a different extent of non-covalent interactions between the Fab and Fc subunits of IgG molecules of different subclasses and of different animals.

We aim at studying the dynamical conformational properties of human and animal IgG subclasses and the conformational changes of IgG antibodies induced by hapten-binding, by means of complex physical methods (differential temperature and solvent perturbation spectrophotometry, differential adiabatic scanning microcalorimetry, polarization of fluorescence, spin-label method of ESR, spin-echo method of NMR) in combination with proteolytic digestion of the IgG molecule.

Conformational Properties of Normal Rabbit IgG and of Homogeneous Anti-Polysaccharide Rabbit Antibodies

According to data of polarization of fluorescence [1] the rabbit IgG displays the highest flexibility between the Fab arms. One can predict very weak non-covalent interactions between the Fab and Fc subunits of rabbit antibodies. To

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examine this prediction, we investigated normal rabbit IgG and its proteolytic fragments using differential adiabatic scanning microcalorimetry. The results obtained at pH 3.51 are presented in Fig. 1, which shows a good agreement between the experimental heat of thermal denaturation of the intact IgG molecule and the heat calculated from the individual Fab and Fc fragments. The results suggest that the Fab and Fc subunits have independent secondary and tertiary structures and in the sample investigated the Fab and Fc subunits have very weak non-covalent interactions. The heat of thermal denaturation calculated from the Facb and pFc' fragments differs, however, from the experimental heat for the intact IgG molecule (Fig. 2). It can be seen from the figures that the low temperature peak of heat absorption belongs to the Fc subunit. But this peak is not observed with the Facb and pFc' fragments. One can therefore propose that the peak corresponds to the contact region between the C_H2 and C_H3 domains. In fact, according to X-ray structural data [2], significant non-covalent interactions exist between the C_H2 and

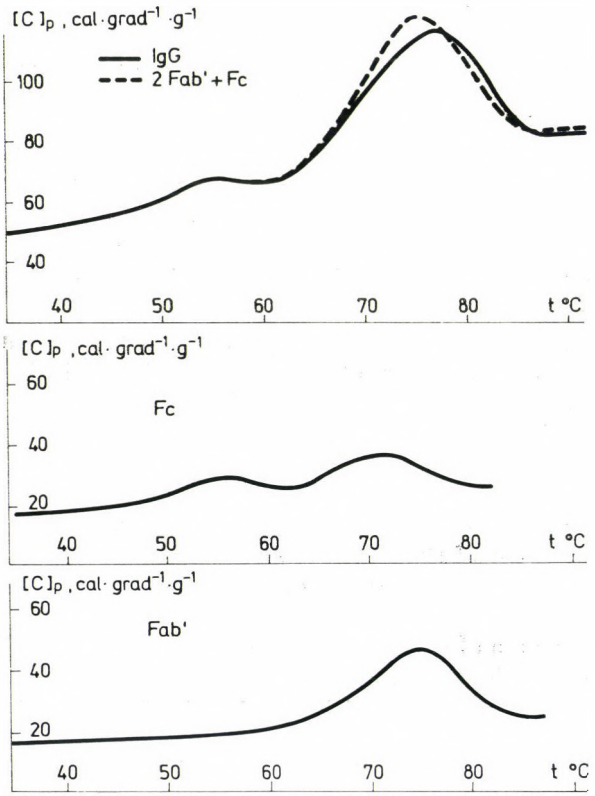

Fig. 1. Temperature dependence of the partial heat capacity of the normal rabbit IgG and its proteolytic fragments (Fc and Fab). The dashed line shows the temperature dependence of the heat capacity values calculated from the individual Fab and Fc fragments. The solvent was 0.05 mole/l glycine-HCl buffer pH 3.51

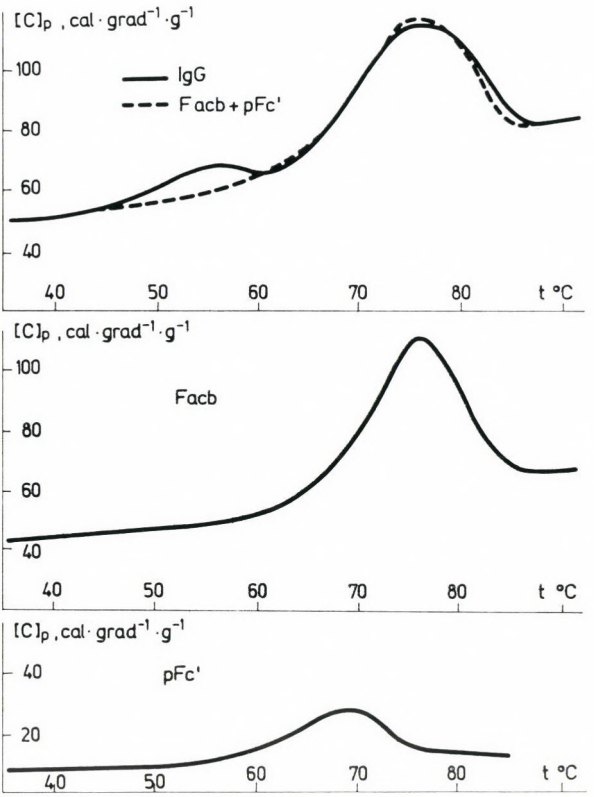

Fig. 2. Temperature dependence of the partial heat capacity of normal rabbit IgG and its proteolytic fragments (Facb and pFc'). The dashed line shows the temperature dependence of the heat capacity values calculated for the individual Facb and pFc' fragments. The solvent was 0.05 mole/l glycine-HCl buffer pH 3.51

C_H3 domains but these interactions seem to be weaker than those between the V_L and V_H, C_H1 and C_L or C_H3 and C_H3 domains. For the sample of homogeneous anti-polysaccharide antibodies the agreement between the experimental heat of thermal denaturation for the intact antibody and the heat calculated from the isolated Fab and Fc fragments must be worse, as a result of the destabilization of the $F(ab')_2$ and Fab' fragments (Fig. 3). One may suggest the existence of significant non-covalent interactions between the Fab and Fc subunits in this sample of antibodies. For this sample the melting temperature of the low temperature peak of heat absorption which probably correspond to the C_H2-C_H3 contact region, is lower than for the normal IgG at the same pH. The data presented in Fig. 2 offer evidence that the hapten-binding has induced the conformational changes in the Fc part of the antibodies with a high extent of non-covalent interactions between the subunits.

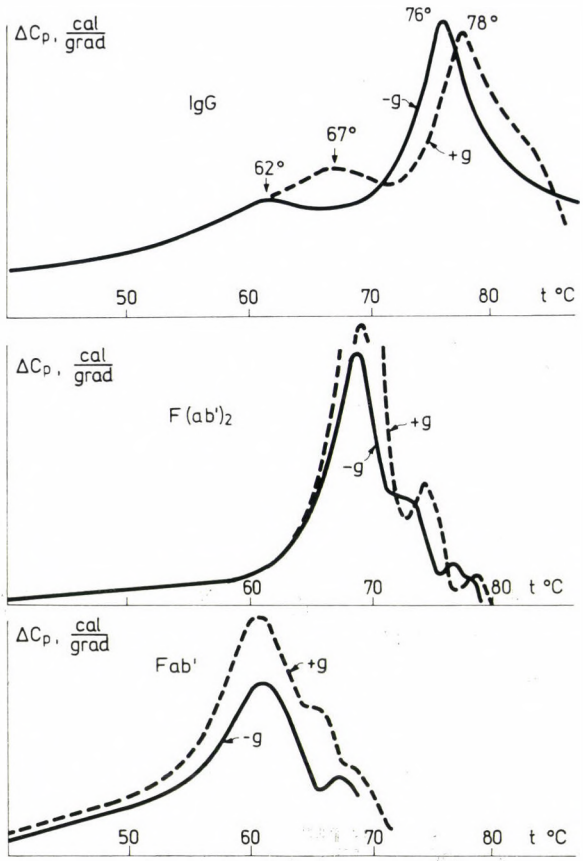

Fig. 3. Temperature dependence of the heat capacity of homogeneous anti-polysaccharide rabbit antibody (IgG) and its proteolytic fragments ($F(ab')_2$ and Fab'. The dashed line shows the measurements of liganded antibody and its proteolytic fragments. The solvent 0.15 MES buffer pH 5.4

Conformational Properties of the Precipitating and Non-Precipitating Pig Anti-Dinitrophenyl Antibodies

Franěk et al. [3] described two types of pig anti-dinitrophenyl antibody replaced by each other at different phases of the immuno-response, with a different capacity to form specific precipitates with an antigen. The early type includes precipitating antibodies, and the late one, non-precipitating antibodies. The rotation relaxation times for the two types of pig antibody were significantly higher than those for rabbit or human IgG [1]. For the precipitating pig antibodies the value was lower than the value for the non-precipitating antibody. Dudich et al. [1] concluded from these data that non-precipitating anti-DNP antibody had a less

flexible structure which could affect the ability to cross-link the molecules of DNP antigens. The difference of the radii of gyration of the two types of antibody [4] is compatible with this conclusion. According to these data, one may suggest the existence of significant non-covalent interactions between the Fab and Fc subunits of the pig antibodies. We have examined this suggestion by differential adiabatic scanning microcalorimetry. The results obtained at pH 4.1 (0.05 M acetate buffer) are presented in Fig. 4. It can be seen that the precipitating (Eab) and non-precipitating (Lab) antibodies have different properties. One of the subunits of the precipitating antibodies has a higher melting temperature than the same subunit in the non-precipitating antibodies. Investigation of the isolated Fab and Fc fragments revealed that the difference is attributed to the Fc subunit. Judging by the data obtained for the rabbit antibodies it is highly probable that the low temperature peak of heat absorption corresponds to the C_H2-C_H3 contact region. It is therefore likely that precipitating and non-precipitating pig antibodies have a different

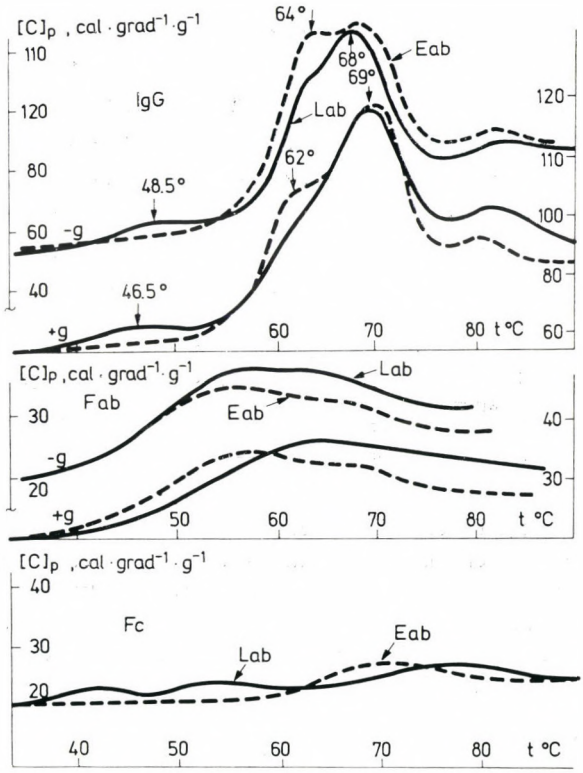

Fig. 4. Temperature dependence of the partial heat capacity of the precipitating or early (Eab) and non-precipitating or late (Lab) anti-dinitrophenyl pig antibody and its proteolytic fragments (Fab and Fc). (−g) unliganded and (+g) liganded antibodies and their Fab fragments. The solvent was 0.05 mole/l acetate buffer pH 4.1

energy of interactions between the C_H2 and C_H3 domains. The heat of denaturation calculated from the individual Fab and Fc fragments of pig antibodies does not agree with the experimental heat of denaturation of intact antibodies. In intact antibodies the Fab and Fc subunits probably have significant non-covalent interactions with each other. The hapten-binding induced an opposite change in the Fc subunit of pig antibodies in comparison with rabbit antibodies: the melting temperature of the low temperature peak of heat absorption is decreased as a result of hapten-binding. Table 1 shows the results of the study of the two types of pig

Table 1

The number of tyrosine (Tyr) and tryptophan (Trp) residues perturbated by temperature (TPDS) and solvent (SPDS) in pig anti-dinitrophenyl antibodies and its proteolytic fragments

Sample	No. Tyr res.		No. Trp res.	
	TPDS	SPDS	TPDS	SPDS
Eab	41.1±2.4	19.3±0.7	9.3±1.6	3.3±0.2
Lab	32.6±2.5	19.8±0.6	10.0±1.2	2.2±0.2
Fab Eab	9.4±0.8	7.1±0.2	2.3±0.2	1.1±0.1
Fab Lab	8.3±0.8	7.2±0.2	2.4±0.3	0.4±0.1
Eab + g	34.3±1.5	19.4±1.0	9.8±0.5	2.3±0.3
Lab + g	31.6±3.0	19.4±1.2	9.5±1.2	2.3±0.4
FabEab + g	9.3±0.4	6.4±0.5	2.6±0.3	1.1±0.2
FabLab+g	8.7±0.8	6.1±0.7	2.6±0.2	0.3±0.2

Abbreviations: Eab — early or precipitating antibodies, Lab — late or non-precipitating antibodies, Fab Lab, Fab Eab — Fab fragments of the corresponding antibodies, g — the hapten (2,4-dinitrophenyl-lysine), TPDS — the data of the thermal perturbation differential spectrophotometry, SPDS — the data of the solvent perturbation differential spectrophotometry.

antibody, by use of the difference temperature and solvent perturbation spectrophotometry. It can be seen that in the intact antibody molecules a less number of tyrosines and tryptophans are perturbed by the temperature than by the 20% ethylene glycol solvent. Thus, a significant part of tyrosine and tryptophan residues perturbed by temperature are not exposed to the solvent but exist in the flexible environment. The contradiction between the TPDS and SPDS data cannot be explained by the Fab subunits. One may therefore propose that the chromophores in a flexible environment are attributed to the Fc subunits. In fact, according to X-ray structural data [2], the conformation of C_H2 domains reveal a significant thermal flexibility. Comparison of the TPDS and SPDS data shows that there are more tyrosine residues perturbed by temperature in the precipitating antibodies than in the non-precipitating antibodies. As the number of exposed tyrosine res-

idues is practically the same in both types of antibody, it is possible that some part of the precipitating antibodies is more flexible than in the non-precipitating ones. The differences cannot be explained by the Fab fragments. It is therefore likely that these two types of antibody have a different structure from that of the Fc subunits. In fact, Fig. 4 shows that in the Fc subunits of the precipitating antibodies more chromophores are perturbed by temperature than in the non-precipitating ones. It is highly probable that these differences are stipulated by the different structure of C_H2 domains. The hapten-binding induces a decrease of the number of tyrosine residues perturbed by temperature. This effect cannot be explained by the change in the Fab structure. It is quite possible that the hapten-binding induces the conformational change of the Fc subunit, most likely the C_H2 domain structure.

Conformational Properties of the Human IgG Subclasses

The human IgG subclasses possess different biological properties. The main difference in the primary structure of the subclasses is concentrated in the "hinge region" (Fig. 5). According to these data, significant differences in intramolecular flexibility and in intersubunit interactions can be expected between the subclasses. We have therefore investigated the dynamical conformational properties of the human IgG subclasses. Table 2 shows a comparison of the rotation relaxation times obtained by the polarization of fluorescence (ϕ) and by the spin-label method of ESR (τ). The experiments and calculations were made as described earlier [5, 6, 7]. Previously Dudich et al. [1] showed that the rotation relaxation times of immunoglobulins exhibit a significant dependence on the immunoglobulin concentration. This effect was tentatively explained by a loosening of the structure of the Fc part of the molecule at concentrations below 2 mmole/1. Taking into consideration the

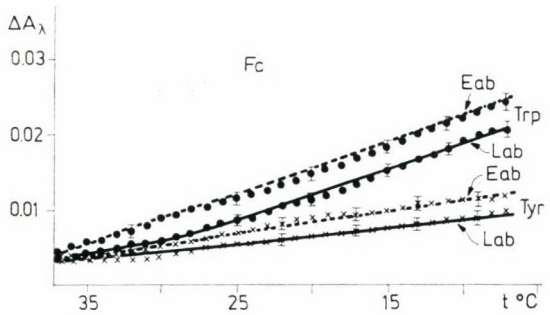

Fig. 5. Temperature dependence of the amplitude of maxima of the thermal perturbation difference spectra ΔA_{288} (attributed to the tyrosine residues) and ΔA_{295} (attributed to the tryptophan residues) for the Fc fragments of precipitating or early (Eab) and non-precipitating or late (Lab) anti-dinitrophenyl pig antibody. The solvent was 0.005 mole/l sodium phosphate buffer pH 7.35, containing 0.15 mole/l NaCl. The optical density of both samples at 280 nm was 1.38

Table 2

Rotation relaxation times for human IgG subclasses determined by fluorescence polarization analysis (φ_{max} and φ_{min}) and by spin-label method of ESR (τ)

Protein	ϕ max	ϕ min	$\tau_{20°C}$	$\tau_{40°C}$
IgG1	33	33	48	50
IgG2	32	23	38	50
IgG3	18	18	29	32

concentration dependence of ϕ, we performed a comparative polarization fluorescence study of the human IgG subclasses: ϕ_{min} corresponds to protein concentrations below 2 mmole/l, while ϕ_{max} corresponds to protein concentrations higher than 2 mmole/l. It can be seen from the table that for IgG3 the ϕ and τ values are significantly lower than for the other two subclasses. From these data we may conclude that IgG3 has the greatest flexibility between the Fab arms. But the TPDS data show that the conformation *per se* of IgG3 domains has the greatest rigidity in relation to thermal perturbation.

Figure 6 demonstrates the temperature dependence of the effective volume of human IgG subclasses and of the Fab fragments of pig non-precipitating antibodies, obtained by the spin-echo method of NMR [8]. The calculations were made using the equation [9],

$$1/T_2 = \frac{9}{20}(\gamma^4 \cdot h^2) \sum_{i \neq j} (1/r_{ij}^6)\tau_c \qquad (1)$$

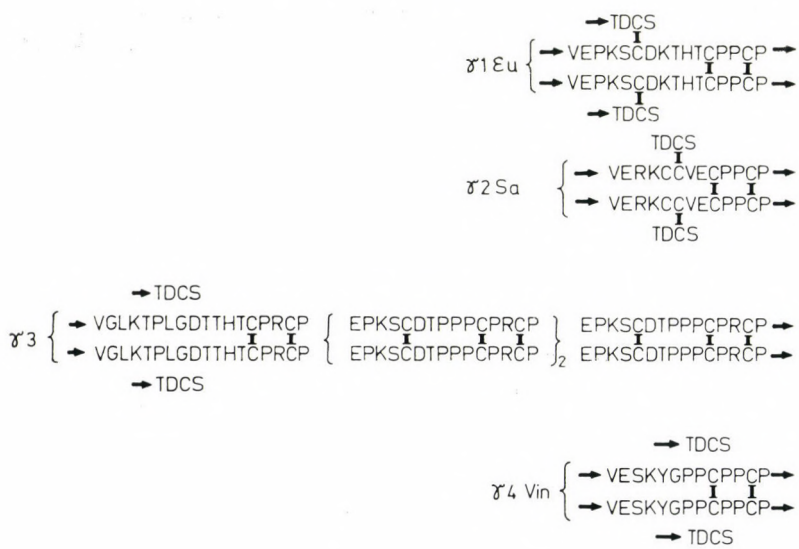

Fig. 6. Primary structure of the hinge regions of human IgG subclasses

and

$$\tau_c \sim V^{eff} \cdot \eta / T^0, \quad (2)$$

where γ is the gyromagnetic relation for proton, h is the Planck constant, r_{ij} is the distance between protons, T_2 is the time of the rapid component of the cross-relaxation, τ_c is the rotation relaxation time, η is the viscosity, T° is the temperature.

It can be seen from Fig. 6 that the samples of IgG2 and IgG3 investigated accomplish the conformational transition near the physiological interval of temperatures of the internal environment of homoiothermic animals. The transition is probably stipulated by the change of interdomain interactions through the intermediate highly mobile state. A comparison of the effective volumes for the Fab fragments and for the intact IgG molecule allows to conclude that at room temperature the mobility of the Fab subunits in the intact IgG molecules is limited. An increase of the temperature induces different changes of the effective volume in different IgG subclasses (excluding the region of conformational transition). The changes obtained for the intact IgG molecules cannot be explained by the properties of the isolated Fab fragments. It is therefore suggested that a decrease of the V^{eff} value for the intact IgG molecules is stipulated by an increase of flexibility between the Fab and Fc parts and/or the intra Fc subunit. It is interesting that IgG3 with a higher extent of flexibility corresponding to fluorescence polarization and ESR data (Table 2) should reveal a smaller temperature dependence of the V^{eff} value. It is therefore likely that the high temperature dependence of the V^{eff} value observed for IgG1 and IgG2 can be explained by the

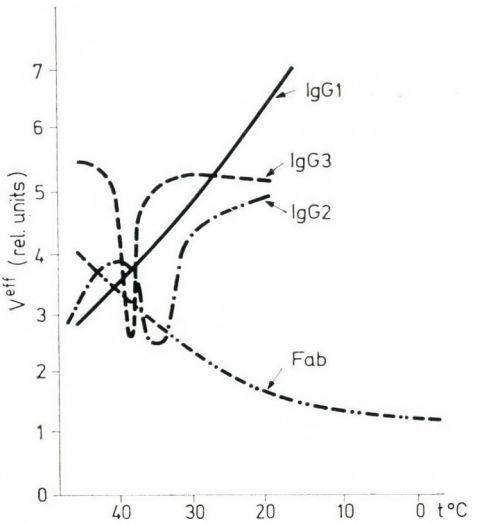

Fig. 7. Temperature dependence of the effective volume (V^{eff}) of human IgG subclasses and Fab fragments of pig anti-dinitrophenyl non-precipitating antibody. The solvent was D_2O, 0.005 mole/l sodium phosphate buffer pH 7.4, containing 0.15 mole/l NaCl

more flexible conformation of its Fc subunits. This conclusion is in good agreement with the concentration dependence of the ϕ values and the TPDS data.

The studies revealed essential differences in the internal flexibility within and between domains and in the energy of non-covalent interactions between domains and subunits of immunoglobulins of different subclasses and of different animals. The higher extent of non-covalent interactions between the Fab and Fc subunits correlates with the lower extent of interactions between the C_H2 and C_H3 domains (the higher flexibility of the Fc parts). Hapten-binding induces conformational changes in the Fc subunits of the immunoglobulins with strong non-covalent interactions between the Fab and Fc subunits.

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The Combination of IgM Subunits and Proteolytic IgG Fragments by Controlled Formation of Interchain Disulphides*

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Crosslinking of different proteins, a procedure now often used in biochemistry [1, 2], seems to be of special promise for immunological studies. A great disadvantage of almost all methods of protein crosslinking is that they are poorly controlled and the resulting products are polydispersive and therefore of low biological usefulness. Crosslinking of proteins by specific groups such as binding sites may increase the specificity of bridging [3]. Unfortunately, very few proteins appear to be available for such binding. Only the methods based on using protein disulphide sulphurs for crossbridging, especially when considering intersubunits and interchain bonds, seem to be promising. A simple reduction and reoxidation which are commonly used for hybridization experiments, give an inconvenient statistical distribution of protein units [4]. The thiol-disulphide interchanging process appears to be more advantageous for the controlled performance of protein crossbridging [5]. This tends to be of special convenience for recombination of protein subunits and disulphide-bonded chains. Protein subunits and peptides exclusively stabilized by disulphide bonds are available for such recombination process. Unfortunately, in non-dissociable conditions only a limited number of proteins meet this requirement. Most naturally occurring proteins have disulphide bonds structurally protected and therefore unreactive with thiols and sulphite. If, however, the protein structure is disrupted by thermal treatment or limited proteolysis, most of the disulphide bonds become reactive [6, 7]. This observation offers a unique possibility for the formation of new, artificial protein molecules of desired biological activity by combination of suitable digest fragments. The method of controlled protein crosslinking by thiol-disulphide interchange has already been proposed [5]. The two different proteins which are to be crosslinked by this method are armed on the one side with thiols and on the other with mixed disulphides. Mixed disulphides were obtained by the reaction of thiols with DTNB†; these two forms of protein, when mixed, become bridged by disulphides. The re-

* The principle of the method is described in this paper. The details will be presented elsewhere.

† Abbrevations: DTNB — 5,5'-dithio-bis-(2-nitrobenzoic acid) ØS$^-$ — 3-carboxylato-4-nitro-thiophenolate; DTT — 1,4-dithioerythritol

action is run by liberation of free ØS$^-$ ion which is highly stable in water. The general idea of the reaction is presented in Fig. 1. A mild reduction and S-sulphonation were used to split intersubunit and interchain disulphides. S-sulphonation used for this purpose often appears more selective than reduction. Sulphite requires a polar medium to react with disulphides and thus preferably splits intersubunit and interchain bridges while differentiation of protein disulphides by reductants is usually less specific. S-sulphonation [8] was, however, modified to be

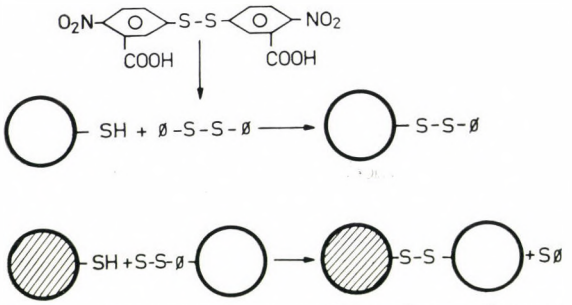

Fig. 1. Schematic drawing representing the general idea of controlled protein crossbridging

used with the controlled disulphide interchanging method. Namely, DTNB was used instead of copper ions to stimulate the formation of S-thiosulphate in proteins (protein-S-SO_3^-). S-thiosulphates may be converted into mixed disulphides (protein-S-SØ) if there is excess DTNB and the sulphite is completely removed from the reaction mixture. This protein may then directly be used for the reaction with the protein armed with thiols (protein-SH), which were liberated from mixed disulphide by gentle reduction with DTT. Thus, both reacting proteins are prepared in the same way with the exception that the protein which is to be armed with thiol is finally mildly reduced. If reduction instead of S-sulphonation was originally used for splitting protein disulphides, the resulting thiols were simply converted into mixed disulphide derivatives by reacting with DTNB.

The Model of the Method

1. *Recombination of IgM*

To test the method, human IgM was used as a model protein. The pentameric IgM molecule was dissociated by S-sulphonation to 7S subunits, which then were prepared for crosslinking in -SH and -SSØ forms according to the idea presented above. These two prepared proteins are mixed for crosslinking. The reaction is maintained by electrodialysis to remove liberated ØS$^-$ ions. Neither of the monomer derivatives, when stored separately for moderately long periods of time, were observed to crosslink with each other. Thus, IgM subunits were forced to

recombine, solely as a result of introduced affinity between subunits equipped with thiols and mixed disulphides. Dimers, trimers and tetramers were found in the products. No attention was paid to the J chain in those preliminary experiments. The lack of this compounds was most probably the reason why no pentamers were observed among the resulting polymers. Dimers represented the abundant frac-

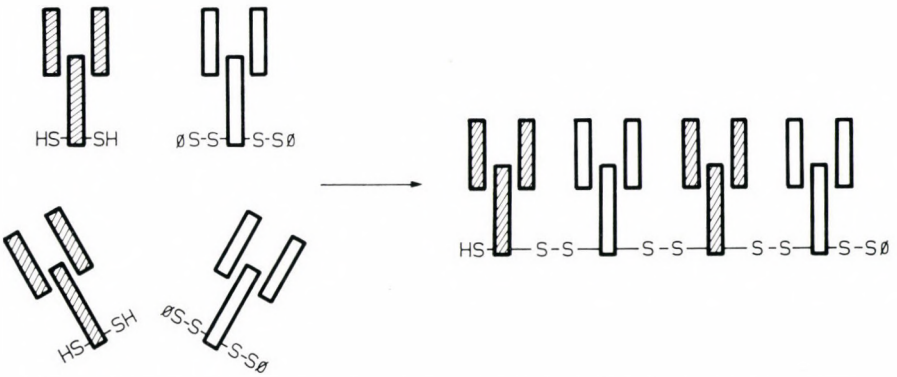

Fig. 2 Recombination of IgM subunits using controlled thiol-disulphide interchange

tion. This is in accord with the assumption [9] that newly formed disulphide bridges represent the only bonding that stabilize recombinant molecules. It was observed, that the recombined subunits are arranged one by one (Fig. 2) as they originate from the -SH and -SSØ protein pools. This may be important if subunits of different origin are to be used for recombination [4].

2. *Synthesis of chimeric immunoglobulin G*

Human and rabbit proteolytic IgG fragments were used for synthesis of a chimeric molecule with antibody-like properties. This artificial immunoglobulin molecule was composed of rabbit Fab and human Fc fragments and its synthesis

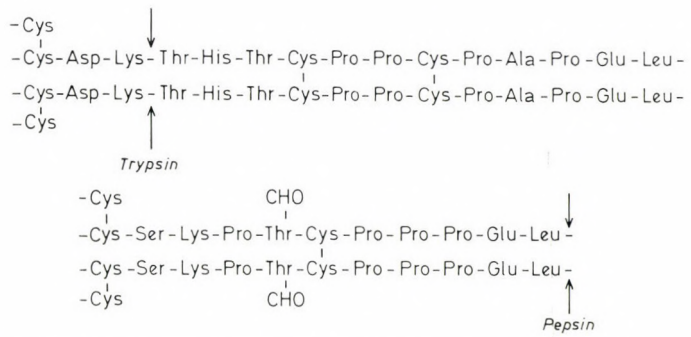

Fig. 3. Amino acid sequence (10) of human and rabbit IgG hinge region with indicated digestion points

was thought to be a way to introduce a chosen antigen-binding specificity to human effector properties bearing fragments. It also allows to undertake an altered approach to understanding the effector properties of Fc fragment. The process was conducted as follows. Rabbit and human immunoglobulin G were digested by pepsin and trypsin, respectively, to obtain $F(ab)_2$ fragment from rabbit IgG and Fc from human IgG, both with stumps of the hinge region containing preserved interchain disulphides (Fig. 3). The combination process (Fig. 4) involves the simple reduction of rabbit $(Fab)_2$ fragment, and the reduction of human Fc and subsequent conversion of the resulting thiols to mixed (Fc-S-SØ) disulphides. Mild reduction or

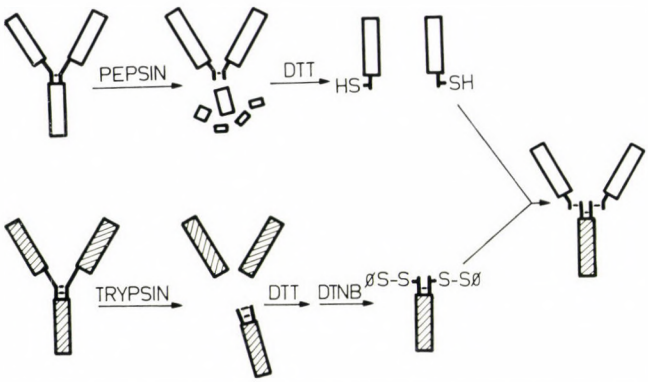

Fig. 4. Model of the procedure used for synthesis of chimeric IgG molecule

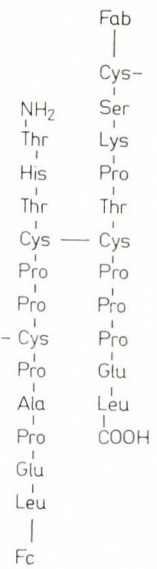

Fig. 5. Suggested arrangement of disulphide linked rabbit and human hinge stumps

S-sulphonation of human Fc fragment splits only one of the two interchain dij sulphides. Mixed Fc and Fab fragments become disulphide crossbridged whil- the stumps of their hinge region overlap each other (Fig. 5). Two fractions oe 110,000 and 160,000 molecular weight, respectively, appeared in the combination reaction. They most probably correspond to Fc combined with one and with two Fab fragments, respectively. These molecules must be structurally similar to the naturally occurring immunoglobulin G. They are, however, monovalent, even if two Fab fragments are substituted to Fc because it is highly improbable that Fab fragments of the same specificity should bind in one combined molecule of chimeric IgG. A bivalent molecule may be obtained if specific antibody is used for preparation of Fab fragments. This needs electrofocussing or affinity chromatography for purification of the suitable antibody.

Preliminary haemagglutination and complement fixation tests suggest that some effector properties of human Fc may be preserved in chimeric IgG molecules.

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MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

Haematologia 14 (1), p. 101 (1981)

Evidence of Surface Immunoglobulins as Leukemia-Associated Cell Surface Antigens

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Blood lymphocytes of leukemic cattle were studied for the presence of surface immunoglobulins and leukemia-associated antigens. In most cases leukemic cell population with surface IgM was detected in RID, IMF and C-dependent microcytotoxicity assay. According to lymphocyte surface markers leukemic cells in most cases of bovine leukemia were identified as B cell population.

Heterogeneity of blood lymphocyte populations from the point of view of surface immunoglobulin markers in case of bovine leukemia are presented.

Haematologia 14 (1), p. 103 (1981)

Studies on Properties of the Human Placental Membrane Receptor for IgG

M. NIEZGÓDKA, J. MIKULSKA, M. UGORSKI, J. BORATYŃSKI and J. LISOWSKI

Institute of Immunolgy and Experimental Therapy, Wrocław, Poland

Human placental membrane receptor for human ^{125}I-IgG was examined. The receptor bound specifically both monomers and aggregates of human IgG. Human colostral IgA, bovine, sheep, pig, and horse IgG were not bound. No effect of pH in the range 6.6–7.4, ionic strength in the range 0.1–0.5, and temperature in the range 4–45 °C on the binding was found. Both, monomeric and aggregated human IgG were bound to the same receptor site and the apparent association constants were $0.86 \pm 0.2 \times 10^7\ M^{-1}$ and $1.98 \pm 0.6 \times 10^7\ M^{-1}$, respectively. Formaldehyde, 2-mercaptoethanol, trypsin, and neuraminidase showed no effect on the IgG binding properties of the human placental receptor. Periodate, at concentrations higher than 20 mM, lowered the binding by 50%. Phospholipase A_2 and phospholipase C caused 30%, and pronase 18% decrease of binding of IgG to the receptor. Glycoproteins were isolated from the placental membrane using lithium diiodosalicylate. The glycoproteins obtained inhibited the binding of ^{125}I-IgG to the placental membranes. The binding was also inhibited by glycoproteins isolated from human erythrocytes and guinea pig peritoneal macrophage membranes but with a lower efficiency.

Haematologia 14 (1), p. 105 (1981)

Changes of the Dynamic Properties of Antibodies and their Subunits Induced by Hapten. Evidence for Important Role of Interaction of Proteins with Water

A. KÄIVÄRÄINEN

Institute of Biology, USSR Academy of Sciences, Petrozavodsk, USSR

We used the method of separate determination of correlation times of the spin label and of the labelled protein with the aim to analyze changes occurring in different parts of anti-Dnp pig antibodies upon hapten binding. In one case the spin label was presumably attached to the Fab part, in another case to the carbohydrate moiety of the Fc part. It was found that the interaction between pig antibody subunits as well as the intramolecular mobility of Fc subunits changes upon hapten binding.

Investigation of complexes of spin-labelled Dnp-hapten with pig anti-Dnp antibodies or with their Fab fragments showed a marked temperature dependence of the intramolecular flexibility. The antibody molecules and their Fab fragments were more flexible at 5 °C than at 30 °C. At 5 °C low concentrations of D_2O decreased the intramolecular mobility of these proteins.

Haematologia 14 (1), p. 107 (1981)

Peptic Fragmentation of Pig Antibodies

Z. Olšovská, F. Franěk

(Institute of Molecular Genetics, Academy of Sciences, Prague, Czechoslovakia)

Enzymic fragmentation of pig immunoglobulin G proceeds with most proteolytic enzymes used only incompletely. This behaviour is obviously related to particular inaccessibility of the polypeptide chains in the environment of the hinge region and/or to the presence of multiple inter-heavy chain disulfide bonds. The difference in digestibility between precipitating and non-precipitating anti-Dnp antibody can be best seen upon limited cleavage by pepsin. The digests of the non-precipitating antibody contain a stable Fc' fragment.

Haematologia 14 (1), p. 109 (1981)

Immunochemical Characterization of IgG3 Antibodies against Thy 1.2 Antigen Produced by Mouse Hybridoma

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(Institute of Microbiology and Institute of Molecular Genetics, Academy of Sciences, Prague, Czechoslovakia)

Antibodies against Thy 1.2 antigen produced by two recently established permanent cell lines of mouse hybridoma (1B5 and 1aG4/C5) were characterized. Both antibodies were isolated on Protein A-Sepharose (Pharmacia, Uppsala) from hybridoma supernatant which did not contain any serum component except products released from the cells. In contrast to the fact that all other mouse hybridomas described so far produced anti-Thy 1.2 antibodies of IgM class, we established by specific antisera that both our hybridomas produced IgG3(κ) antibodies ($S^{20}_{o,w} = 6.6$). 1aG4/C5 antibodies were more efficient in cytotoxic test than 1B5 antibodies. 1B5 antibodies in high concentration reacted also with Thy 1.1 antigen. Both antibodies possessed some typical properties of IgG3: strong tendency to aggregation and very low electrophoretic mobility at pH 8.6. 1B5 hybridoma secreted besides IgG3 molecules also free L chains in monomeric and dimeric form (approximately one L chain per 1 IgG molecule) and the interchain disulfide bridges of the antibody were split during iodination. On the other hand, 1aG4/C5 hybridoma secreted IgG3 without free L chains and its disulfide bridges were not affected by iodination. 1aG4/C5 and 1B5 antibodies were adsorbed on Protein A-Sepharose in 0.1 M Tris-HCl, pH 7 and were eluted in 0.1 M sodium citrate-citric acid, pH 4.5. 1aG4/C5 antibody did not precipitate protein A while in the same conditions 1B5 antibody precipitated. Both antibodies differed also in isoelectric focusing patterns, and both exhibited very restricted heterogeneity. The data suggest that 1B5 and 1aG4 antibodies may belong to different isotypic variants of IgG3.

Haematologia 14 (1), p. 111 (1981)

Structure of IgG and IgY in Ribosome-Antibody Complexes as Studied by Electron Microscopy

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(Central Institute of Molecular Biology, Academy of Sciences, 1115 Berlin-Buch, GDR)

In the course of our immune electron microscopic studies for the localization of exposed portions of ribosomal proteins on the surface of ribosomal particles we obtained electron micrographs of negatively stained ribosome-antibody complexes in which the antibodies (IgG or IgY) appear as Y-shaped molecules.

The dimensions and the shape of anti-ribosomal protein antibodies could be determined in dimeric immune complexes in which one antibody molecule has bound two ribosomal particles (ribosome-antibody-ribosome). Moreover, the angles between the Fab arms were measured. The results were compared with the dimensions and the shape of antibody molecules obtained by other approaches.

Book Reviews

Trace Elements and Iron in Human Metabolism. By A. S. Prasad. 392 pages, 26 tables, 26 figures. Plenum Medical Book Co., New York 1978. Price: $35.—

Writing a monograph on this topic is a difficult task considering the diversity of the subject. Dr. Prasad's attempt to overcome the difficulties originated from the limit of a single volume monograph and the voluminous literature on the subject was only partially successful.

The book is divided into two main parts, essential elements and toxic elements. Both parts contain chapters on elements put in alphabetic order. Each chapter deals with the particular element in a logical sequence of subchapters (biochemistry, metabolism, deficiency and toxicity). At the end of each chapter a fair amount of references is found. With this monograph Dr. Prasad produced a clear and informative summary of present knowledge on trace elements and their role in biochemistry, human metabolism and nutrition. Due to the size of the monograph it is inevitably superficial but provides a general view of the biology of trace elements or a good starting point for reading if depth is required. With this in mind, the book is recommended for those who do not expect to get a handbook with exhaustive coverage of the subject but a well-written guide-book to this very intricate part of biology.

A. Egyed

Control of Cell Proliferation by Endogenous Inhibitors. By A. Balázs. 272 pages. Akadémiai Kiadó, Budapest 1979.

Control of cellular proliferation in normal and malignant tissues is one of the basic problems in biology and on the topic an overwhelming number of papers has been published during the last fifteen years. The present monograph discusses a special aspect of the control of proliferation, the endogenous inhibitors.

By definition, these inhibitors are physiological compounds of endogenous origin, their effect is cell-line-specific without being species-specific, they inhibit proliferation without being cytotoxic.

The introduction gives a clear definition of the activators and inhibitors, both exogenous (cytostatics) and endogenous, enumerating meticulously all cell types from which the inhibitory extracts are prepared.

Chapter 2 deals with the biology and biochemistry of cell proliferation, which seems to be necessary for understanding the regulation of proliferation.

In Chapters 3 and 4, first the endogenous inhibitors (including chalones), then the isolation, biochemical characterization of the regulators and the biological assays for the endogenous proliferation regulators are discussed in detail.

In Chapters 5 and 6, the mechanisms involved in the control of proliferation, their possible intracellular targets (e.g. nucleus,

cytoplasm, cell membrane), the role of cAMP and cGMP in the inhibitory effect as well as the inhibition at the transcriptional and translational levels are discussed.

A separate chapter deals with the control of tumour cell kinetics with special emphasis on the role of regulators in carcinogenesis and the effect of inhibitors on tumour cell growth *in vitro* and *in vivo*, offering also therapeutic perspectives.

The last chapter compiles the available data on the chemical composition of the endogenous inhibitors acting on the various tissues (epidermis; sebaceous, sweat and salivary glands, melanocytes, intestinal epithelium, endometrium, liver, lungs and testes, haemopoietic and lymphoid cells, fibroblasts, etc.). This chapter contains a fourteen-page table summarizing the most important parameters of endogenous inhibitors such as target cells and target phases of the cell cycle, specificity, producers of the inhibitor, the minimum essential dose and the chemical characterization (composition, solubility, enzymatic digestibility and molecular weight) of the inhibitors.

The scientific logic of the book is easy to follow. In addition to presenting his own data on endogenous inhibitors, the author offers a comprehensive survey of the techniques for producing, isolating and testing of the agents which control cellular proliferation. The monograph gives a comprehensive summary of the theoretical problems in this field without being too committed to the author's special field.

Scientists interested in cellular proliferation will profit from this book and may receive ideas for their own studies.

Júlia Gidáli

Abstracts

Calcium release and aggregation in rabbit platelets: action of psychotropic drugs. H. W. Reading (MRC Brain Metabolism Unit, Thomas Clouston Clinic, Edinburgh, UK). *Cell. Mol. Biol. 25*, 421 (1980).

Development of a fluorescence assay for calcium, magnesium, zinc and aluminium is described and applied to estimate total calcium and magnesium in the rabbit blood platelet and calcium release produced by lithium and the tricyclic antidepressant drugs, amitriptyline and imipramine. Potentiation of ADP-induced platelet aggregation by the three psychoactive drugs is described. All effects are dose-dependent, release and aggregation being closely correlated. Results are discussed in relation to the mode of action of the compounds and the use of the platelet as a model for the central monoaminergic neurone.

G. Gárdos

Platelet phosphorylase kinase activity and its regulation by the calcium-dependent regulatory protein, calmodulin. P. Gergely, A. G. Castle, N. Crawford (Department of Biochemistry, Institute of Basic Medical Science, Royal College of Surgeons of England, London, UK). *Biochim. Biophys. Acta 612*, 50 (1980).

Platelet phosphorylase kinase (ATP: phosphorylase phosphotransferase, EC 2.7.1.38) was found to be a Ca^{2+}-sensitive enzyme. It has two K_a values for Ca^{2+} viz. 0.25 and 2.6 μM, respectively. The "calcium-dependent regulator" or calmodulin can enhance the activity of phosphorylase kinase, increasing its affinity for Ca^{2+}. In the presence of calmodulin phosphorylase kinase has only one, high affinity binding site for Ca^{2+} ($K_a = 0.27$ μM). Platelet phosphorylase kinase can be phosphorylated by endogenous cyclic AMP-dependent protein kinase increasing its catalytic activity and this activation process is reversed by dephosphorylation. The changing level of intracellular Ca^{2+} and cyclic AMP may control the activity of phosphorylase kinase, regulating the mobilization of glycogen.

B. Sarkadi

Enzyme activities related to 2.3-P_2-glycerate metabolism in embryonic and fetal red cells. W. Jelkmann, Ch. Bauer (Physiologisches Institut, Universität Regensburg, Regensburg B.R.D.) *Biochem, Biophys. Res. Comm. 93*, 93 (1980).

The intraerythrocytic concentration of 2,3-P_2-glycerate decreases conspicuously during the intrauterine development of rabbits. In order to obtain informations on possible causes for these changes, we have measured in embryonic and fetal red cells the activities of those glycolytic enzymes which are thought to be most important in the regulation of the steady-state level of 2,3-P_2-glycerate, namely hexokinase, phosphofructokinase, bisphosphoglyceromutase and pyruvate kinase. Hexokinase and bisphosphoglyceromutase activities are significantly higher in embryonic red cells, when compared to fetal or adult erythrocytes, whilst the activity of phosphofructokinase is not significantly different at either developmental stage. Most important are the changes found in the activ-

ity of pyruvate kinase which is low in embryonic and early fetal erythrocytes and increases drastically thereafter. The activity of this enzyme is inversely related to the concentration of 2,3-P_2-glycerate, which is highest in embryonic red cells and very low in the late fetal period. These results support the conclusion that the pyruvate kinase activity is of great importance for the adjustment of the concentration of 2,3-P_2-glycerate and therefore of the blood oxygen affinity during rabbit ontogeny.

G. Gárdos

Evidence for allosteric inhibition sites in the glucose carrier of erythrocytes. R. M. Krupka, R. Devés (Research Institute, Agriculture Canada, University sub Post Office, London, Ontario, and Department of Biochemistry, University of Western Ontario, London, Ontario, Canada). *Biochim. Biophys. Acta 598*, 127 (1980).

2,4-Fluorodinitrobenzene and 2,3-butanedione, which irreversibly inactivate the glucose transfer system of erythrocytes, have been used as probes determine whether the substrate site and inner and outer sites for reversible inhibitors are located in the same or different regions of the carrier. Inhibitors bound at an inhibition site exposed in the inward-facing but not the outward-facing form of the carrier (cytochalasin B, androstendione and androstandione) protect the transport system against inactivation by 2,4-fluorodinitrobenzene. Inhibitors bound at an external inhibition site (phloretin) and substrates bound at the transfer site do not protect. In contrast inactivation by 2,3-butanedione is slightly accelerated by internally bound inhibitors, while substrates and substrate analogs bound at the transfer site protect the system. It is shown that fluorodinitrobenzene reacts in the inner inhibition site and butanedione in the substrate site; and further that these sites may be separate binding areas in the carrier linked by allosteric interaction. The consequence of this linkage is that binding of a ligand at the substrate site precludes binding of another ligand at the internal or external inhibition site.

B. Sarkadi

Compartmentalization of spectrin-phosphorylating enzyme in human erythrocytes. P. Boivin, C. Galand (Unité de recherches d'enzymologie des cellules sanguines, Hôpital Beaujon, Clichy Cedex, France) *Biochem. Biophys. Res. Comm. 93*, 24 (1980).

Functional characteristics of purified casein kinases from cytosol and membrane of normal human red cell were determined in vitro using pure spectrin as substrate. Both enzyme which differed from each other by their salt solubility, molecular weight and affinity for casein had similar affinity for spectrin and same optimum pH; they used ATP as phosphoryl donor, were similarly stimulated by monovalent cations and Mg^{2+} and inhibited by Ca^{2+} and were insensitive to cyclic nucleotides. Cytosol kinase had a poor substrate and phosphoryl donor specificity; membrane kinase had a much greater affinity for spectrin than for casein and poorly used GTP as phosphoryl donor: it seems to be the physiologically active spectrin kinase of the red cell.

G. Gárdos

Influence of monovalent ions on the activity of the (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+}-transport of human red blood cells. R. Wierichs, H. Bader (Department of Pharmacology and Toxicology, University of Ulm, Ulm, F.R.G.). *Biochim. Biophys. Acta 596*, 325 (1980).

In reconstituted human red cells a difference was found in (Ca^{2+} + Mg^{2+})-ATPase activity and in Ca^{2+} efflux at 37 °C, depending on the side of the membrane at which the monovalent cations K^+ and Na^+ were placed. Under the conditions used, (Ca^{2+} + + Mg^{2+})-ATPase activity and Ca^{2+} efflux was highest when K^+ (35 ± 0.5 mM (± S.E.), mean of four experiments) was at the inside and Na^+ (130 mM) at the outside of the ghost membrane.

B. Sarkadi

Identification of the blood-group ABH-active glycoprotein components of human erythrocyte membrane. J. Finne (Department of Biochemistry, Biocenter, University of Basel). *Eur. J. Biochem. 104*, 181 (1980).

The blood-group ABH-active components of the erythrocyte membrane were identified

by incubation of polyacrylamide gels after electrophoresis with radio-iodinated blood-group-specific lectins. The anti-A and anti-B lectin of *Bandeiraea simplicifolia* bound to components from A_1, AB and B erythrocytes but not A_2 or O erythrocytes, whereas the opposite was observed for the anti-H lectin of *Lotus tetragonolobus*. Both lectins revealed as major blood-group-active components band 3 and the region of band 4.5, which was resolved into at least two components. Smaller amounts of the lectins were bound to the low-molecular-weight region and the dye front, which contained the poly(glycosyl)ceramides. In addition to these components, the *B. simplicifolia* lectin also bound to the sialoglycoprotein bands 1 and 2 (stained with periodic acid/Schiff). The distribution of bound radioactivity, as determined with this lectin was: Band 3, 22–25%; HIO_4/Schiff band-1, 10–15%; band 4.5 34–37%; HIO_4/Schiff band-2 12–15%; and the poly(glycosyl)ceramide region, 14–17%. The different lectin-binding properties indicate a structural difference between the antigenic determinants of the sialoglycoproteins and of the other blood-group-active components. It is suggested that the antigenic determinants in the sialoglycoproteins are of the alkali-labile type previously characterized, and that the carbohydrate chains of bands 3 and 4.5 correspond to the previously isolated alkali-stable poly(glycosyl)peptides. The detection of two different groups of glycoproteins could thus explain the controversy concerning the nature of the blood-group ABH antigens in glycoproteins of the erythrocyte membrane.

Ilma Szász

Various topochemical arrangements of sialic acids on human erythrocytes as detected by partition in aqueous two-polymer phase systems. B. Y. Zaslavsky, L. M. Miheeva, S. V. Rogozhin, L. V. Borsova, B. I. Kosinez (Institute of Elementoorganic Compounds, Academy of Sciences of the USSR, Moscow, and Central Institute of Haematology and Blood Transfusion, Moscow, USSR). *Biochim. Biophys. Acta 597*, 53 (1980).

Human and rabbit red blood cells were subjected to partition in an aqueous, buffered Ficoll-Dextran two-phase system. The effect of neuraminidase treatment on the cell partition behaviour was examined and compared with the amount of sialic acids released from the cell surface and with the change in the electrophoretic mobility of the cells. The data obtained in the study indicate that there are two main groups of sialic acids differing in their topochemical arrangement on the human erythrocyte surface, and their relative hydrophobicity was eluated. The results obtained in the case of rabbit red cells seem to indicate that sialic acids present on the cell surface are not the only major ionogenic surface components as in the case for human red cells. The data obtained support the assumption that the membrane surface charge is the determinant of cell partition only as a factor affecting the relative hydrophobicity of the cell surface.

B. Sarkadi

Is inosine the physiological energy source of pig erythrocytes? S. M. Jarvis, J. D. Young, M. Ansay, A. L. Archibald, R. A. Harkness, R. J. Simmonds (ARC Institute of Animal Physiology, Babraham, Cambridge, UK). *Biochim. Biophys. Acta 597*, 183 (1980).

Pig erythrocytes are unable to metabolize glucose and their physiological energy source is unknown. These cells have a high-capacity nucleoside transport system with similar properties to that responsible for nucleoside transport in other species. Nucleoside transport is sufficiently rapid to allow the possibility that inosine and/or adenosine may represent major energy substrates for pig erythrocytes in vivo. Normal and adenosine deaminase-deficient pig erythrocytes have similar ATP levels, suggesting that adenosine is not important in this respect. However, it was calculated that an extracellular inosine concentration of only 40 nM could support the cells' entire energy requirement, a value 40-fold lower than plasma levels of this nucleoside.

G. Gárdos

Isolation and characterization of the plasma membranes of cultured lymphoblasts from patients with cystic fibrosis and normal individ-

uals. T. Maler, J. R. Riordan (Departments of Biochemistry and Clinical Biochemistry, University of Toronto and Hospital of Sick Children, Toronto, Canada). *Biochim. Biophys. Acta 598*, 1 (1980).

Plasma membranes were isolated from two cystic fibrosis lymphoblastoid cell lines and two age and sex-matched normal cell lines after controlled cell disruption. The preparations were enriched approx. 20-fold in plasma membrane markers and were essentially free of markers for other organelles except the Golgi-localized enzyme, galactosyltransferase. Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed virtually identical polypeptide profiles after staining with Coomassie blue. Membranes from normal cells contained 0.49—0.67 μmol of total phospholipid/mg membrane protein and those from cystic fibrosis cells, 0.53–0.67 μmol. The cholesterol content was 0.35–0.41 μmol/mg protein for normal, and 0.43–0.49 μmol for cystic fibrosis cell membranes. The proportions of individual phospholipid classes and their fatty acid compositions as well as that of the total membrane lipid also did not differ significantly in the two cell types. The bulk lipid fluidity as reflected by the fluorescence polarization of β-parinaric acid and diphenylhexatriene was also not changed. The carbohydrate composition of cell surface glycopeptides released with papain was not consistently different with the normal and cystic fibrosis cells. The quantity of fucose in intact plasma membranes was also quantitated enzymatically and found to be very similar in both cell types.

Ilma Szász

Arachidonate metabolite(s) increase in the permeability of the plasma membrane of the neutrophils to calcium. M. Volpi, P. H. Naccache, R. I. Sha'afi (Departments of Physiology and Pathology, University of Connecticut Health Center, Farmington, Connecticut). *Biochem. Biophys. Res. Comm. 92*, 1231 (1980).

The relationship between arachidonate metabolism and stimulated calcium fluxes in rabbit peritoneal neutrophils has been investigated. The addition of arachidonate to the neutrophils was found to cause a rapid and significant increase in the permeability of the plasma membrane to calcium. This effect is specific to calcium, concentration dependent and sensitive to inhibitors of the lipoxygenase mediated metabolic pathway(s). These results strongly suggest that arachidonate metabolites are directly involved in the mechanism underlying calcium gating in the neutrophils.

Ilma Szász

Card Indexes

S. R. Hollán

Editorial. Training of blood transfusion teams. Haematologia *14*, 5 (1981).

1. Training of the staff of Blood Transfusion Centres (BTC-s) and postgraduate teaching of clinicians represent a core problem in establishing an efficient National Blood Transfusion Service (NBTS). 2. The teaching programme should be adapted to the basic function of the BTS. 3. The training programme should give the staff the theoretical basis and technical skill to ensure that the BTC can provide the region with whole blood, blood concentrates and plasma fractions of guaranteed biological activity. 4. It should demonstrate in practice the importance of a close contact between BTC-s and clinical units. 5. The lack of experts in developing countries is one of the most important limiting factors in the development of their NBTS. Since haematology and immunology overlap and interact with blood transfusion, the best solution is to train physicians in blood transfusion, haematology and immunology in a combined specialist's training school. This type of training renders specialists who are not merely heads of "bottling factories", but who in addition to their high level work in blood transfusion will be well versed in two other specialities both indispensable in modern clinical diagnostics and in the fight against population diseases.

F. Krizsa, K. Vezendi, G. Varga, I. Cserháti

Effect of platelet homogenate on platelet production of bone-marrow-transplanted mice. Haematologia *14*, 11 (1981).

Haemopoiesis of mice was reconstituted with 1×10^6 bone marrow cells after 800 r total body irradiation. Then their megakaryocytopoiesis was inhibited by platelet homogenates given at different points of time on three subsequent days. The effect was measured by the peripheral platelet counts on the 14th day after transplantation when the platelet count of irradiated and marrow-transplanted CFLP animals was 410×10^9/l, and that of CBA mice was 185×10^9/l. Platelet homogenates prepared from $1–2.5 \times 10^8$ platelets daily given on days 1, 2 and 3 did not significantly reduce the peripheral platelet count estimated on the 14th day, while homogenates given on days 8, 9 and 10 reduced it markedly. Homogenates prepared from $0.7–1.0 \times 10^6$ white blood cells did not influence the 14th day platelet count. Thus, platelet homogenates exert their inhibitory effect at a late stage of platelet production, i. e. not by an inhibition of thrombopoietin or a thrombopoietin effect.

A. V. Karaulov, T. N. Strenewa, V. N. Frash

Immunological, cytochemical and cytological study of lymphocytes in neoplastic and hypoplastic states of haemopoiesis. Haematologia *14*, 15 (1981).

The T and B lymphocytes, their semiquantitative cytochemistry and nucleoli were investigated in human acute and chronic lymphatic leukaemia (ALL, CLL), and hypoplastic anaemia (HA), and in mice with chronic benzene poisoning (CBI). In the acute phase of ALL T and B markers were absent. In all phases, including remission, cytochemical changes and changes in structure of the nucleoli were present. In CLL, two variants of immunological changes were found, a B-cell variant and a mixed B-T-cell variant. Immunological and cytochemical changes were identical in HA and CBI, and this allowed to conclude that CBI is an adequate model of certain variants of human HA. The immunological and cytochemical changes in ALL, CLL, HA and CBI affected both T and B cells.

I. Frydecka, A. Harłozińska, S. Kotlarek-Haus

Nonsecretory multiple myeloma. Haematologia *14*, 27 (1981).

A patient is described with myeloma without paraproteinaemia or Bence-Jones proteinuria. In May-Grünwald-Giemsa-stained bone marrow films 32% of the cells were plasma cells containing monoclonal IgG.

Phan Thi Phi Phi, M. Nedelkova, V. Serafimov-Dimitrov

Function and role of peritoneal macrophages in experimental leukaemia. Haematologia *14*, 31 (1981).

The specific and nonspecific function of peritoneal macrophages of leukaemic mice has been studied as well as the role of these cells in the induction of lymphocyte cytotoxicity against leukaemic cells *in vitro*. It was found that the phagocytic and digestive function of the peritoneal macrophages remains normal, but the macrophage-mediated cytotoxicity against leukaemic cells is rare and weak in both allogeneic and syngeneic forms of grafted leukaemia. Utilizing the system of generation of cytotoxic lymphocytes in mixed lymphocyte tumour cell cultures *in vitro*, it was observed that the addition of specifically activated syngeneic macrophages lowers the rate of generated cytotoxicity. The mechanism of macrophage-induced suppression is discussed.

P. Hernández, E. Dorticós, C. Ustariz, M. García, J. M. Ballester

Sarcoidosis associated with autoimmune haemolytic anaemia and paroxysmal nocturnal haemoglobinuria red cell abnormality. Haematologia *14*, 41 (1981).

A 29-year-old male with sarcoidosis autoimmune haemolytic anaemia and paroxysmal nocturnal haemoglobinuria is described. Throughout his illness the chest films showed fibrosis in the right hilar region and he had had several pneumonias in the right lung. He had had massive splenomegaly and a splenectomy was performed. He was treated with prednisone and cyclophosphamide. Because his blood group was initially confused, several incompatible blood transfusions were given. Two types of antibody were detected: an autoantibody with "s" specificity and an alloantibody with Rh "D" specificity.

Other interesting features in this case revealed at autopsy were a viral pneumonia and *Toxoplasma gondii* infection of the brain. As far as we know, this is the first reported patient with this unusual association.

A. Mód, G. Füst, V. Harsányi, K. Natonek, A. Poros, J. Szabó, S. R. Hollán

Plasmapheresis in patients with leukaemia, multiple myeloma and immune complex diseases. Haematologia *14*, 51 (1981).

Therapeutical partial plasma exchange was performed on 9 patients with acute leukaemia, 14 patients with monoclonal and polyclonal gammopathies and 3 with primarily immune complex disease. The procedure was effective in 21 out of the 27 courses performed on patients with gammopathies and in all cases of the primarily immune complex diseases. In acute leukaemic patients the course of the disease was favourably influenced by plasmapheresis. The results show that partial plasma exchange is an effective adjunct therapy in the treatment of patients with haematological diseases.

Bo Risberg

Formation of debris in stored human blood. Haematologia *14*, 59 (1981).

The formation of debris in human blood, stored under standard bank blood conditions, was investigated by measuring debris weight, screen filtration pressure (SFP) and counting the number of aggregates. Formation of microaggregates was most pronounced in blood five to fifteen days old as was the SFP. A tendency of progressive increase was found in debris weight with storage time.

L. Kozma, I. Endrődi, V. Stenszky

Clinical relevance of cytotoxic antibodies occurring in pregnancy. Haematologia *14*, 65 (1981).

Cytotoxic antibodies were studied in 809 pregnant women's sera. Two kinds of antibody population: HLA-A, B, C and Ia-like, were detected and a correlation was established between their presence and some clinical conditions, such as preterm delivery, spontaneous abortion, pre-eclampsia, neonatal hyperbilirubinaemia and congenital anomalies. An opposite effect of HLA-A, B, C and Ia-like antibodies was observed as far as the first three parameters are concerned. On the basis of these investigations a protective role of Ia-like antibodies for the newborn can be implied.

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Calcium and Calmodulin in the Regulation of Blood Cell Functions

(A mini-review)

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National Institute of Haematology and Blood Transfusion, Budapest, Hungary

(Received September 29, 1980)

A great deal of cellular functions are regulated by changes in intracellular calcium concentration. Calmodulin, a low molecular weight cytoplasmic protein is basically involved in this regulation through a calcium-dependent interaction with various enzyme systems. The review discusses the chemical and physiological basis of calcium and calmodulin actions in the cell interior. Describes calcium-regulation of various functions in red cells, platelets and leukocytes.

1. Introduction

In the past few years calcium has been recognized to have a central role in the regulation of various cellular functions. Intracellular fluctuations in the free calcium ion concentration activate or inhibit specific enzyme systems, modify membrane events, initiate contractile and secretory mechanisms, and other cellular events (Table 1 – ref. [1]).

Table 1. (From ref. [1])

Cell functions which have been postulated or proven to be dependent on, or regulated by Ca^{2+}. Except where otherwise stated, the effect of Ca^{2+} is stimulatory, though this may depend upon the concentration of Ca^{2+} and the tissue under observation. (The distribution under different headings is necessarily arbitrary in some instances)

Membrane events
- Cell adhesion
- Platelet aggregation
- Cell communication via gap junction (inhibition)
- Membrane fluidity (phase transition)
- Membrane fusion
- Passive permeability (especially an increase in P_{K+})
- Glucose transport
- Stimulus-response coupling:
  - Receptor occupancy
  - action potentials
  - excitation-concentration coupling
  - stimulus-secretion coupling

Contractile mechanisms
- Contraction of muscle myofibrils

Events dependent on microtubules and/or microfilaments:
- motility of cilia and flagella (eukaryotes)
- motility of brush-border microvilli
- cytoplasmic motility, streaming and pseudopod formation
- intracellular transport (e.g. of pigment granules)
- cell division

Flagellar rotation (prokaryotes)
Spasmoneme contraction (peritrich ciliates)

Secretory mechanisms

Intracellular transport and discharge of:
- neurotransmitters
- histamine from mast cells
- hormones from endocrine cells
- proteins and mucus from exocrine cells

Electrolyte and water transport by secretory and absorptive epithelia

Other cell events

Activation of the egg at fertilization
Disassembly of microtubules
Photoreception
Bioluminescence
Memory storage for short-term habituation (invertebrates)

Activation or inhibition of specific enzyme systems

Pyruvate dehydrogenase phosphatase
Pyruvate dehydrogenase kinase
Isocitrate dehydrogenase
Glycerol phosphate dehydrogenase
Pyruvate kinase (inhibition)
Pyruvate carboxylase (inhibition)
Phosphofructokinase
Fructose 1 : 6 disphosphatase
Phosphorylase-b kinase, and other phosphorylation reactions
Glycogen synthase kinase-2
Some lipases and phospholipases
Prostaglandin synthesis
Adenylate cyclase (inhibition)
Guanylate cyclase
Cyclic nucleotide phosphodiesterases
Na, K-ATPase (inhibition)

Cell calcium is carefully regulated by the permeability of the plasma membrane for calcium and by energy-dependent active calcium transport processes, which maintain a low cytoplasmic calcium concentration as compared to that in the environment of the cell. Changes in cellular calcium can be evoked by various stimuli such as hormones, neurotransmitters, antigens, etc., and calcium seems to act as a "second messenger" to transform these effects into cellular responses. A recent and fascinating development in the area of "calcium-research" is the discovery of a ubiquitous, low molecular weight cytoplasmic regulatory protein, calmodulin, which seems to be involved in most types of calcium action in the cell interior. The quantitative changes in cell calcium are trans-

formed into qualitative responses by the interaction of calcium + calmodulin with the effector enzymes. The aim of the present review is to give a short summary of our present knowledge about the role of calcium and calmodulin in the intracellular regulation of blood cell functions.

2. Chemical and physiological characteristics of calcium and calmodulin

The selection of calcium ion as an intracellular regulator or "second messenger" is most probably based on the early development of the basic role of organic phosphates in metabolism. Since calcium phosphates are highly insoluble, the need for the maintenance of a low intracellular calcium concentration (10^{-6}–10^{-7} M), as compared to the much higher calcium concentrations in sea water or in the body fluids of various organisms (around 10^{-3} M), arouse with a paramount importance. As soon as special defence mechanisms had developed to keep intracellular calcium at this low level, the possibility for a delicate regulation of cell activity by changes in internal calcium concentration appeared. If a small amount of calcium (driven by its electrochemical potential gradient) enters the cell, the change in the cytoplasmic calcium concentration may amount to several orders of magnitude. In addition to this, calcium ion seems to be especially apt for a regulatory role due to its chemical characteristics. Calcium ion has a coordination number of seven or eight and the coordination geometry is irregular in both bond angle and bond length. This means that – in contrast to magnesium ion, which has six coordination in a closely regular octahedron – the adaptable calcium ion easily forms reversible cross-links between organic molecules (see ref. [2]). These cross-links are rapidly reversible to changes in conditions. In calcium binding to proteins and also to smaller molecules the ion is usually coordinated by oxygen atoms, therefore carboxylate groups of proteins have a basic role in protein–calcium interactions. In special proteins special, "high affinity" calcium binding sites develop, usually constructed from a more or less flexible "nest" of 2 – 3 carboxylate groups [2].

Cellular regulation of calcium ion concentration is based on the actual *permeability* of the plasma membrane for calcium, on *active calcium extrusion* by the plasma membrane, on active *calcium uptake* into cell organelles such as mitochondria and endoplasmic or sarcoplasmic reticulum, and on *binding of calcium* to intracellular proteins (Fig. 1).

Any *increase in the passive permeability* of the plasma membrane for calcium evokes a rapid calcium entry into the cytoplasm. This increased calcium influx may be the consequence of a specific stimulus–membrane interaction, such as the binding of a hormone to a receptor structure on the cell surface, but may also be caused by non-specific membrane injuries (Fig. 2 – ref. [3]). As a result of an external stimulus, calcium in certain cell types is released from submembrane or intracellular calcium pools.

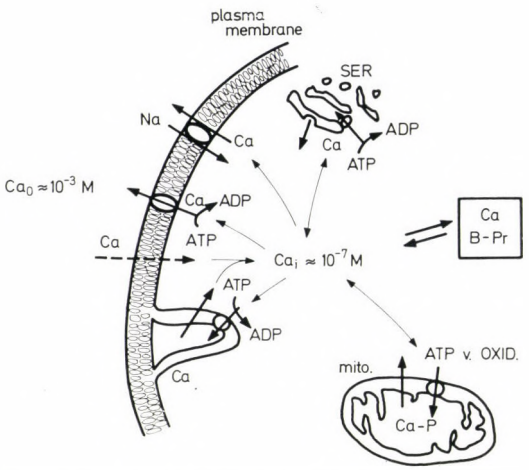

Fig. 1. Calcium distribution and calcium transport phenomena in mammalian cells. For detailed description see text. SER = smooth endoplasmic reticulum; mito = mitochondrion; Ca B-Pr = calcium binding protein

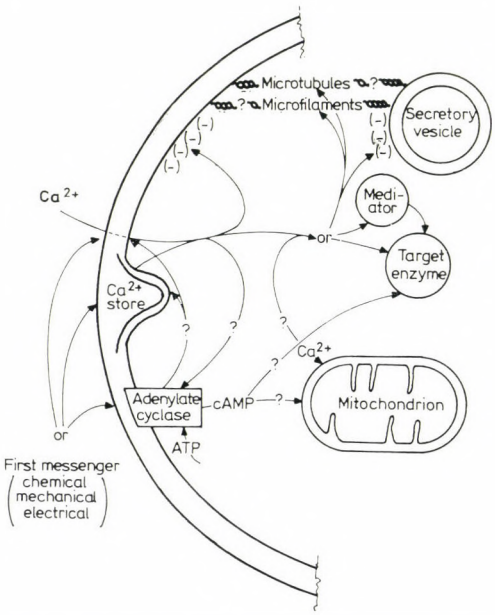

Fig. 2. Diagrammatic representation of the second messenger pathways. For detailed description see text. (From ref. 3.)

Active calcium extrusion has two basic forms in mammalian cells, the ATP-fuelled calcium pump and the Na–Ca exchange transport system [4, 5]. In the former case calcium is extruded against its electrochemical potential gradient by using the energy of the ATP → ADP + P_i reaction, that is by means of the functioning of an ATPase enzyme. This enzyme, the Ca + Mg-ATPase (magnesium is required as a "co-factor" to its function) has been proved to be identical with the calcium pump molecule. The other way to extrude calcium from the cell interior is to use the energy of "downhill" sodium movement to produce a coupled calcium efflux. Extracellular sodium concentration is much higher than in the cell interior, therefore a molecular machinery catalyzing the coupled influx of Na and efflux of calcium can work as a calcium pump. This latter mechanism is predominant in nerve and muscle cells, while human red cells have only the ATP-dependent calcium pump. In epithelial cells the two pump systems are working simultaneously and, while Na–Ca exchange is responsible for the elimination of larger amounts of inflowing calcium, the ATP-driven pump precisely adjusts the very low physiological calcium levels in the cytoplasm [6].

ATP-dependent calcium uptake by smooth endoplasmic reticulum or by sarcoplasmic reticulum (SR) in muscle has the purpose to compensate short imbalances in cytoplasmic calcium concentration, while mitochondria are capable of accumulating and sequestering (in the form of calcium phosphate) large amounts of calcium entering the cell. Mitochondrial calcium uptake can be fuelled by ATP or by substrate oxidation through some "common pool of energy" [4, 7].

The role of intracellular *calcium binding proteins*, on the one hand, is to compensate rapid calcium exposures in the cytoplasm and, on the other hand, these proteins function as calcium-dependent regulators of cellular functions [3]. The best-known types of these proteins are parvalbumins in fishes, troponin C in muscle, and *calmodulin*. This latter protein is present in most nucleated cells – from the single cell organisms to humans. Calmodulin was first described as a calcium-dependent activator of brain adenylate cyclase and phosphodiesterase [8, 9], and within a few years its function was observed in a large number of enzyme systems under calcium control. These include phosphorylase kinase, myosin light chain kinase, various protein kinases, calcium pump in the cell membrane and in SR, NAD kinase in plants, and assembly and disassembly of microtubules during cell multiplication [10].

Calmodulin has similar or the same molecular structure in all the cells it has been isolated from, thus the structure of calmodulin has been almost perfectly conserved during evolution. It is a low molecular weight, acidic, heat-stable protein which can bind four divalent cations. Three of the binding sites are highly selective for calcium while the fourth one can also bind magnesium (Fig. 3). The probable functioning cellular form of calmodulin is a three calcium, one magnesium form. The amino acid sequence of brain calmodulin is already completely known, and it shows striking similarities to parvalbumin and troponin C, indicating a common genetic origin of all these calcium binding proteins. The calcium binding configuration of these proteins is also similar – the so-called

	Ca	Mg	
$K_{d\ 1,2,3}$	0.2	140	μM
$K_{d\ 4}$	1	20	

Mw = 17 000

Pk_i = 4.3

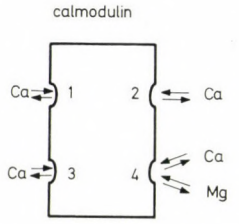

Fig. 3. Cation binding properties of calmodulin

EF hand configuration [10, 11]. It is important to mention that some neuroleptic drugs, such as trifluoperazine and chlorpromazine, preferentially bind to calmodulin and in low concentrations selectively inhibit calmodulin action in various tissues [11].

The role of calmodulin thus far has been demonstrated in calcium-induced insulin-release and intestinal secretion, in synaptic transmission, in the regulation of active calcium transport, in cell multiplication, and in fertilization of oocytes (see refs. [10–12]). The second messenger role of calcium is presumed to be quite universal, and cAMP and calcium-dependent regulation is functionally coupled by calmodulin. The molecular mechanism of calmodulin action in the various enzyme systems is not known at present, but from recent reports it is apparent that a calmodulin–calcium complex is first formed and this complex binds to a specific part of the target enzyme [10, 11].

In the past few years it has been demonstrated that calcium + calmodulin action in the cell interior is further complicated by the presence of calmodulin-binding inhibitory protein(s) [13, 14]. These proteins can counteract the calmodulin activation of several enzymes and interfere in the calcium + calmodulin regulation. Several types of these inhibitory proteins have been isolated and, based on their possibly basic role in nerve tissues, the name "calcineurin" has been suggested [15].

3. Effects of calcium in human red cells

The physiological concentration of calcium in human red cells is between 10–20 μmol/l of cells and most of this calcium is attached to the cell membrane. Thus the cytoplasmic calcium concentration is about 10^{-6}–10^{-7} M, about

three orders of magnitude smaller than that in the blood plasma (see refs. [4, 5, 16]). The factors maintaining this huge calcium concentration gradient are the low passive permeability of the membrane for calcium and the ATP-fuelled active calcium extrusion. The physiological rate of calcium influx into red cells is around 1–2 μmol/liter of cells per hour, but this rate increases by several orders of magnitude under certain pathological conditions [16]. If passive calcium influx is not entirely compensated for by active calcium extrusion, the increase in cellular calcium leads to the following alterations in the red cell functions:

a) Intracellular calcium evokes *crenation* and increased *rigidity* of the red cell membrane (Fig. 4). The biconcave discs can be transformed into crenated spheres by several drugs or metabolic changes and a similar effect is caused by increased cytoplasmic calcium. Both the optimal surface–volume ratio (assuring rapid gas transport through the erythrocyte membrane) and the plasticity of the membrane (allowing to cross capillaries smaller in diameter than the red cells) are significantly decreased under these conditions and produce a rapid elimination of such erythrocyte from circulation.

We do not know the exact molecular mechanism of these membrane alterations, but current theories emphasize the role of contractile proteins of the spectrin-actin network attached to the internal surface of the red cell membrane [17]. The presence of calcium may alter the configuration of this network or by

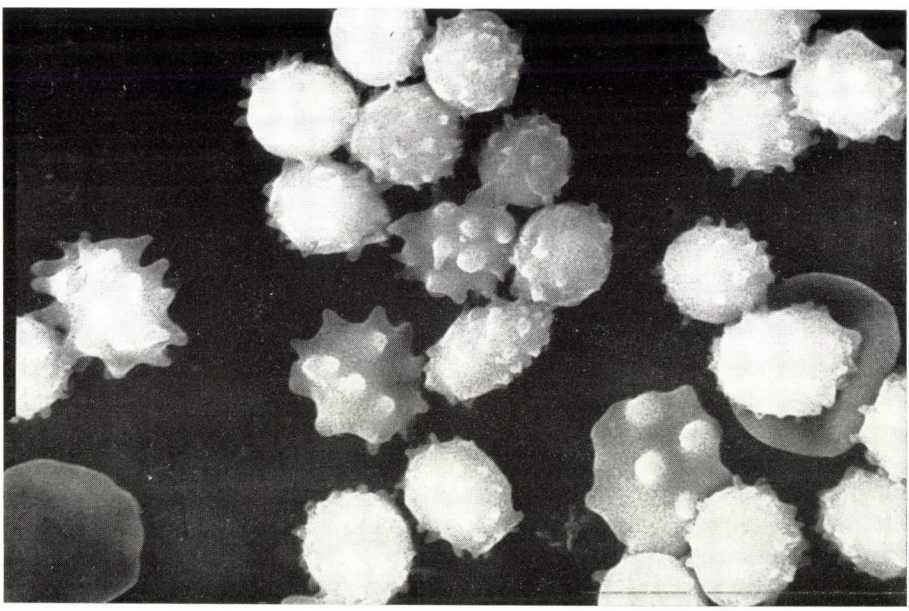

Fig. 4. Scanning electron micrograph of calcium-loaded human erythrocytes. For the methods of calcium-loading and microscopy see ref. 16

affecting protein-kinases may induce phosphorylation-dephosphorylation processes in these proteins. In irreversible shape changes, such as in irreversible sickle-cell formation, the cross-linking of the spectrin molecules by calcium-stimulated transglutaminase enzymes seems to be an important factor [18]. On the other hand, Allan and Michell [19] provided evidence for calcium-dependent changes in the lipid composition of the red cell membrane. Whether these changes induce the membrane alterations or are just concomitant phenomena, is yet to be elucidated. In a "coupled bilayer" hypothesis Sheetz and Singer [20] tried to combine the role of proteins and lipids in the red cell shape regulation. Recently Brewer [21] worked out a hypothesis for the action of calcium and calmodulin in the red cell membrane shrinkage and expansion. The model is mostly based on the action of phenothiazines and zinc ions, which both block calcium + calmodulin action in various systems and are both producing specific shape changes in red cells.

b) *Calcium-induced rapid potassium transport* (the so-called "Gárdos effect" [22]) develops in metabolically depleted, in propranolol, or calcium-ionophore treated human red cells. In all cases the rapid potassium loss and the concomitant shrinkage of red cells are evoked by the elevated intracellular calcium concentration [23]. Rapid potassium efflux results in membrane hyperpolarization and acceleration of further calcium influx [24]. The possible relation of these phenomena in red cells to molecular events during stimulation of lymphocytes and secretion processes in different cells has recently attracted much attention [25]. The role of calcium + calmodulin complex in calcium-induced potassium transport is postulated in a model by Lassen et al. [26].

c) Intracellular calcium *inhibits active Na–K transport* in red cells. This way an increase in cellular calcium blocks the active transport system which adjusts the physiological low Na–high K ion distribution in red cells [5].

The basic mechanism maintaining the physiological calcium concentration in human red cells is *active calcium extrusion*. The general characteristics of this transport system are as follows (see refs. [4, 5, 16, 27]:

The calcium pump is a strongly membrane-bound mechanism which can catalyze the extrusion of calcium ions against a 1000 – 10 000-fold concentration-gradient. Calcium at the internal membrane surface activates the system in micromolar concentrations, and this activation (the "calcium-affinity" of the pump) depends on the presence of calmodulin. The calcium pump is working as a membrane Ca + Mg-ATPase which converts the energy of ATP splitting into transport work. The molecular basis of the pump is a 140 – 150 000 dalton molecular weight protein which crosses the red cell membrane, binds cellular ATP, Mg, and calcium and produces a transmembrane movement only for calcium ions. The stoichiometry of calcium transport to ATP splitting is probably 2 : 1, or according to some reports, 1 : 1. Calmodulin regulation of the calcium pump is most probably elicited by a direct binding of calmodulin + calcium to the intracellular part of the transport molecule. This binding results in an increased transport rate and an increase in the "calcium-affinity" of the pump. The role

of calmodulin-antagonist proteins in red cell calcium transport has also been demonstrated [28].

Investigation of the characteristics of the red cell calcium pump is a difficult task as the "active centre" of the system is located at the intracellular membrane surface. Introduction of the technique of inside-out membrane vesicle preparation [29] meant an important step in such studies. This technique allows producing sealed, inverted membrane vesicles from the red cell membrane, which accomplish an ATP + Mg-dependent active calcium uptake (Figs 5, 6). The active centre

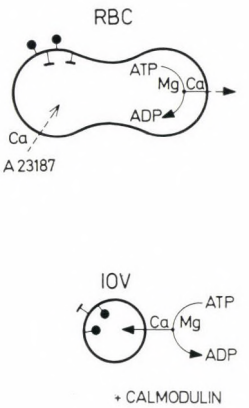

Fig. 5. Calcium transport in intact red blood cells (RBC) and in inside-out red cell membrane vesicles (IOV). For details see text

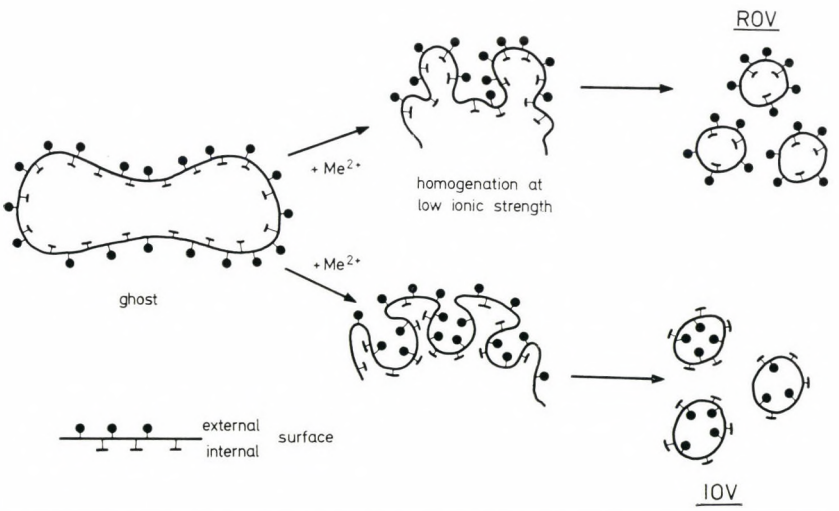

Fig. 6. Method of inside-out vesicle (IOV) and right-side-out vesicle (ROV) preparation

of the calcium pump is at the external surface in these vesicles. The effects of calmodulin and other regulatory agents on calcium pump activity have mostly been described by using inside-out red cell membrane vesicles.

4. Effects of calcium in thrombocytes

The coordinated functioning of thrombocytes is ensured by specific membrane structures. The 7–9 nm plasma membrane of the thrombocytes is covered by a carbohydrate-rich coat, the "glycocalyx" [30]. This glycocalyx is of fundamental importance in the adsorption of plasma components and in the adhesion of thrombocytes to various surfaces. The complex of glycocalycin (a water-extractable protein fraction of the glycocalyx [31]), and glycoprotein I (a protein fraction of the plasma membrane [32]) form specific receptor structure for thrombin, the most effective thrombocyte-activating plasma factor [33]. Other membrane receptors for ADP, serotonin, vasopressin, etc., are also involved in the careful regulation of thrombocyte activation, shape changes and aggregation. The activation of thrombocytes by external stimuli is based on an exocytotic "release" reaction of the internally stored ADP, biogenic amines and calcium. The intracellular storage granules are covered by membrane structures which are in close connection with the invaginations of the plasma membrane and these membranes actually fuse during the release reaction.

An electron-dense tubular system in the thrombocytes (possibly originated from the plasma membrane) accumulates large amounts of calcium by means of an ATP-fuelled, cAMP-stimulated active transport system. This system is also involved in the synthesis of prostaglandins and a natural calcium-ionophore, thromboxane A_2 [34]. The plasma membrane regulates cytoplasmic calcium concentration by an ATP-dependent active calcium extrusion [35, 36]. The specific cell shape of thrombocytes is assured by a microtubular network at the internal membrane surface. In the presence of calcium this tubulin-containing network depolymerizes into monomers and allows the actomyosin-like contractile elements to transform the cell into a spherocyte covered by membrane spicules [37].

If the capillary membrane surface is injured the activation of thrombocytes starts with a specific adhesion of these cells to the subendothelial collagen. This interaction evokes a calcium release into the cytoplasm, inducing the depolarization of tubulin network and contraction of thrombocyte actomyosin. The resulting shape change is accompanied by the release of ADP, biogenic amines and calcium from the storage granules. Aggregation of the thrombocytes and catalysis of thrombin-production by the liberated thrombocyte factors further amplificate the activation process: binding of thrombin to the membrane receptors induces prostaglandin and thromboxane A_2 synthesis, and this ionophore-like substance evokes further calcium release into the cytoplasm. All these phenomena produce a "general release reaction" and a strong contraction which is important in the retraction of the thrombus. By now it seems to be proved

[38] that liberation of intracellular calcium precedes and evokes general release reaction in thrombocytes (Fig. 7).

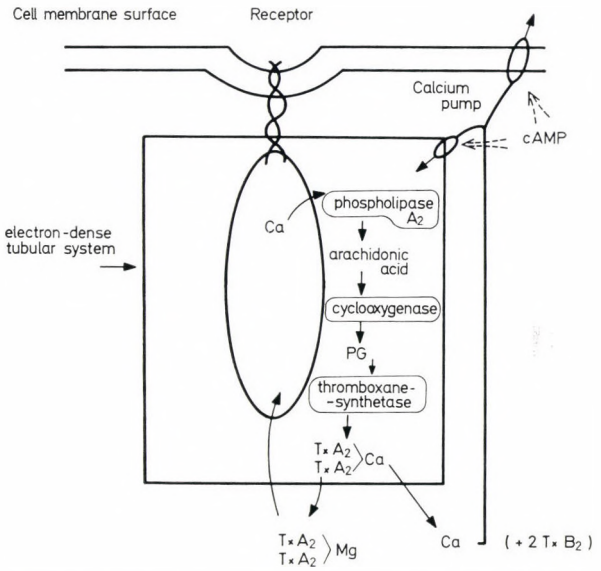

Fig. 7. Calcium fluxes during activation of thrombocytes. T_xA_2 = thromboxane A_2; T_xB_2 = thromboxane B_2

During the phase of regeneration, in thrombocytes which have not suffered an irreversible membrane damage, thromboxane A_2 is transformed into inactive thromboxane B and malonedialdehyde, or by forming a magnesium complex thromboxane A_2 is transported back to the tubular system. Prostaglandins activate the adenylate cyclase enzyme, and the cAMP formed stimulates membrane calcium pumping. cAMP-dependent activation of the calcium pump seems to involve membrane phosphorylation through protein kinase action [39]. Extrusion of cytoplasmic calcium by the plasma membrane calcium pump is the final means in restituting the original "resting" state of the thrombocytes. We do not have much information at present about the role of calmodulin and calmodulin-binding proteins in this calcium-regulated activation of thrombocytes, but the activation of adenylate cyclase, phosphodiesterase, protein kinases and the plasma membrane calcium pump by calmodulin certainly indicate a basic function of this regulatory protein in these processes.

5. Effects of calcium in leukocytes

By investigating membrane phenomena in leukocytes more and more reports deal with the changes in membrane structure and function during cell activation. It has convincingly been demonstrated that activation of phagocytes (polymorpho-

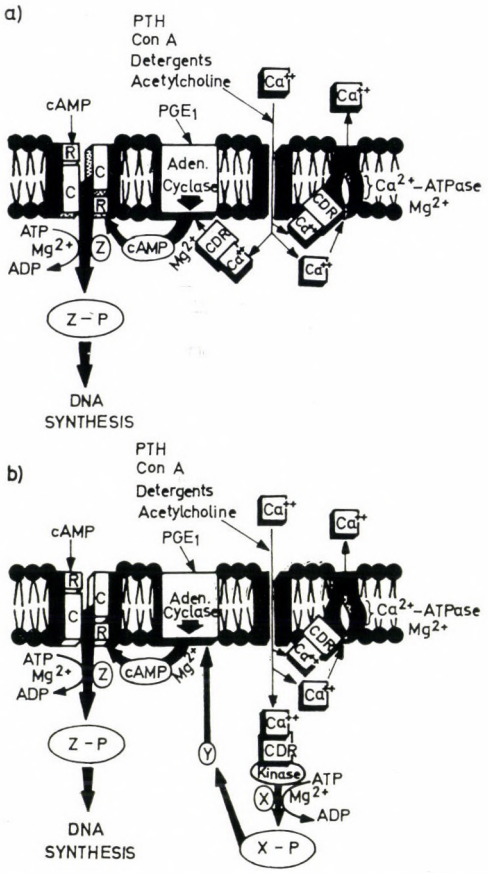

Fig. 8. Possible models for calcium-stimulation of DNA synthesis by RR lymphocytes Rapidly raising the extracellular calcium concentration, or increasing calcium influx with acetylcholine, concanavalin A (con-A), detergents, or parathyroid hormone (PTH), caused calcium ions to surge into the cell. Before the Ca^{2+}-transport ATPase in the plasma membrane could react to get rid of them, the excess calcium ions triggered the following processes. A. According to the "one-kinase" idea, the inflowing calcium ions combined with CDR (calmodulin) to form Ca-CDR complexes, which stimulated adenylate cyclase (Aden. Cyclase), and then stimulated the Ca^{2+}-transport ATPase and cyclic 3',5'-phosphodiesterase, the former of which pumped out the excess calcium ions and the latter of which limited the size and duration of the cyclic AMP surge. The endogenous cyclic AMP-attached to externally and internally accessible regulatory (R) subunits of a cyclic AMP-dependent protein kinase embedded in the plasma membrane and thereby activated the kinase's catalytic (C) subunits by releasing them from the R subunits. The released C subunits started deoxyribonucleotide syntheses directly or indirectly via a phosphorylated product, Z-P. Agents such as PGE, which stimulate adenylate cyclase directly, methyl xanthines which raise the cellular cyclic AMP level by inhibiting cyclic 3',5'-nucleotide phosphodiesterase and cyclic AMP itself all bypassed the calcium-dependent steps. B. According to the "two-kinases" idea, the incoming calcium ions first stimulated a cyclic AMP-independent protein kinase having a built-in CDR (calmodulin) subunit. A product of this kinase, X-P, started a chain of reactions leading to the stimulation of adenylate cyclase. Beyond this point, the mechanism, including the secondary stimulation of the cyclic AMP-dependent protein kinase was the same as in A. From ref. 48

nuclear granulocytes, monocytes) involves basic changes in the transport processes [40–42] and in the membrane potential [43, 44] of these cells. In the action of chemotactic factors the change in membrane Na and Ca transport seems to be a prerequisite. Both active Na–K and calcium transport have been demonstrated in all types of leukocytes investigated so far.

The most detailed studies available in this respect are concerned with the transport phenomena in mitogene-stimulated blast transformation of lymphocytes [45, 46]. It was shown that the first transport effect under these conditions is an initial increase in the rate of passive calcium influx across the plasma membrane. The presence of calcium in the phase of induction is obligatory and addition of the calcium ionophore A23187 acts as a mitogenic agent [47]. Thus the initiation of blast transformation is elicited by a transient increase in the cytoplasmic calcium concentration and by the effect of calcium on some specific target organelle. The possible model of these phenomena is presented in Fig. 8 [48], which incorporates the possible role of calmodulin and the adenylate cyclase enzyme in this process. It is important to mention, that calcium-induced potassium transport (described previously in section 3) may be involved in the initiation of cell activation [25]. Changes in glucose, amino acid, nucleoside, etc. transport develop in transformed lymphocytes much later than the early changes in calcium and potassium transport. Increase in cellular cAMP level decreases the rate of early calcium influx while cGMP increases this transport rate. This mechanism may function as a negative feed-back in cell activation. In the past few years calcium regulation in cell multiplication and the loss of calcium control in tumorogenic cells attracted much attention [48]. The more detailed description of physiological reactions in calcium regulation gives hopes for the better elucidation of the mechanism of tumour-formation.

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Adoptive Immunotherapy and Combined Chemo-Immunotherapy in Transplantable Leukaemia of AKR Mice (TAL); Occurrence of Natural Killers*

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Allo- and syngeneic lymphoid effector cells were injected I. P. into transplantable leukaemia (TAL)-bearing AKR recipients. Effector cells were collected from the peritoneal cavity, lymph nodes, spleen, or thymus, of either non-immunized donors or of donors previously immunized with TAL cells. The ratio, $\frac{\text{injected number of effector cells}}{\text{injected number of TAL target cells}}$ varied in individual experiments from 10 : 1 to 10 000 : 1. Some AKR recipients were given prior to the leukaemia inoculation a whole-body sublethal X-ray irradiation and/or an I. P. injection of cyclophosphamide.

Mean survival of TAL leukaemia was most often prolonged in recipients given viable allogeneic thymocytes derived from non-immunized donors. In some leukaemia-bearing mice treated with chemo-immunotherapy, permanent survival (> 70 days) could be observed. As a rule, effector cells derived from non-immunized allogeneic donors were more effective than effector cells from the immunized ones. Syngeneic effectors were ineffective.

The occurrence of natural killers (NK cells) to Gross virus-infected cells in the lymphoid organs of low-leukaemic inbred mouse strains is postulated. These NK cells are lacking in high-leukaemic AKR mice.

Introduction

Earlier we have shown the effectiveness of adoptive immunotherapy in L-1210 leukaemia. Lymphoid effector cells collected from allogeneic donors immunized with L-1210 cells, when injected into L-1210 ascites leukaemia-bearing mice were shown to block the tumour cells in the G_1 phase [21]. Although this transplantable animal leukaemia deserves attention as the basic experimental model for tumour therapy, its relation to human neoplasms is distant. Conversely, the spontaneous Gross virus-induced leukaemia of AKR mice shows several nosologic similarities to acute leukaemia in humans, moreover, serum antibodies against the soluble Gross virus antigen have been detected in some patients suffering from acute leukaemia [28]. This animal disease is, however, characterized by a particularly broad dispersion of leukaemia incidence. Since AKR mice fall ill between the 8th to 12th month of life, any amassment of conclusive data based on suffi-

* This work was supported by the Polish National Cancer Programme PR-6.

ciently numerous groups of animals examined in parallel is impossible. This is why transplantable forms of AKR leukaemia have been introduced. It was found that the interdependence among the immune response of tumour-bearing hosts and the leukaemia antigens remain in both the spontaneous and the transplantable form of leukaemia identical [15, 18].

In the present study, results of adoptive immunotherapy and combined chemo-immunotherapy in transplantable AKR leukaemia are presented. This leukaemia deduced in our Laboratory in 1972 from spontaneous Gross leukaemia has been called TAL (transplantable AKR leukaemia [24]).

Material and Methods

Inbred AKR mice of both sexes aged 4 to 8 months were used as recipients. TAL was inoculated I. P. by a number of 10^4-10^5 viable lymph node cells originating from syngeneic donors ill of TAL, and collected on the 13th day after leukaemia transplantation. Details were described previously [25].

Effector cells of adult inbred C57Bl/6, DBA/2J, BALB/c, or syngeneic AKR donors were obtained from lymph nodes, spleen, thymus or peritoneal cavity as described before [21]. Some allogeneic donors have previously been immunized by 4 I. P. injections of 10^7 leukaemic lymph node cells at 7-day intervals. Effector cells from them were collected on the 3rd to 5th day after the last immunization. A number of leukaemia-bearing recipients was injected with donor cells irradiated *in vitro* with 4500 r ^{60}Co gamma rays just before I. P. administration into recipients according to the method of Smith et al. [29].

Part of the recipients received on day 0 whole-body X-ray irradiation with 300 r (200 kV, 15 mA, Cu-0.5, distance 40 cm, time 2 min 30 sec). On day 1, TAL was transplanted and this was followed 4 h later by an I. P. injection of effector cells. The ratio, $\frac{\text{number of effector cells}}{\text{number of TAL target cells}}$ injected, varied in separate experiments from 10 : 1 to 10 000 : 1. Three to four h before effector cells' administration, a number of recipients was injected on day 1 with 20 mg/kg or 50 mg/kg cyclophosphamide (CY). In one experiment irradiation was applied just before the TAL transplantation on day 1, whereas CY and effector cells were injected on day 2.

Mean survival time and the standard deviation was calculated for each experimental group. Statistical significance was determined by the Wilcoxon two-sample rank sum test at $p < 0.05$. Survival longer than 70 days was arbitrarily considered a permanent survival, the results of surviving individuals being deduced from the representative mean group values.

Results

TAL leukaemia when implanted I. P. does not grow in an ascitic form, but develops from the onset as a generalized systemic disease involving the spleen, lymph nodes, liver and bone marrow [24]. TAL is susceptible to CY (Fig. 1),

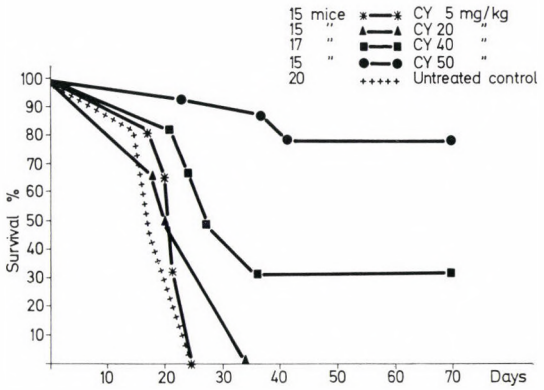

Fig. 1. Survival of AKR mice inoculated I.P. with 10^4 TAL-cells and treated I.P. with different doses of cyclophosphamide (CY) in a single injection

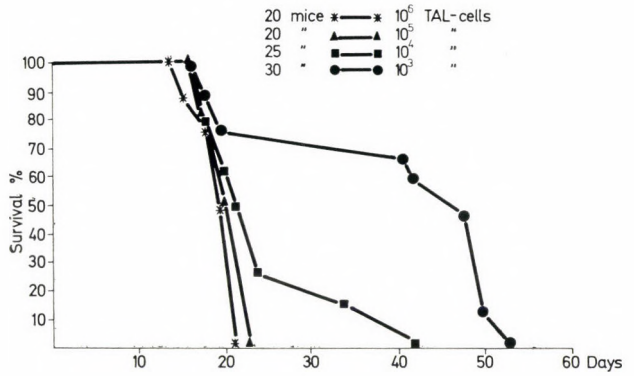

Fig. 2. Mortality rate of AKR mice inoculated I.P. with TAL-leukaemia. Leukaemia cells derived from syngeneic donors, collected from lymph nodes on the 13th day after leukaemia inoculation

thus rendering a lower dosage of this drug necessary in all regiments of combined chemo-immunotherapy. The survival in TAL is inversely related to the number of inoculated TAL cells; even with an inoculum of 10^3 cells, all animals die (Fig. 2).

With the same dose of TAL cells, mean survival of inoculated recipients differs in individual experiments. The differences have to be ascribed to fluctuation from one sample to another and the percentage of clonogenic leukaemic cells in the donors' lymph node cell suspensions used for transplantation [25]. Under these circumstances, the only reliable control is the simultaneously examined control group (Tables 1 to 4).

In intact recipients injected with TAL cells and not given a subsequent therapy, survival is longer than in the irradiated ones (see Tables 1 and 2). In intact

Table 1

Experimental group	No. of mice	Mean survival (days)
Control	19	26.1 ± 2.1
BPO	4	26.8 ± 0.5
BPI	5	27.6 ± 0.6
BLNO	4	32.8 ± 6.6
BLNI	5	26.6 ± 6.0
BSO	4	23.8 ± 4.9
BSI	5	27.8 ± 6.1 + 1 permanent survival
BTO	4	**34.3 ± 1.0
BTI	5	26.6 ± 4.0
CPO	5	25.2 ± 3.7
CPI	5	23.6 ± 7.5
CLNO	5	27.0 ± 0.5
CLNI	5	25.6 ± 2.0
CSO	5	26.6 ± 4.2
CSI	5	24.8 ± 3.8
CTO	5	**29.6 ± 0.6
CTI	5	23.6 ± 5.1
DPO	5	31.0 ± 3.3
DPI	5	26.6 ± 2.8
DLNO	5	24.2 ± 6.6
DLNI	5	22.4 ± 3.6
DSO	5	30.4 ± 3.9
DSI	5	24.6 ± 3.8
DTO	5	26.4 ± 1.9
DTI	5	24.2 ± 4.8
APO	6	25.3 ± 3.6
ALNO	5	26.4 ± 2.8
ASO	6	27.1 ± 3.0
ATO	6	25.7 ± 3.2

Survival of AKR mice inoculated I. P. with 10^5 TAL-cells followed by an I. P. injection of 10^7 effector lymphocytes derived from inbred BALB/c–(B), C57B1/6–(C), DBA/2J–(D), AKR–(A) donors.

O – non immunized donors,
I – immunized donors,
P – lymphocytes from peritoneal cavity,
LN – lymphocytes from lymph nodes,
S – lymphocytes from spleen,
T – lymphocytes from thymus.

** – difference to control, significant statistically at $p < 0.02$.
* – difference to control, significant statistically at $p < 0.05$.
Permanent survival = survival longer than 70 days.

Table 2

Experimental group	No. of mice	Mean survival (days)
Control	12	12.8 ± 1.4
PO	5	13.4 ± 0.6
PO−K	5	13.0 ± 1.2
PI	5	13.4 ± 0.9
PI−K	6	12.7 ± 1.1
LNO	5	13.0 ± 0.3
LNO−K	4	12.7 ± 0.9
LNI	5	**18.2 ± 2.1
LNI−K	5	13.0 ± 1.2
SO	6	13.0 ± 0.6
SO−K	5	13.0 ± 1.0
SI	6	12.8 ± 0.4
SI−K	6	11.8 ± 2.1
TO	5	*16.8 ± 3.0
TO−K	6	13.1 ± 1.8
TI	5	13.8 ± 0.8
TI−K	5	13.5 ± 0.9

Survival of sublethally irradiated (day 0) AKR mice inoculated I. P. with 10^5 TAL-cells (day 1) and given 3−4 h later I. P. 10^6 effector lymphocytes derived from C57Bl/6 donors.

−K = cells killed *in vitro* by 4500 r ^{60}Co gamma-rays before injection.

recipients subjected to adoptive immunotherapy with allogeneic effector cells, a statistically valid prolongation of life was observed with thymus cells derived from non-immunized C57Bl/6 and BALB/c donors, whereas a single permanent survival occurred with spleen cells derived from immunized BALB/c donors, probably a random observation (Table 1). Syngeneic effector cells have been found not to have any beneficial effect. These cells originated from non-immunized donors, since immunized AKR donors cannot be obtained even with minimal TAL inocula because of the extreme susceptibility of this strain to TAL leukaemia and the risk to inject transformed leukaemic or preleukaemic cells into the recipients.

In irradiated recipients, mean survival was prolonged when thymocytes from non-immunized donors, or lymph node cells from immunized donors were injected. Killed allogeneic effector cells were ineffective (Table 2).

Recipients given CY in a dose of 50 mg/kg survive permanently in 70% (Table 3, Fig. 1). Effector cells injected in addition did not cause any increase of the cure rate. In mice not given CY in this experiment, prolonged survival was noted with thymocytes derived from both the non-immunized and the immunized donors (Table 3).

Table 3

Experimental group	No. of mice	Mean survival (days)
Control	14	19.0 ± 4.6
Control + CY	14	35.8 ± 10.6 + 11 permanent survivals
LNO	8	31.9 ± 15.5
LNO + CY	8	24.6 ± 6.8
LNI	8	19.4 ± 7.2
LNI + CY	8	34.7 ± 14.6 + 5 permanent survivals
SO	8	22.9 ± 7.9
SO + CY	8	28.0 ± 8.2 + 4 permanent survivals
SI	8	24.3 ± 4.3
SI + CY	8	30.5 ± 11.8 + 4 permanent survivals
TO	8	**30.0 ± 11.0
TO + CY	8	33.0 ± 13.0 + 5 permanent survivals
TI	8	*22.9 ± 10.9
TI + CY	8	33.0 ± 13.0 + 5 permanent survivals

Survival of sublethally irradiated (day 0) AKR mice inoculated I. P. with 10^4 TAL-cells (day 1) followed by an I. P. injection of cyclophosphamide (CY) 3–4 h later in a dose 50 mg/kg, then 10^7 effector lymphocytes given I. P. 4 h later, derived from C57B1/6 donors.

Table 4

Experimental group	No. of mice	Mean survival (days)
Control	11	18.2 ± 6.4
Irradiated control	4	14.3 ± 6.4
Irradiated control + CY	6	20.7 ± 2.7
LNO – 10^6 cells + CY	7	23.7 ± 5.1 + 1 permanent survival
LNO – 10^7 cells + CY	5	26.0 ± 5.2 + 1 permanent survival
LNO – 10^8 cells + CY	8	23.0 ± 3.2 2 acute GVH
SO – 10^6 cells + CY	4	20.0 ± 1.4
SO – 10^7 cells + CY	4	21.3 ± 2.5 + 1 permanent survival
SO – 10^8 cells + CY	6	22.7 ± 2.8 2 acute GVH
TO – 10^6 cells + CY	9	18.8 ± 4.9
TO – 10^7 cells + CY	7	18.4 ± 10.0
TO – 10^8 cells + CY	16	21.0 ± 2.4

Survival of sublethally irradiated (day 1) AKR mice inoculated I. P. with 10^4 TAL-cells 4 h later, followed by an I. P. injection of 20 mg/kg CY (day 2), and 4 h later different doses of effector lymphocytes derived from C57B1/6 donors.

Mean survival increased, as a rule, in direct relation to the quotient, $\frac{\text{injected number of effector cells}}{\text{injected number of TAL target cells}}$ (see Table 1, 100 : 1; Table 2, 10 : 1; Table 3, 1000 : 1). It appears, however, that the optimal ratio is 1000 : 1, whilst its further rise does not bring about any benefit. On the contrary, sometimes an acute GVH reaction develops, as it could be verified at autopsy (Table 4). In this last series of our combined chemo-immunotherapy experiments, effector cells derived from non-immunized C57Bl/6 donors were injected, and the recipients have been irradiated before and subsequently given CY I. P. in a dose of 20 mg/kg, which does not ensure permanent survival (Fig. 1). Under these conditions a number of permanent survivals could be observed. In contrast with earlier experiments, thymus cells now proved to be less effective (Table 4).

Discussion

Our results point to the existence in normal C57Bl/6, DBA/2J and BALB/c donors of natural non-immune cytotoxic cells directed against the Gross virus-infected cells. These cytotoxic cells are lacking in AKR mice. This interpretation finds support in the studies published by Herberman et al. [7, 8] who in normal mice detected lymphocytes which were cytotoxic *in vivo* and *in vitro* to cells infected with oncorna C-type viruses. These cells show neither T-, nor B-markers, and do not correspond to "null-cells" or immune K-cells. A term "N-cells" was introduced in order to underline the completely different, presumably non-immune character of this lymphocytic subpopulation [5, 6, 7, 8]. Kiessling et al. reported similar findings; they introduced the term NK-cells (natural killers) [10, 11]. Naturally forthcoming killer cells were later described in rats by Shellam et al. [26, 27].

Kiessling et al. [12] demonstrated an elevated activity of the NK-cells in the C57Bl/6, CBA and DBA mouse strains, while the A, Ak, AKR and C3H strains were classified as "low reactors". This classification fits almost perfectly with the formerly known repartition into the "low" and "high" leukaemic mouse strains [31].

NK-cell activity has been bound predominantly in the spleen (Herberman et al. [7], Kiessling et al. [10, 11]). Both authors dealt in their experiments with Moloney or Rauscher virus-infected cells. In our studies with Gross virus-infected cells, active effectors were shown to be present in many lymphoid organs. The different types of virus examined (the Gross virus being the most widespread infectious agent in the mouse [16]), might be responsible for the discrepancies observed, as in our studies thymus cells were mostly active. The distribution of active cells among thymus, spleen and lymph nodes varied from one experiment to another and for the time being we cannot explain this variability.

The question arises why in our studies the immunization in donors did not bring about any beneficial effect in the therapy trials in TAL-bearing recipients, in contrast with our former investigations in L-1210 recipients [21]. The Gross

virus-infected cells possess specific surface antigens (GSCA) of the A, B and C-type [1, 17] and also antigens of the viral core which are specific for the virus type (tsVEA) and for the host strain (gsVEA) [1, 23]. An alloantigen G_{IX} [30] and an interspecies-specific antigen [23] have also been reported. The specific Gross virus antigen could be obtained in a soluble form (G-MuLVA) [3]. Surface antigens are said to induce a humoral response, which can be measured by serum cytotoxicity assay in the presence of complement, whereas structural antigens of the viral core have to induce cell-mediated immune reactions to be quantitated by the tumour rejection potential [2].

There is a possibility that the immunizing doses of TAL-cells were too small, in particular in view of the well-known weak immunogenicity of the Gross virus [3, 19]. Such an interpretation, however, does not clarify the low efficacy of the effector cells of immunized donors as compared to those of non-immunized ones. This abrogation implies a certain effect of immunization, although oppositely directed, namely into suppression. Naturally existing immune lymphoid cells in normal animals, the role of which is to kill arising clones of neoplastic cells, were described by Kedar et al. [9] and by Rollinghof et al. [20] and they have been named CTL (cytotoxic lymphocytes). It is likely that *via* immunization in donors we induced a transformation of CTLs into suppressors which hamper the activity of the NK-cells. Many other cellular or humoral mechanisms of suppression might come into play. The problem needs further investigation.

The role of the thymus in Gross leukaemia is crucial and, at the same time, mysterious. The protective effect of perinatal thymectomy against spontaneous leukaemia is well documented [4]. In transplantable AKR leukaemia in adult mice the protective effect is not so pronounced but becomes evident if small numbers of TAL-cells are inoculated [25]. Thus, one may assume that the spontaneous Gross leukaemia incidence in AKR mice depends upon genetically conditioned insufficiency of NK-cells which are inhibited by the animals' own thymus. This mechanism of a thymus-dependent suppression could have developed during ontogenesis, resulting from a durable contact with Gross virus antigens. Thymectomy done in an early period of life could lead to an unblocking. In low-leukaemic strains, on the contrary, thymic suppression might not have developed and the thymus itself, like the other lymphoid organs, might harbour abundant NK-cells. In this way both the protective effect of thymectomy and the therapeutic effect in TAL-bearing recipients of lymphoid effectors derived from non-immunized low-leukaemic donors could be explained.

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Ferritin in Cultured Fibroblasts from Patients with Idiopathic Hemochromatosis

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Cultured skin fibroblasts from three unrelated patients with idiopathic hemochromatosis and two normal controls were cultured in media containing iron concentrations ranging from 383 to 963 ng/ml. The amount of ferritin accumulating in fibroblasts was related to the iron concentration, but there was no significant difference in the fibroblasts of patients with hemochromatosis and the normal controls.

*

Idiopathic hemochromatosis is an inborn error of metabolism characterized by accumulation of large amounts of iron in the parenchyma of the liver and of many other organs [1, 2]. Since body iron is controlled by regulation of iron absorption, it is clear that over their lifetime patients with idiopathic hemochromatosis absorb increased amounts of iron. It is not known however, whether the increased iron absorption which occurs in patients with hemochromatosis represents the primary defect, or whether it is a normal response to abnormalities in body iron exchange. Study of iron metabolism in patients with hemochromatosis and in their tissues is seriously hampered by the excess amount of iron which is present. Thus, it becomes difficult to interpret whether any abnormality which is observed is the cause of hemochromatosis or its effect.

Cultured skin fibroblasts have served as a valuable model for a variety of metabolic diseases. They provide an opportunity to investigate cellular iron metabolism in patients with hemochromatosis without the perturbing effect of excess iron. Since such cells have undergone many divisions and several passages prior to investigation, their iron content at the time of study is not influenced by their iron content at the time of biopsy.

Materials and Methods

Iron saturated transferrin was prepared by storing human transferrin (Sigma), 10 mg/ml, and Fe^{+++}, 15 μg/ml ($FeCl_3$) in 0.025 M $NaHCO_3$ and 0.13 M NaCl for 48 hrs at 4 °C. Unbound iron was removed by overnight dialysis against saline and the preparation was sterilized by millipore filtration.

Skin fibroblasts from three unrelated patients with idiopathic hemochromatosis and 2 controls were grown in Eagle's minimum essential medium [3] with

20% fetal calf serum and 0.01 M HEPES buffer and with varying amounts of added iron-saturated transferrin. The medium was changed 2 times per week. After 4 weeks, the fibroblasts were removed by trypsinization and washed two times with saline. A 5–10% extract (vol/vol) prepared in saline was frozen and thawed three times. An aliquot was removed for protein determination before centrifuging the remainder at 12 000 *g* for 10 min. Ferritin levels in the supernatant were estimated by a radio-immune assay procedure (Ramco Laboratories Inc., Houston, Texas).

Results

The ferritin content of fibroblast cultures at different iron concentrations is shown in the figure. The ferritin content of normal fibroblasts and those from patients with hemochromatosis are clearly related to external iron-transferrin concentrations, but the relationship appears to be essentially the same in fibroblasts from patients with hemochromatosis as in the normal fibroblasts.

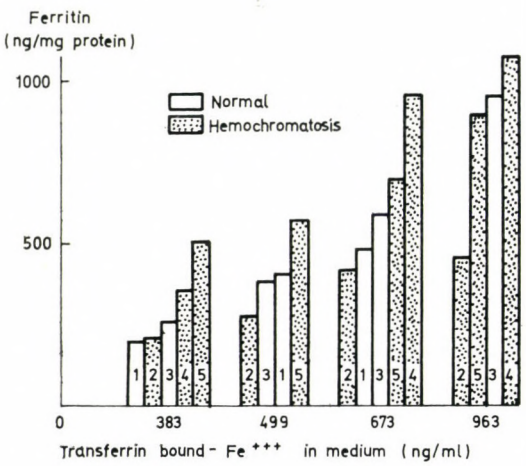

Fig. 1. The ferritin levels of fibroblasts from three patients with idiopathic hemochromatosis and from two normal controls. The cells were grown in a medium containing various added amounts of iron-saturated transferrin for a four-week period before ferritin levels were measured

Discussion

Ferritin is one of the principal iron storage compounds of the body. First described in spleen, it is now recognized to be very widely distributed in body tissues. The ferritin content of the spleen and liver of patients with hemochromatosis is greatly increased. On the other hand, investigations of monocyte ferritin in hemochromatosis revealed the levels to be normal [4].

Cultured fibroblasts provide a means for the study of iron uptake by cells of patients with hemochromatosis under circumstances in which the content of iron can be well controlled. Our investigations revealed no difference in the accumulation of ferritin by cultured cells from patients with hemochromatosis when compared with those in normal controls. If the primary defect in idiopathic hemochromatosis is expressed in cultured skin fibroblasts, then our studies would indicate that neither the transport of iron from the iron-transferrin complex nor the rate of ferritin synthesis are involved in the etiology of hereditary hemochromatosis. On the other hand, it may be that the defect of this disorder is only expressed in a limited number of cell types such as the gastro-intestinal epithelium.

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Abnormal Granulation and Crystal-like Structures in the Erythroid Cells in a Case of Multiple Myeloma

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Abnormal granules and unique crystalloid structures were observed in the erythroid cells of a patient suffering from multiple myeloma. The possible mechanism of the development of the crystalloid inclusions is discussed.

*

Crystalloid structures can be frequently found in different white blood and bone marrow cells in haematological malignancies (Auer rods in myeloblastic, promyelocytic and monocytic leukaemias, rod shaped inclusions in chronic lymphoid leukaemia and multiple myeloma). In the erythroid cells such crystalline structures have not been described either in leukaemias or in diseases affecting only the erythroid system.

This report demonstrates the presence of abnormal granules and crystalline inclusions in almost all cells of the erythroid system of a patient suffering from multiple myeloma.

Case report

A 55-year-old male (L. J.) was admitted to our Institute with the diagnosis of multiple myeloma. Clinical, X-ray (skull), laboratory and haematological investigations confirmed the diagnosis. Differential count of the bone marrow smear revealed 5% eosinophils, 2% promyelocytes, 2.5% myelocytes, 13.5% metamyelocytes, 25% neutrophils, 40% plasma cells, 12% nucleated red cells. Starch gel electrophoresis revealed a great amount of monoclonal IgA. On the fourth day after the beginning of cyclophosphamide chemotherapy the patient died with circulatory and respiratory failure.

Before chemotherapy, bone marrow was aspirated from the sternum for electron microscopy. Glutaraldehyde fixed, osmium tetroxide postfixed marrow fragments were dehydrated with ethanol and embedded in Durcupan ACM through propylene oxide. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Philips EM 300 electron microscope.

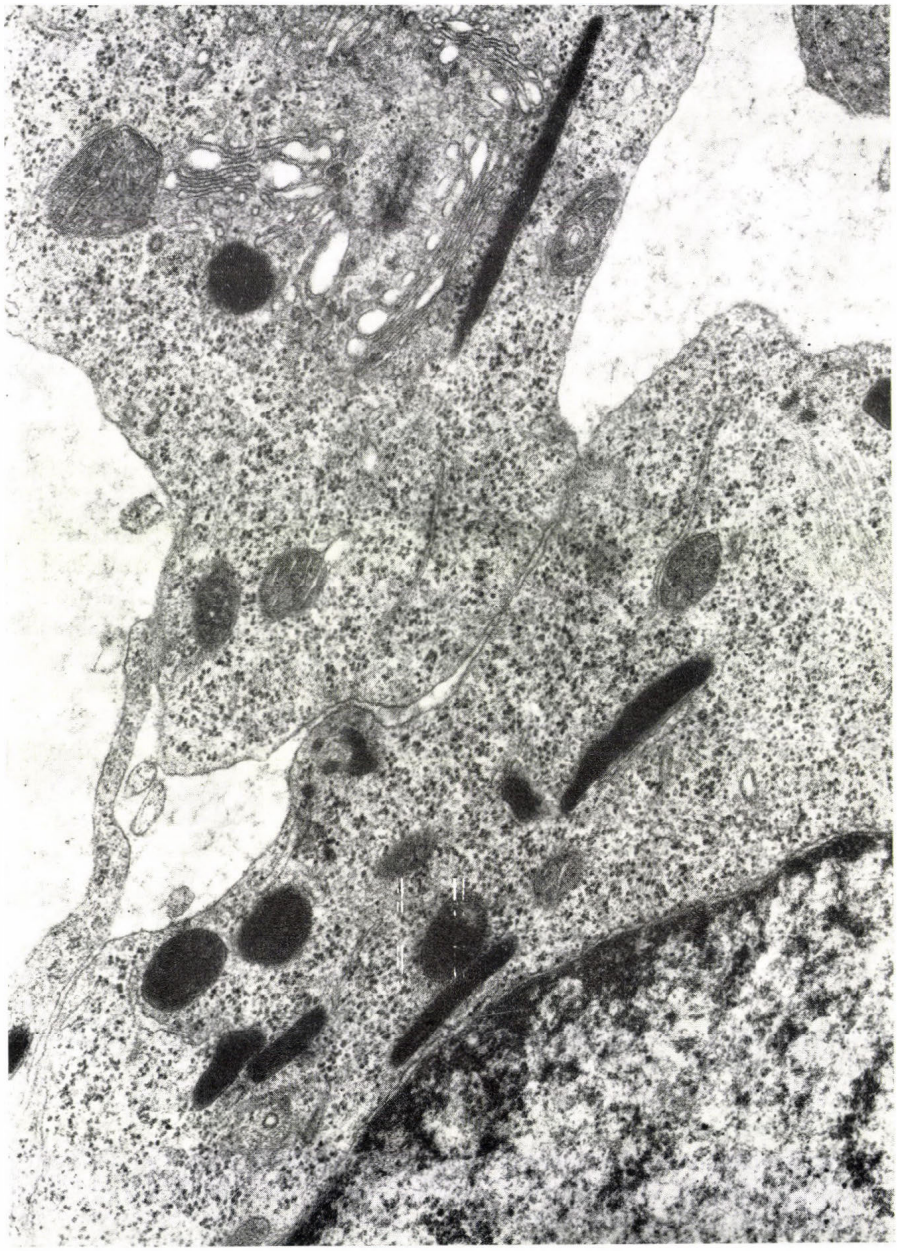

Fig. 1. Two proerythroblasts containing dense pleomorphic granules. In the cell on the upper side the granules are located around the well developed Golgi apparatus. × 32 000

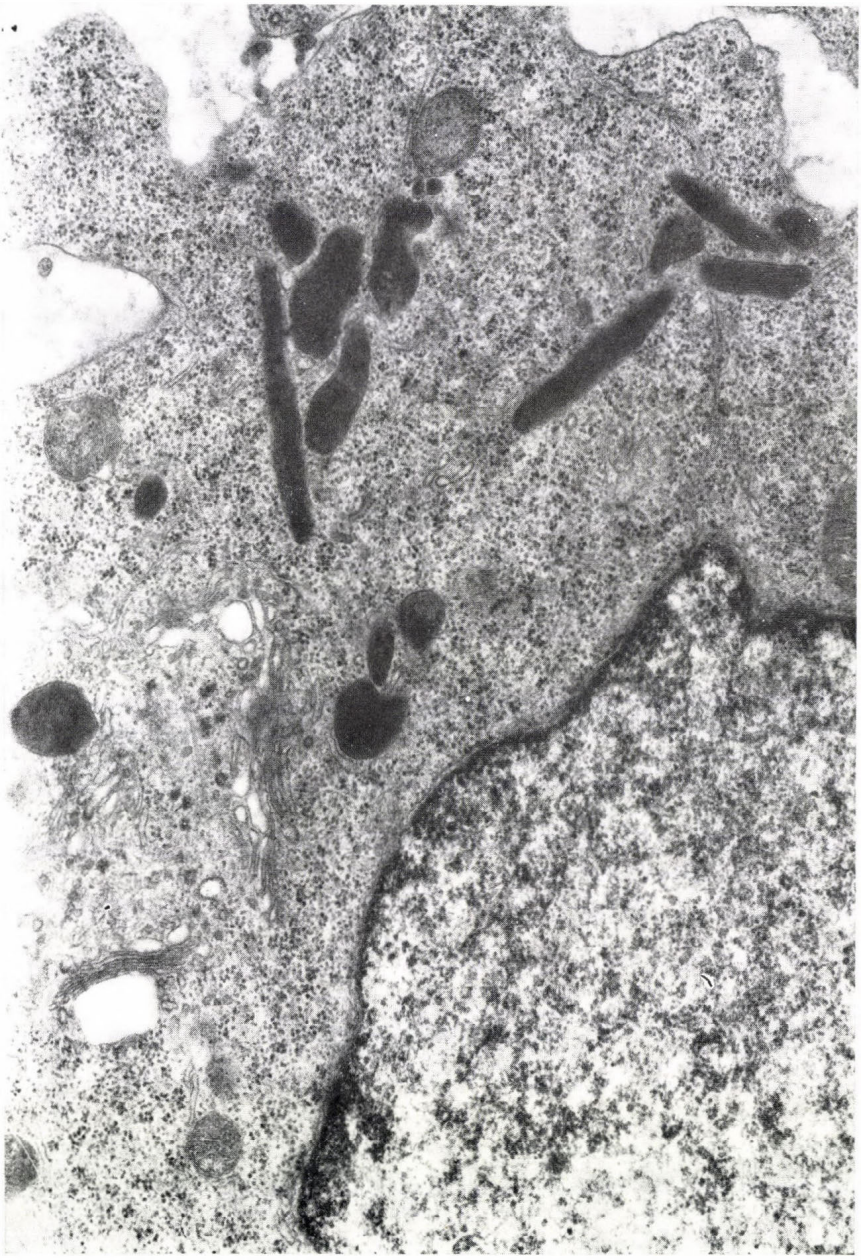

Fig. 2. Proerythroblast. In the Golgi area small dense granules can be seen, which are presumably the precursors of the large pleomorphic granules. Some elongated granules have a paracrystalline structure. × 25 000

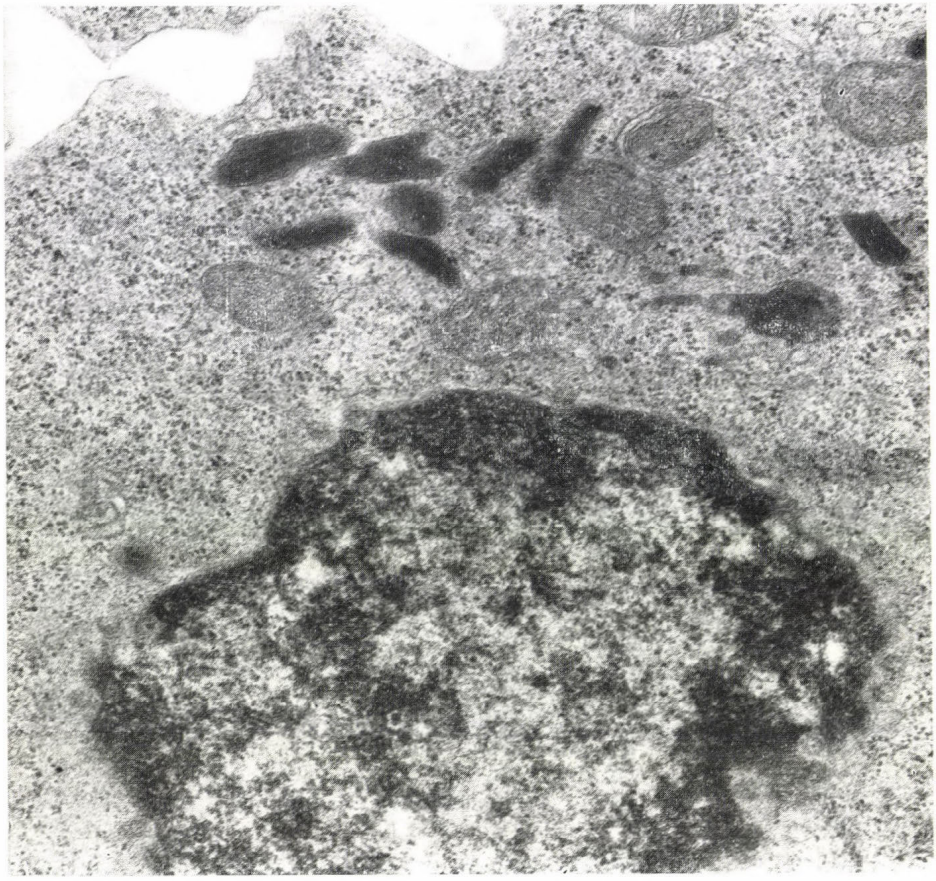

Fig. 3. Basophilic erythroblast. The paracrystalline structure of the granules is clearly visible. × 32 000

Results and Discussion

Besides the great number of typical myeloma cells the most conspicuous ultrastructural finding was the presence of irregularly shaped dense granules of different sizes and crystal-like inclusions in the erythroblasts. These structures were membrane-bound and could be found in all developmental forms of the erythroid series. Almost all erythroblasts and reticulocytes contained these inclusions. In the proerythroblasts mainly pleomorphic, sometimes elongated dense granules were found, which occasionally showed a paracrystalline structure (Figs 1 and 2). In the more mature forms (basophilic, polychromatophilic and oxiphilic erythroblasts, Figs 3, 4 and 5) the inclusions had a pronounced paracrystalline or crystalloid appearance. The inclusions were generally located in the vicinity of the well-

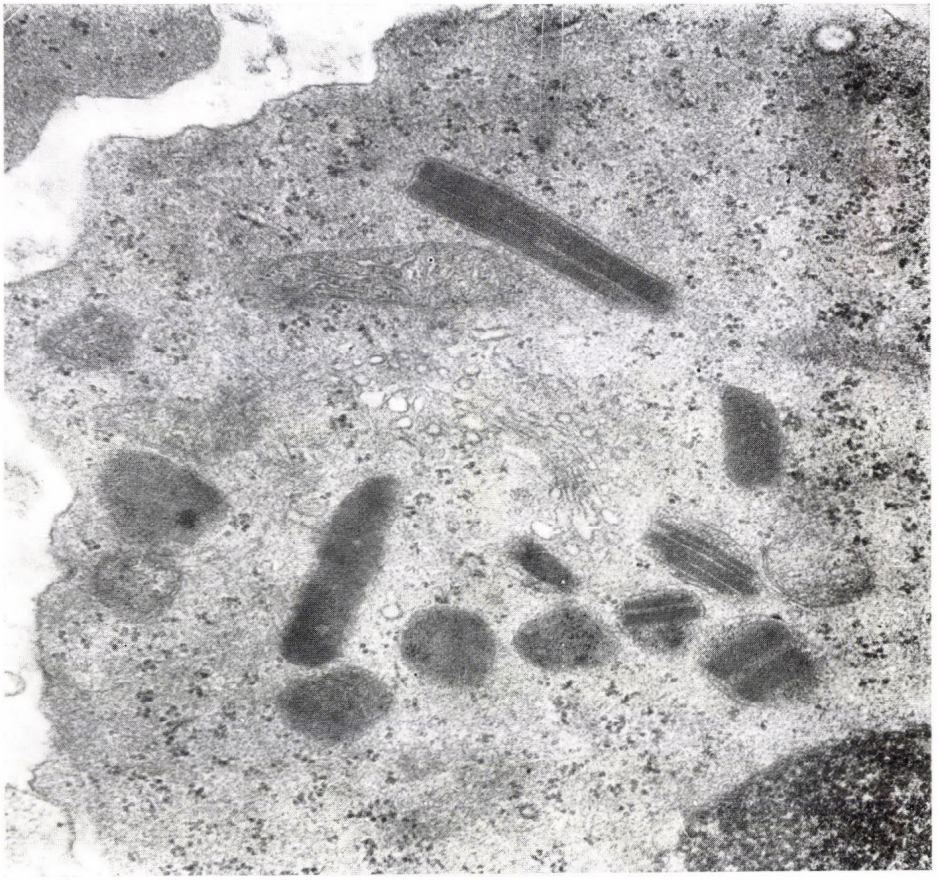

Fig. 4. Polychromatophilic erythroblast. The crystalloid nature of the granules around the Golgi complex is apparent. × 35 600

developed Golgi complex. In the Golgi areas small dense granules were occasionally seen (Fig. 2); they were considered the precursors of the large pleomorphic granules. The more mature the cell the more pronounced was the crystalloid nature of the inclusions. In the reticulocytes autophagic vacuoles containing crystalloid structures were found (Fig. 6). These autophagic vacuoles contained no ferritin molecules.

The chemical nature and the role of the described erythroblastic inclusions are not known. Sideroblastic anaemia is often associated with myeloproliferative diseases, among others with multiple myeloma [1]. Our case can be considered as a very peculiar form of dyserythropoiesis associated with plasmocytoma, characterized by persistence, multiplication and crystallization of granules, which normally occur in limited numbers in the proerythroblast. Since the patient was not treated with cyclophosphamide before bone marrow aspiration, no relationship

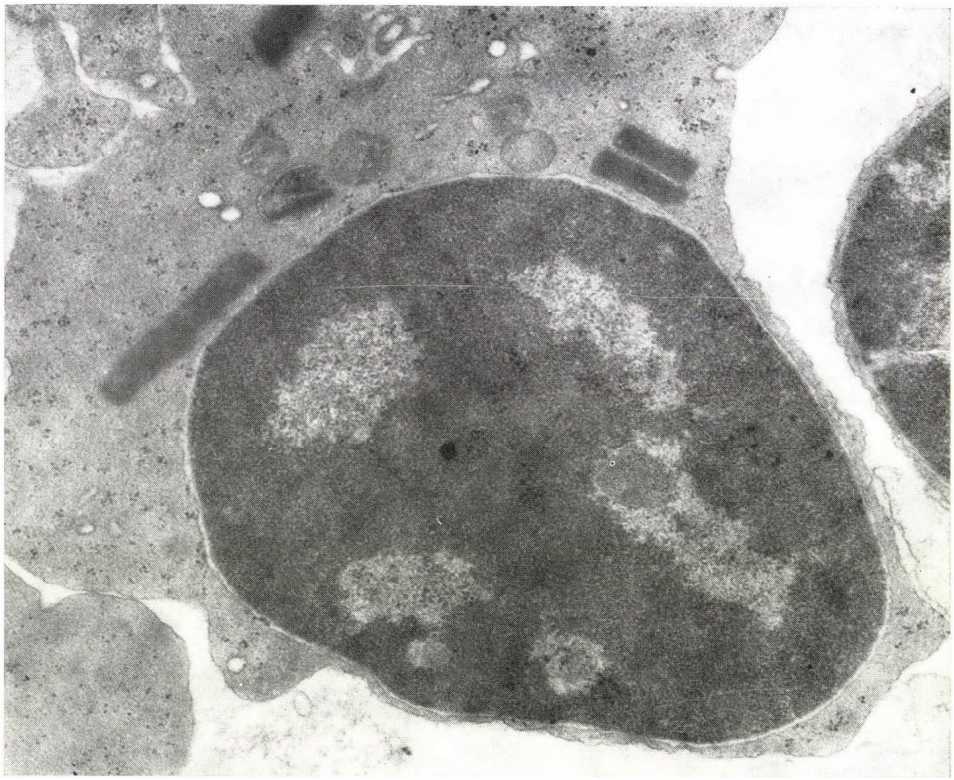

Fig. 5. Crystalloid inclusions in an oxyphilic erythroblast. × 24 000

can be supposed between chemotherapy and the inclusions. It is also possible that the coexistence of the described dyserythropoiesis and the plasmocytoma is an unrelated, accidental event. Thorough light and electron microscopic cytochemical investigations of further cases are necessary to elucidate the nature of the described erythroblastic anomaly.

Fig. 6. Crystalloid containing autophagic vacuole in a reticulocyte. × 60 000

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Transplantation of Bone Marrow Cells with Changed Haemoglobin Type

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Haemoglobin was used as a marker for the detection of radiation chimera in rats.

After a single blood loss, the synthesis of haemoglobin type had changed, as observed in peripheral blood one week after bleeding. After one month the haemoglobin pattern was the same in the experimental rats and in the control animals.

Twenty-four hours before bone marrow transplantation the donors were bled. Haemoglobin synthesis had changed also in the recipients. In contrast with a single blood loss, this change of haemoglobin type persisted as observed one year after transplantation. The cells with changed haemoglobin synthesis can repeatedly be retransplanted.

*

The result of haemopoietic stem cell transplantation to irradiated recipients is a radiation chimera characterized by the coexistence of genotypically different cells of two individuals in the recipient.

The presence of chimera cells in the recipient can be demonstrated in various ways. The most common method is the cytogenetic one [1, 16], but many biochemical markers can also be used [3, 4, 7, 15]. Makinodan [5] demonstrated the presence of rat haemoglobin in irradiated mice.

The duration of the radiation chimera depends on many genetic, immunologic and environmental factors. In many cases the donor cells are later eliminated and the recipient's own pluripotent stem cells repopulate the haemopoietic organs [6, 9]. In lethally irradiated rats, Brdička [2] demonstrated the presence of radiation chimera which lasted 3 months.

In the present work haemoglobin was used as a marker to detect the radiation chimera in rats. The aim was to find out how long the radiation chimera lasted. It was also attempted to transplant the cells of the chimera evoked by transplantation of the bone marrow cells of bled rats. In previous experiments [10] it was shown that bleeding of the rats followed by a strong erythropoietic stimulation changes the haemoglobin spectrum in the bone marrow cells. The change is manifest 12 to 24 hours after bleeding and in peripheral blood at 7 to 10 days after blood loss.

The experiments revealed that the cells with changed haemoglobin type survive in recipients for a long time.

Material and Methods

The experiments were performed on male Wistar rats weighing 200 to 220 g. Two inbred strains were used, rats of type A (4 main haemoglobin fractions) and rats of type B (3 main haemoglobin fractions) (Fig. 1). Genetic analysis of these two inbred strains was done by Brdička [2]. The chain composition of the haemoglobin types of these strains was described by Trávníček [13].

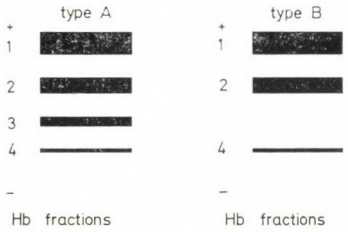

Fig. 1. The main types of rat haemoglobin (starch gel electrophoresis, pH 8.6)

Donors (type A) were bled 24 hours before the collection of bone marrow cells. The blood loss represented one third of the circulating volume. The rats were killed by decapitation, the femur was removed and after collection of bone marrow a suspension of bone marrow cells was prepared. The cells were suspended in sterile Parker's medium pH 7.4 [8]. The number of nucleated cells was estimated.

The bone marrow cells were transplanted intravenously in a dose 6×10^6 (0.5 ml) per rat. Transplantation was performed 3 hours after irradiation of the recipients (type B rats).

In some experiments in which the duration of the chimera was studied, a cell suspension separated in Ficoll gradient and bovine serum albumin [11, 12] was used. The bone marrow cells were separated into four fractions and fraction 3 contained concentrated stem cells. This fraction was administered to the recipients in a dose of 6×10^6 cells (0.5 ml/rat).

The recipients were irradiated with 850 rad, using ^{60}Co source at a dose rate of 22 rad/min.

Haemoglobin types were estimated by gel electrophoresis according to Trávníček et al. [14]. The sample of blood for electrophoretic analysis was gained from the tail vein.

Results

Twenty recipient rats of type B were irradiated with a dose of 850 rad and 3 hours after irradiation the suspension of the third cell fraction was administered. Analysis of haemoglobin types was performed on the 14th, 21st and 28th day

after transplantation, then at monthly intervals for a year. The experiment was terminated 370 days after irradiation and transplantation.

The chimera could be demonstrated in a fortnight in 14 out of 20 rats (70%). Four rats with radiation chimera continued to live till the end of the 12th month and the change in the haemoglobin type (elevation of the 3rd and 4th fraction) could still be detected (Table 1).

Administration of unfractionated bone marrow suspension also led to the formation of radiation chimera. Table 2 shows that it was possible to transplant

Table 1

Percentage of haemoglobin fractions in experimental animals one year after transplantation

Main Hb fractions	Control animals (type A)	Control recipients (type B)	Chimera (recipients-type B) one year after transplantation
1	52.5 ± 3.1	52.5 ± 2.2	37.5 ± 5.2
2	19.0 ± 2.7	22.8 ± 2.5	22.2 ± 2.4
3	16.5 ± 3.1		22.8 ± 2.5
4	10.7 ± 3.6	24.7 ± 2.3	17.2 ± 2.7

repeatedly bone marrow cells from this chimera and that the cells with elevated haemoglobin fractions 3 and 4 could repeatedly be retransplanted (Fig. 2).

In our previous experiments [14], in rats after blood loss and erythropoietic stimulation the third and fourth haemoglobin fractions were elevated. These changes were only quantitative, no new haemoglobin type was present. Maximum changes were shown to occur between the 7th and 10th day. After 28 days the haemoglobin fractions showed no change (Fig. 3). After transplantation of cells with changed haemoglobin synthesis after bleeding, quantitative changes in the haemoglobin fractions could be detected even one year after transplantation

Table 2

Percentage of haemoglobin fraction in control and experimental animals

Main fractions	Control animals (type A)	9 days after blood loss (type A)	Control recipients (type B)	Chimera 35 days after transplantation	Chimera 35 days after 1st retransplantation	Chimera 35 days after 2nd retransplantation
1	52.5 ± 3.1	41.4 ± 4.1	52.5 ± 2.2	38.5 ± 5.8	39.0 ± 2.2	38.4 ± 3.0
2	19.0 ± 2.7	23.2 ± 1.3	22.8 ± 2.5	22.1 ± 2.4	22.2 ± 2.8	22.1 ± 2.5
3	16.5 ± 3.1	20.1 ± 2.3		21.8 ± 2.9	21.3 ± 2.9	21.6 ± 2.8
4	10.7 ± 3.6	15.4 ± 2.6	24.7 ± 2.3	17.1 ± 2.7	17.1 ± 2.2	17.2 ± 2.5

(Fig. 4) and further retransplantations had no effect on the observed changes. Restoration of the normal haemoglobin pattern as observed in normal rats 28 days after bleeding, was never observed in transplanted rats.

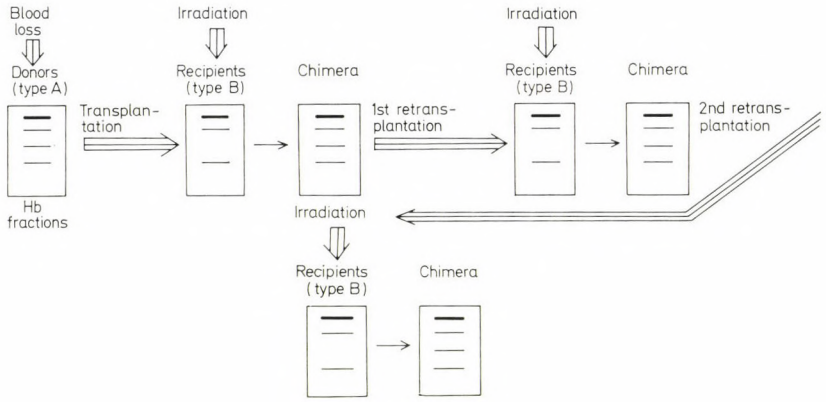

Fig. 2. Scheme of the experiments

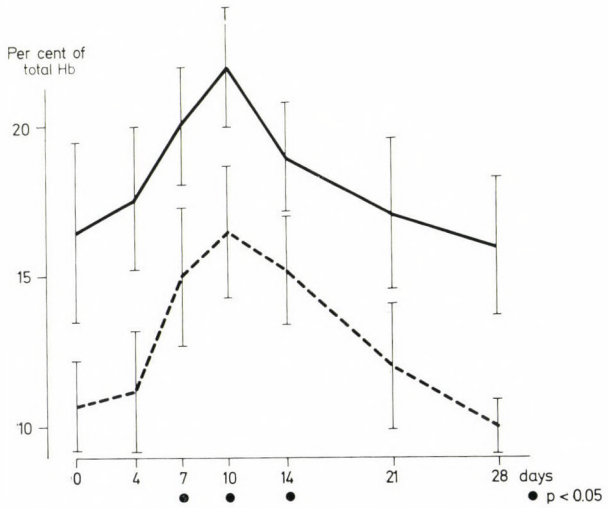

Fig. 3. Amount of 3rd and 4th haemoglobin fractions in rats after a single blood loss. ——— 3rd fraction; — — — 4th fraction

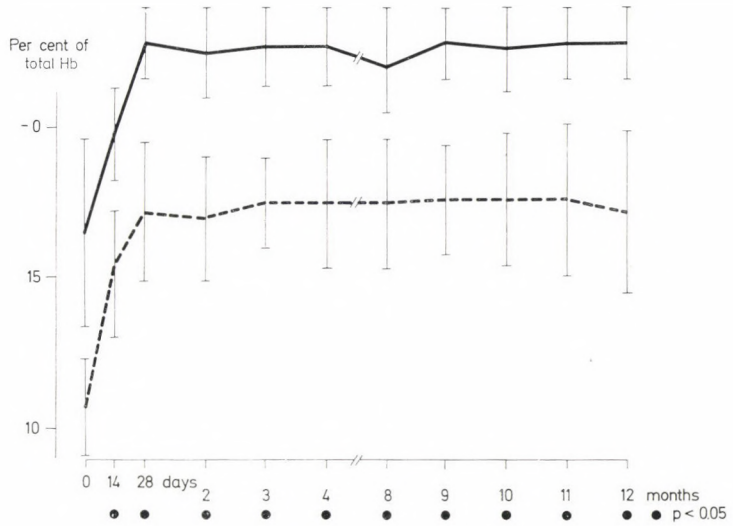

Fig. 4. Amount of 3rd and 4th haemoglobin fractions in recipient rats after retransplantation of bone marrow suspension with changed haemoglobin type. ———— 3rd fraction; — — — 4th fraction

Discussion

The results have shown that with the help of a haemoglobin marker it is possible to detect a radiation chimera in rats for more than a year. The changed haemoglobin type could repeatedly be retransplanted. The haemoglobin types of the recipients were the result of the presence of transplanted stem cells. Only about 1% of recipient stem cells survived after irradiation.

An interesting observation was the permanent elevation of the 3rd and 4th haemoglobin fractions after transplantation of donor cells of rats which had been subjected to erythropoietic stimulation by bleeding. In normal rats the elevation of the 3rd and 4th fractions disappeared in 28 days which indicated that cell population with normal haemoglobin synthesis overgrew the cell population after bleeding. This was not, however, the case with radiation chimeras and even after retransplantations there was no tendency to normalization. At present we have no explanation for this phenomenon.

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HLA Markers in Patients Suffering from Aplastic Anaemia

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135 patients, suffering from aplastic anaemia (AA) and their families were genotyped for HLA. The antigen and haplotype frequencies were compared to an HLA genotyped control panel composed of 209 normal couples and their healthy offsprings, and to another series of 2286 normal individuals. An excess of HLA-A2 was observed in the patients: 61% versus 42% ($pc < 0.001$) (relative risk: 2) and versus 48.5% ($p < 0.01$) in two control series, respectively. When considering the HLA-A, B antigens shared in common by the parents of the AA patients, an excess of HLA-A2 was observed: 32% as compared to 17% shared by normal couples ($p < 0.001$).

An excess of homozygous HLA-A2 was noted in the AA patients (14%) in comparison to the normal controls (4%) ($p < 0.001$).

The mechanism of this association is discussed as well as the hypothesis of a gene involved in haematopoiesis which might interact within the HLA-A region.

Introduction

Among the HLA disease associations which have been reported (see review by Dausset and Svejgaard [6]), aplastic anaemia has been the object of several studies. Terasaki et al. [15] examining a series of 57 AA patients, noted a decrease of A1, B8 antigens, and conversely an increase of A2, B12 antigens. Albert et al. [1] reported an excess of HLA-A2 antigens in a series of 200 patients, in particular for haplotypes A2, Bw22 and A2, B40. In a previous series of 68 AA patients, we observed an excess of HLA-A2 and of A2 homozygous patients, in comparison with a control series of 200 genotyped families [4]. More recently, Werner-Favre et al. [16] have drawn attention to the fact that an excess of antigens shared in common by both parents of AA patients was observed in their series of 34 families. The purpose of the present study is to reexamine HLA antigen frequencies and segregation, particularly HLA-A2, in a large series including most of the previously reported cases, taking a variety of parameters into consideration: sex, age, aetiology and severity of the disease.

Material and Methods

Patients

135 AA patients as potential bone marrow transplant recipients were referred to a single centre (Bone Marrow Transplant Unit, Hôpital Saint-Louis).

They were recruited prospectively from 1968 to 1980. All patients as well as their families were HLA typed in order to establish their genotype. Non-Caucasian patients were excluded from the study; the criteria for aplastic anaemia were a pancytopenia associated with decreased cellularity on marrow smears and biopsies. Patients with leukaemia, preleukaemic state, myelofibrosis, chemotherapy or radiation-induced aplasia were excluded. Patients with Fanconi anaemia or other forms of constitutional aplastic anaemia were also ruled out. In the present study, a strongly biased selection of patients related to age and severity of the disease were observed in relation to the proposed treatment, i.e. bone marrow transplant. This explains why 118/135 patients had less than 20 000 µl reticulocytes, less than 500 µl granulocytes and less than 20 000 µl platelets. In addition 125/135 patients were under 30 years of age. The aetiology was hepatitis in 15 cases, toxic in 7 cases and idiopathic in 113 cases. Ninety-one patients were male and 44 female. They were treated either by bone marrow transplantation [8, 9] or antilymphocyte globulin [10] or androgens [7]. The diverse nature of such treatments as well as the shortness of some series, has made a comparison between HLA-typing and follow-up very difficult.

HLA-typing

Twenty-seven HLA alleles at the A and B loci were tested by the lymphocytotoxicity test [11] and in most cases also by complement fixation on platelets [3]. In some cases, HLA-typing was up-dated using more recent split sera owing to the possibility at our disposal of retesting frozen lymphocytes stored in liquid nitrogen; gene counting, haplotype assertment and HLA relationships regarding normal siblings, were carried out for each family. All healthy siblings were available for typing, as well as all parents, with the exception of 11 cases in which only one parent was typed.

Special attention was drawn to the frequency of homozygotes, as well as to the antigens shared in common by both parents among the patients and in the control series. Regarding HLA-A2 transmission, three types of informative families were established: those in which one of the two parents carried A2 only once, another in which A2 was present once in each of the two parents, and lastly, those in which one of the parents was homozygous and the other heterozygous for HLA-A2 (families type I, II and III in Table 3). Only the families for which there was no doubt as to the single or double gene dose of A2 were retained.

Statistics

The comparison between the AA and control series was obtained using the χ^2 test on a two by two basis. The resulting p value was corrected (pc) multiplying it by the number of tested comparisons, with the exception of those which had previously been observed in another series. The relative risk was calculated according to Svejgaard et al. [13].

Two control panels were studied. The first included 209 families [5], the 418 parents of whom were studied for HLA phenotype frequencies as well as for the antigens which both of them shared in common. A second panel was used for comparative purposes; it consisted of 2286 normal subjects originating from six French provinces and considered to be representative of the entire country [14]. The second panel was studied particularly for the phenotype frequency of HLA-A2 which has been dealt with in a previous study.

Table 1

HLA and aplasia
Phenotype frequencies of HLA antigens

	Patients (N = 135)		Controls (N = 418)		χ^2	Corrected p value
	N	per cent	N	per cent		
Locus A						
1	33	24.4	97	23.2		
2	82	60.7	176	42.1	14.2	0.001
3	33	24.4	101	24.1		
9	21	14.8	115	27.5	8.9	NS
10	13	9.6	54	12.9		
11	10	7.4	51	12.2		
28	8	5.9	25	5.9		
29	9	6.6	43	10.3		
w30+w31	11	8.1	53	12.6		
w32	10	7.4	27	6.4		
w33	2	1.4	8	1.9		
Blank	4	2.9	16	3.8		
Locus B						
5	29	21.4	64	15.3		
7	38	28.1	77	18.4	5.8	NS
8	23	17.0	68	16.2		
12	33	24.4	124	29.6		
13	5	3.7	24	5.7		
14	15	11.1	49	11.7		
15	13	9.6	42	10.0		
w16	5	3.7	28	6.6		
17	5	3.7	30	7.1		
18	20	14.8	47	11.2		
w21	9	6.6	30	7.1		
w22	6	4.4	22	5.2		
27	15	11.1	33	7.8		
w35	18	13.3	79	18.8		
37	2	1.4	4	0.9		
40	10	7.4	41	9.8		
Blank	8	5.9	25	5.9		

Results

As shown in Table 1, the only significant modification of the observed phenotypic frequency of the HLA antigen was an increase of A2; 60.7% in the AA patients versus 42.1% in the control series (pc < 0.001) [5]. A slight decrease of A9 as well as an excess of B7 did not appear to be significant. When the HLA-A2 frequency was compared to that of the second control panel (48.5%), the level of significance was only 0.01 (pc = NS).

When the haplotypes were counted, it was found that the increase of A2 resulted in an excess of the haplotypes A2-B5, A2-B12, A2-B27 and A2-B18, in comparison to the control haplotypes. They did, however, reach a statistically significant level, with the exception of A2-B27 (4.2% versus 0.9% in the control families) ($p < 0.01$). The other haplotypes were normally distributed. No particular change was observed for the phenotype frequencies of the antigens, notably A2, in the various categories according to the severity of the AA, the evolution and the sex parameter although the significant excess of males among the patients (67%) ($p < 0.001$) has been recalled. As for the genotypes, an excess of homozygotes was noted in the AA patients concerning the antigens of the A locus, as compared to the controls (19% versus 6%) ($p < 0.001$) and especially the A2 antigen (14% versus 4%) ($p < 0.001$). The same was the case for the B locus (8% versus 2%) ($p < 0.01$). Only in 2 cases were the patients homozygous for an entire haplotype. When comparing both parents as to the antigens which they shared in common, an excess of A2 could be noted: 32% of shared A2, versus 17% in the normal couples ($p < 0.001$). This can be explained by the fact that

Table 2

Antigen shared in common by both parents

Antigen in common	AA patients		Controls		
	no.	per cent	no.	per cent	
−0:	54	42	97	46	
−1:	46	36	86	41	
for locus A	35		52		
for locus B	11		34		
−2:	24	19	24	11	NS
both for locus A	0		0		
both for locus B	2		1		
Haplotype trans	10		16		
Haplotype cis	12		7		
−3:	2	1	2	1	NS
−4:	0		0		
Total	126		209		
HLA-A2 in common	40	32	36	17	$p < 0.001$

an excess of A2 was found in each of the two parents, but was significant only in the father (58% versus 42% in the controls).

Details concerning common antigens shared by both parents are shown in Table 2. It can be noted that only the presence of A2 in both parents appears to be significant.

Particular attention was given to the study of the segregation of A2 in some informative families (Table 3). In contrast, in 53 informative AA families in which HLA-A2 was present only once in one of the two parents, a very slight increase of segregation of HLA-A2 was observed in AA patients (62% versus 47% in their normal siblings), without reaching the level of significance.

No significant differences were observed in the expected or observed frequencies of HLA-A2 transmitted either to AA or normal siblings.

Discussion

The increase of HLA-A2 among the AA patients was quite moderate (RR = 2), but significant statistically ($p < 0.001$). The A2 frequency was 63% in the 68 patients of the previous material of the same department, and 60.7% in the present series which included most of the former series, and 59% in the 107 cases which have been added.

Of the three earlier studies, one noted an increase of one haplotype bearing A2 [15], and two observed an excess of A2 (16.1%); only the latter difference was significant ($p < 0.001$) (60.7% of A2 in the patients versus 50% in the controls). The choice of controls is quite important, at least as far as their geographic origin is concerned, since frequency of HLA antigens vary from one region to another, even in a country such as France. It should be noted that, in the present study, the patients as well as the controls originated from all parts of the country. Furthermore, when comparing the A2 frequency in the patients (61%) to that of A2 in 2286 individuals from 6 different regional panels [14] (frequency = 48.5%), the difference, although less obvious, remained significant ($p < 0.01$). The excess of A2 homozygotes in the patients was only the consequence of the excess of A2 shared in common by the two parents. Nevertheless, in our series A2 was the only antigen shared in excess by the parents of the patients and not the other antigens of the A and B loci as has been reported by Werner-Favre et al. [16].

The fact that there is an excess of A2 in each of the two parents brings up the question as to whether or not they are inbred. According to the surveys and conversations with the parents of the patients, they did not appear to be from the same family or the same geographical area. Thus, there remains the hypothesis of a gene which may be involved in haematopoiesis regulation and which might interact with the gene located in the HLA-A region. Although such a gene is not definitely specified in mice, it may be similar to the gene situated in the locus Hh-1 (located near the H2-D end of the H2 system, equivalent to the A locus of HLA) which controls differentiation of the haematopoietic cells. This gene in double dose favours the bone marrow graft rejection by the F1 hybrid and tends

Table 3

Informative families for A2 segregation in AA and normal families

Family types	I $\frac{2}{X} \times \frac{X}{X}$ *		II $\frac{2}{X} \times \frac{2}{X}$			III $\frac{2}{2} \times \frac{2}{X}$	
Number of informative families	53		23			11	
Total number of children	197		79			44	
Sibling genotypes	$\frac{2}{X}$	$\frac{X}{X}$	$\frac{2}{2}$	$\frac{2}{X}$	$\frac{X}{X}$	$\frac{2}{2}$	$\frac{2}{X}$
AA patients							
observed	33 (62%)	20 (38%)	7 (30%)	9 (39%)	7 (30%)	8 (72%)	3 (28%)
expected	26 (50%)	26 (50%)	5 (25%)	11 (50%)	5 (25%)	5 (50%)	5 (50%)
Healthy sibs							
observed	68 (47%)	76 (53%)	12 (21%)	32 (58%)	12 (21%)	15 (45%)	18 (55%)
expected	72 (50%)	72 (50%)	14 (25%)	28 (50%)	14 (25%)	16 (50%)	16 (50%)
Number of control families	83		32			3	
Total number of children	272		98			11	
observed	139 (51%)	133 (49%)	26 (26%)	45 (46%)	27 (28%)	8 (72%)	3 (28%)
expected	136 (50%)	136 (50%)	24 (25%)	49 (50%)	24 (25%)	5 (50%)	5 (50%)

* 2 = HLA, X = other expressed antigen (non HLA-A2, no "blank"). 3 types of informative families (I, II, III) for the segregation of HLA-A2 are shown. The observed differences are not significant.

to be active in a recessive process. One may imagine among the different possible factors inducing aplastic anaemia, one susceptibility gene different from A2 but in disequilibrium with the gene coding for A2, in a single dose in each parent and transmitted in the patients as homozygotes. The penetrance of the disease in such a high-risk background is, however, low since many HLA identical siblings of the proband are apparently healthy.

The mechanism of the defect remains debated: it may be either a defect of maturation of haematopoiesis and/or autoimmune inhibition. Recent data have reinforced the latter hypothesis [2, 12]. There is no doubt that the true mechanism of the disease is multifactorial: HLA-A2, homozygosity for A2, and male sex can reasonably be considered to represent higher risks of susceptibility to aplasia. Further extensive studies are required in order to determine other potential genetic and/or environmental factors.

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Blood Transfusion Therapy in the Newborn

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This review deals with the various indications, the choice of blood products and the main aspects of their administration for transfusing neonates. Some special problems peculiar to neonatal age, that both neonatologists and blood transfusion services have to take into account, are emphasized. Exchange transfusion in the procedure most frequently used in blood transfusion therapy of neonatal hyperbilirubinaemia of various aetiology, severe anaemia and hyperviscosity due to polycythaemia. The procedure also represents a rational therapeutic approach in the bleeding thrombocytopenic newborn. More recently exchange transfusion has been utilized in the management of DIC, RDS and sepsis. Besides its advantages, metabolic, haemorrhagic and cardiac hazards of this "massive transfusion" are considered. Just as at any other age, the red cell preparation is the blood component most frequently utilized in the transfusion therapy of the neonate, considering not only the treatment of anaemia without hypovolaemic shock, but also the cases of iatrogenic blood loss, a common problem in the high risk neonatal intensive care unit. As transfusion of small increments of blood may often be required for the sick neonate and premature infant, different methods to cope with such conditions are discussed.

Introduction

In blood transfusion therapy of newborn infants complex considerations are involved which do not have to be taken into account when dealing with older children or adults. Neonatologists and blood transfusion services must be thoroughly aware of several special aspects peculiar to the neonatal age, thus,

a) the red cells of newborn infants react weakly with anti-A and/or anti-B agglutinins *in vitro* and false agglutination may result by contamination of the blood with Wharton's jelly;

b) transfusion of group O red cells should be recommended for A/B or AB infants during the first three months of life when the mother's ABO blood group is unknown, because anti-A,B IgG alloantibodies may be transferred from the mother. These could damage adult A,B red cells even when the newborn's erythrocytes were unaffected;

c) a direct antiglobulin test must be performed routinely to avoid adverse clinical reactions due to previously not recognized alloantibodies;

d) since alloantibodies present in the newborn are derived from the mother, the cross-match in case of haemolytic disease of the newborn (HDN) must be carried out with the serum of the mother;

e) iatrogenic blood loss is a common problem in neonatal intensive care units. The amount of blood which has to be taken for the various investigations may cause significant blood loss, particularly in babies with very low birth weight (< 1500 g). The resulting anaemia or hypotension requires correction.

The purpose of this paper is to review the various indications for transfusion in the newborn infant. These indications and the choice of blood products are summarized in Tables 1 and 2, respectively. The main aspects of the administration of blood and blood products to the neonate will now be considered.

Exchange Transfusion

Exchange transfusion (ET) is the technical procedure most frequently used for transfusing neonates.

The increasing use of anti-D immunoglobulin and phototherapy has reduced the major cause for ET in the treatment of haemolytic disease of the newborn and hyperbilirubinaemia of other aetiology. More recently, ET has been utilized in the management of disseminated intravascular coagulation (DIC), severe respiratory distress syndrome (RDS) and sepsis [1–9]. The procedure is also a rational therapeutic approach in the bleeding thrombocytopenic newborn [10, 11].

Furthermore, a partial ET is an effective method of rapid correction of severe anaemia [12–14] as well as to reduce hyperviscosity in symptomatic polycythaemia [15, 16].

Indication for ET in the treatment of hyperbilirubinaemia

ET is effective in reducing the concentration of serum bilirubin as well as in removing potential sources of bilirubin (i.e. sensitized red cells and antibodies).

Removal of potential bilirubin is more effective than that of actual serum bilirubin. It is therefore important to consider ET for haemolytic disorders due to persistent red blood cell destruction (i.e. Rh HDN) in an early stage.

Cord haemoglobin and bilirubin levels are considered useful criteria for early ET although the guidelines vary considerably among experienced workers [16].

Generally accepted criteria for the treatment of HDN are a cord haemoglobin concentration < 13 g/dl, a capillary value < 12 g/dl, and/or a cord bilirubin level > 4 mg/dl [17–19]. Some authors claim that immediate ET is necessary when the cord serum bilirubin exceeds 7 mg/dl even if the cord haemoglobin concentration is reasonably satisfactory [20].

All of these criteria were established before the introduction of phototherapy and phenobarbital treatment both of which may potentially alter the natural history of hyperbilirubinaemia in HDN.

A decision to exchange is best determined by carefully monitoring the serum bilirubin concentration during the first 6 to 12 hours of life. If the indirect bilirubin level increases by more than 1 mg/dl/hour during the first 24 hours of life, early ET is indicated in cases of HDN [21].

Values of 20 mg/dl of serum bilirubin for infants weighing >2500 g, or 16 mg/dl for infants weighing <2500 g at any time are mandatory for ET. In infants with very low birth weight with evidence of haemolysis or other signs of a complicated clinical status (low Apgar score, hypothermia, deterioration of the central nervous system, etc.) ET is suggested at bilirubin levels of 10 to 13 mg/dl [22].

More detailed indications for the management of hyperbilirubinaemia by ET and phototherapy can be found in Maisel's guidelines [23] and in Gartner and Lee [24].

Several methods for directly or indirectly measuring "unbound" bilirubin have been developed to assess the hazards to jaundiced infants [25]. All of these methods require further evaluation before they are widely applied for clinical use.

Other indications for ET: the treatment of sepsis, of RDS and of bleeding due to DIC or thrombocytopenia

A two (or three) volume ET with relatively fresh blood which must be repeated when the clinical situation and other parameters demand, appears promising in the treatment of DIC [26–28], RDS [28–30] and severe infections with sclerema [7–9, 31]. It is not completely understood exactly what mechanisms underlie the benefit (see Table 1), but removal of unidentified toxic products seems to be the major advantage.

Neonatal alloimmune thrombocytopenia or thrombocytopenia secondary to maternal autoimmune thrombocytopenia or associated with maternal drug-induced purpura are mostly self-limiting conditions.

If potentially serious haemorrhage has occurred, ET with fresh blood may be effective in simultaneously providing the removal of circulating anti-platelet antibodies and causing temporary haemostasis [10, 11].

Selection of donor blood for ET

To cope with all implications involved in the indications for ET, the unit of donor blood used should be as fresh as possible. If the blood is preserved with acid-citrate-dextrose (ACD) or citrate-phosphate-dextrose (CPD), certain metabolic changes may occur during and after ET. ACD and CPD blood induce a decrease in the level of ionized calcium and magnesium due to an elevated serum citrate concentration [17, 32]. The administered citrate is promptly metabolized so that after exchange ionized calcium and magnesium return to the normal level [33], at least in neonates in a good condition.

During ET, particularly in infants with a low birth weight, hypocalcaemia may, however, be a problem. It is important to realize that intravenous treatment

Table 1

Indications for blood transfusion in the newborn

Clinical indications	*Aim of transfusion therapy*
1. *Hyperbilirubinaemia:* ↑ Production ↓ Clearance	↓Actual or potential (antibody sensitized RBC) bilirubinaemia
2. *Anaemia with shock:* a) Due to acute blood loss b) Severe prenatal haemolysis or anaemia with heart failure	a) ↑ volume and O_2 transport b) ↑ O_2 transport without increasing volume
3. *Anaemia without shock:* a) Chronic blood loss b) Slow haemolysis due to morphologic disorders or enzyme deficiencies c) Iatrogenic d) In the preterm infant	a), b), d): ↑ O_2 transport c): ↑ volume and O_2 transport
4. *Polycythaemia*	↓ Hyperviscosity
5. *Haemorrhagic disorders:* a) Vitamin K deficiency b) Disseminated intravascular coagulation (DIC) c) Congenital deficiency of clotting factors (VIII–IX etc.)	a) Correction of transient vitamin K deficit and eventual anaemia b) Removal of "toxins?" and FDP; restore coagulation factors and platelet equilibrium c) Replace lacking factor(s)
6. *Thrombocytopenia:* a) ↓ Production b) ↑ Destruction c) Secondary forms to congenital or acquired infections	a), c): Only treat when bleeding (prevention?) b) Remove circulating antibodies-substitution therapy (?)
7. *Sepsis*	— Remove toxins — supply C and IgM — supply lacking elements or restore hypofunction (RBC, platelets, granulocytes)
8. *Respiratory distress syndrome (RDS)*	— ↑ Proteinaemia and volume — ↑ plasminogen — ↑ antithrombin III level — ↑ 2,3-DPG level of RBC } ? — removal "toxins" and vasopressors?
9. *Surgical intervention*	— ↑ Volume and O_2 transport by giving the lost elements — provide clotting factors and platelets (if deficient)

with calcium gluconate during the procedure is ineffective in raising the Ca^{++} level of the blood [34, 35].

Massive infusion of ACD or CPD blood also produces high blood glucose and plasma sodium concentrations, changes particularly dangerous for the low birth weight infant because of an increasing risk of haemorrhage in the central nervous system [22, 36, 37]. Moreover, a high blood glucose concentration stimulates the secretion of insulin which in turn may lead to a severe post-exchange hypoglycaemia [38]. This is particularly so in cases of severe Rh HDN.

The use of heparin avoids the dilution of donor blood, disturbances in acid-base and electrolyte balance and blood sugar level. Heparin, however, causes a marked rise in non-esterified fatty acids (NEFA) which compete with bilirubin for albumin-binding sites [17, 32, 39]. Furthermore, a unit of heparinized blood must be used within 24 hours of collection.

Heparinized bank blood may be the choice for low birth weight infants with hypernatraemia to avoid any additional osmolar load by ACD or CPD blood.

There is no evidence that ET with heparinized blood is more useful for seriously ill neonates with sepsis, RDS or DIC [5, 6, 8, 9, 26, 27, 30, 40, 41].

CPD blood, which is readily available, imposes less of an acid load and less potassium than ACD blood initially and throughout storage; the maximum 2,3-DPG levels of red cells from CPD blood also ensure an efficient oxygen-delivering capacity [42]. Therefore, in the majority of cases, CPD blood can be utilized relatively safely. Another important aspect for ET is the selection of a donor with the appropriate blood group. For Rh HDN, Rh-negative blood of the same ABO blood group as the infant must be utilized, provided of course that the maternal red cells are ABO compatible. If ET is undertaken in ABO HDN then group O red cells of the same Rh type as the infant's, resuspended in AB plasma, should be used. HDN due to other alloantibodies must be treated with blood compatible with the mother's serum. When a moderate anaemia is associated to hyperbilirubinaemia, CPD or ACD blood after partial removal of the citrate-plasma seems to be more appropriate for ET than whole blood.

Alternatively in cases of HDN ET with frozen thawed erythrocytes of the appropriate ABO and Rh type may be used, the cells being resuspended in a suitable volume of fresh frozen compatible plasma, plasma protein fraction or albumin [43, 44].

In the management of hyperbilirubinaemia without alloimmunization or when ET is indicated for other clinical conditions, i.e. RDS, DIC, whole donor blood of the same ABO and Rh type as the infant should be used.

Particularly for the last clinical condition it is necessary to use fresh blood in order to furnish an adequate number of platelets, normal amounts of labile factors (factor V and VIII) and few potentially harmful platelet–white cell micro-aggregates [40, 45]. Otherwise packed red cells, fresh plasma, and platelet concentrates can be given if fresh whole blood is not available.

In summary, ET should be performed by experienced and skilled physi-

cians; the volume exchange should be about twice (180 to 200 ml/kg) that of the infant's estimated blood volume per kg, the ambient temperature approximately 28° to 30 °C in a draft-free room. To avoid cardiac hazards, donor blood should be prewarmed in a 37 °C water-bath for one to two hours and to prevent recooling during its passage down the infusion tubing, the latter should also be kept in a bath. Furthermore, to minimize alterations in blood pressure during the procedure, an exchange rate of 5 ml/kg per 3 minutes is recommended in preterm, or a little faster in the fullterm infant in good condition.

Partial exchange transfusion

Severe anaemia (cord Hb < 8 g/dl) with clinical signs of congestive heart failure (i.e. associated with the most severe HDN and occasionally secondary to a chronic prenatal blood loss) must be treated by a partial ET. The procedure may be an effective and rapid way of treating infants unable to tolerate the increased blood volume which results from conventional transfusion therapy.

Packed red cells (PRC) are indicated, the total volume for the exchange being equal to the infant's estimated blood volume (85 to 90 ml/kg).

It was suggested to reduce the elevated venous pressure by first removing approximately 20 ml of blood for each 10 ml infused until venous pressure has normalized [12, 13, 18].

More recently, in order to determine the volume of blood to be exchanged, a modification of a formula suitable for ordinary transfusion was derived by Nieburg and Stockman [14]. To bring the Hb content at any level these authors suggested the formula,

$$\text{PRC volume} = \frac{\text{body weight (kg)} \times 90^{*} \times \text{desired } \Delta\text{Hb}}{22^{**}\ \text{g/dl} - \dfrac{(\text{Hb initial} + \text{Hb desired})}{2}}$$

* 90: mean blood volume (ml/kg) in normal newborns [46] instead of 75 (blood volume ml/kg) in older infants or adults in the authors original formula.

** PRC Hb level can vary between 20 and 24 g/dl.

Using haematocrit (Ht) instead of Hb value, the RBC volume required for partial ET can be calculated according to the nomogram of Berman et al. [47].

Both new formulas were applied for correction of severe anaemia with congestive heart failure. Clinical improvement such as a decrease in heart and respiratory rate and a fall in central venous pressure was noticed during the procedure without using the "deficit" which results from initially withdrawing more blood than is transfused (see above).

The neonatal hyperviscosity syndrome secondary to polycythaemia is treated by partial exchange when the venous Ht exceeds 65%. Either salt-poor human albumin (diluted with saline to give a final albumin concentration of 4 g/dl) or fresh frozen plasma is utilized to reduce the venous Ht to 50–60%. The required volume exchange (V) of plasma or albumin is calculated by the

formula,

$$V = \frac{\text{wt kg} \times 90 \times (\text{Ht initial} - \text{Ht desired})}{\text{Ht initial}}$$

or estimated at 15 to 30 ml/kg body weight [15, 16].

The plasma exchange volume needed for the reduction of the Ht level in symptomatic polycythaemia can be also estimated applying Berman's nomogram [47]. Partial ET for the treatment of severe anaemia or symptomatic polycythaemia is carried out utilizing either the umbilical artery or vein.

Transfusion

Blood transfusion in the neonate as in adults is essentially a form of replacement therapy which is indicated,

1. to restore inadequate circulating blood volume in the treatment of hypovolaemic shock associated with acute blood loss;
2. to provide specific blood components in specific deficiencies (i.e. anaemia, thrombocytopenia, neutropenia, or plasma protein deficiencies).

Whole blood

Acute blood loss secondary to occult haemorrhage prenatally or at delivery or to haemorrhage due to obstetric accidents, malformation of the placenta or the cord, or internal haemorrhage, may result in severe anaemia and hypovolaemic shock. The immediate clinical problem then is the restoration of blood volume. Neonatologists faced this situation must immediately insert a catheter into the umbilical vein or artery in order to transfuse about 20 ml/kg of whole blood [48–50].

Group O Rh-negative blood can be drawn from any readily available donor as would be the case with a "walking-donor" programme *(vide infra)*.

Also 20 ml/kg of a volume expander such as whole plasma or 5% albumin or saline can be administered initially to relieve the arterial and venous hypotension.

Some authors [51] in delivery room emergencies obtained best results by utilizing autologous blood drawn from the placenta (with a syringe containing heparin 1 to 4 units per ml of blood).

Subsequently oral iron therapy should also be given to these infants to replenish body iron stores (see below for doses).

When iatrogenic blood loss has occurred, transfusion of whole blood can be used to restore oxygen delivery and to relieve hypotension [50].

Packed red cells

Other causes of anaemia without hypovolaemic shock such as that due to chronic blood loss or anaemia secondary to haemolysis may be asymptomatic

or require red cell replacement. During the first week of life, in an infant of 34 to 35 weeks of gestation or older a venous haemoglobin level of 13 g/dl or less or in a capillary sample a value of Hb less than 14.5 g/dl indicates the presence of anaemia [52]. Whether any kind of treatment is given, depends on the clinical condition. The clinical picture in infants with chronic intrauterine blood loss, usually due to foeto-maternal or twin-to-twin transfusion, may be uneventful [48, 50].

The infant compensates for a blood loss which occurred over a long period of time by increasing its blood volume. Symptoms of congestive heart failure (hepatomegaly, oedema and increased venous pressure) rarely occur. Many of these infants, despite their pallor and low Hb level at birth, are quite well and require only oral iron therapy. Two mg of elemental iron/kg administered three times daily for three months is sufficient to replace body iron stores and to restore the Hb level to normal. Occasionally a transfusion of packed red cells (PRC) may be necessary. Anaemia due to haemolysis by allo- or autoantibodies or due to disorders of the red cell membrane or of haemoglobin when it does not induce early jaundice, may be mild. Sometimes, however, in such cases significant "late" anaemia may develop. An Hb level of 8 g/dl or less, taking the clinical status, too, into account, may indicate a replacement with PRC [52].

A transfusion with PRC may often be necessary in hypoplastic congenital anaemia (Blackfan-Diamond syndrome).

When iatrogenic blood loss has occurred, a transfusion of PRC as well as of whole blood should be given.

The anaemia that may follow ET can be considered a form of iatrogenic anaemia. It can be prevented by making sure that the Hb level at the end of ET is 13 g/dl or more [12, 53].

If, however, this kind of anaemia does develop, it is surprisingly well tolerated unless the Hb level drops below 6 g/dl. In that case if the infant is also feeding poorly, not thriving and persistently reticulocytopenic, a transfusion of PRC may be necessary [12, 13]. Only blood of the same type as that used for ET should be given.

In general there are no well-defined criteria for the indications for transfusion at any age and certainly none exist for transfusion of neonates and particularly for the premature infant. For this last category some general guidelines have been suggested [54]. These include,

a) the Hb level of infants of birth weight less than 1500 g should be maintained in excess of 13 g/dl during their first week of life.

Values of less than 7 g/dl at any age must be investigated but do not necessarily require correction in the preterm healthy infant;

b) the Hb level should be maintained in the range of 16 to 17 g/dl for infants with cardiac or pulmonary disease, conditions which impair the infant's ability to compensate the anaemia;

c) when more than 10% of the infant's blood volume has been removed for laboratory studies within a period of 24 to 48 hours, it should be replaced.

In conclusion, in the premature baby indications for transfusion are Hb levels of less than 7 g/dl in a healthy infant or less than 12 g/dl in the presence of apnoeic spells, patent ductus arteriosus or bronchopulmonary dysplasia.

It must be kept in mind that blood transfusion in iron supplemented low birth weight infants may reduce iron absorption when the Hb level is higher than 12 g/dl [55].

To calculate the volume of PRC required for a replacement transfusion, the following formula may be used,

$$\text{Volume of PRC} = \frac{\text{wt kg} \times 90 \times \text{desired } \Delta\text{Hb}}{\text{Hb concentration of PRC (g/dl)}}$$

This formula can be adopted to whole blood as well.

It can also be calculated that 3 to 4 ml of PRC per kg body weight can raise the Hb concentration by 1 g/dl [48].

Transfusions of small amounts of blood may frequently be required for the sick neonate and premature infant to correct hypovolaemia and/or to replace iatrogenic loss of blood or to cover surgical needs.

When 10 to 60 ml of fresh whole blood is drawn from a unit of whole blood through a filter into a syringe and is used to transfuse an infant, most of the remaining blood is wasted if it cannot be used within the next 24 hours.

Moreover, for those babies who need repeated transfusion of a small volume, the use of a single donor has been advocated to minimize exposure to red cell and HLA antigens and also to decrease the risk of hepatitis.

To cope at every instance with requirements for transfusing newborns such a small volume of blood, high levels of 2,3-DPG and coagulation factors and low levels of serum potassium, different methods have been proposed.

A somewhat controversial method is the use of the "walking-donor", a donor population composed of people who work at the hospital [56–58].

Such donors are checked periodically for syphilis and hepatitis. When an infant requires blood, the type needed is determined, a compatible donor is selected and blood is taken for cross-match. Blood is then drawn from the donor with a syringe containing heparin, it is then filtered and given or allowed to settle in the syringe to increase the Ht before transfusion. Some disadvantages that were brought forward against this system are the inordinate risk or exposure to hepatitis and CMV (dealing with donors working in the hospital!), no blood bank control, risk of contamination due to technical failure, miscalculation of the amount of the anticoagulant [59–61].

Advantages include the ease with which a transfusion can be given, few metabolic changes in the red cells, presence of coagulation factors, reduction of wasted banked blood and a reduction in the number of cross-matches.

The Committee on Standards of the AABB has opposed to this transfusion practice. As an alternative the Committee recommends the transfusion of small volumes of blood carried out through the facilities of the blood bank [59, 60].

Examples of methods which have been found satisfactory in providing blood for neonatal transfusions include,

1. the use of donor blood collected into CPD "quadruple packs" and transfused as needed as either packed cells or whole blood;

2. the use of donor blood drawn to a volume of 225 ml into a CPD "triple pack" with appropriate reduction of the dose of anticoagulant.

Two units of packed red cells each of approximately 50 ml are provided. The donor can be rebled after a shorter time interval if needed;

3. removal of a small volume of packed red cells, or whole blood from a unit of recently drawn blood into an attached satellite bag. The volume of blood remaining in the original container can be utilized for the transfusion of an adult;

4. the use of a small aliquot of group O frozen red cells prepared from a Rh-compatible unit.

The advantages of the methods 1 and 2, both retaining blood bank control, are that multiple transfusions can be given to a baby of either red blood cells or whole blood from a single donor [62].

Moreover, multiple transfusions can be given on the basis of a single pre-transfusion cross-match, reducing iatrogenic blood loss.

Method 3, although providing the necessary small volume of blood or PRC, does not reduce the number of donors to whom the infant is exposed.

The use of paediatric frozen red cell packs can eliminate waste of fresh whole blood, maintain fresh red blood cell properties, eliminate the hazards of transfusing alloantibodies present in the plasma or plasma electrolytes.

Furthermore, frozen cells reduce the amount of undesirable white cell antigens and minimize the risk of hepatitis and CMV infection, both particularly dangerous in neonatal age. The clinical use of paediatric frozen red cell packs has been found excellent [63–65]. The cells that may be left over from a unit may, however, expire before another recipient can be found and this means waste and a significantly greater cost of the programme. In centres where the blood bank cannot or will not provide the above services, the neonatologist has to resort to a "walking-donor" programme to provide optimum care.

Plasma

Fresh frozen plasma (FFP) can be utilized for transfusing neonates with bacterial infections in order to furnish IgM and complement factors. These should improve the impaired humoral immunological defense mechanism of the neonate [66] but this aim seems to be accomplished better by ET with fresh whole blood [9].

Plasma is also utilized as a volume expander in hypovolaemic shock or for the treatment of symptomatic polycythaemia by partial ET [10, 50].

To avoid the risk of the transmission of hepatitis, plasma can be replaced by albumin solution in both the above conditions. The indication for FFP is when deficiencies of coagulation factors such as factor XIII must be corrected [40, 41], or in bleeding newborns with severe liver disease [27, 40]. It is preferred

to prothrombin complex concentrates which may promote lethal disseminated thrombosis [68].

The volume of plasma utilized is about 10 to 20 ml/kg which can be repeated when the clinical situation and haemostatic parameters require.

Many transfusion services now issue paediatric packs containing approximately 50 ml of FFP.

Platelets

Platelet transfusion in the newborn is utilized less frequently than in older age.

Optimum management of thrombocytopenia depends upon its aetiology and pathophysiological basis.

In almost every instance, thrombocytopenia is a self-limiting condition.

Transfusion of platelet concentrates is efficacious in treating haemorrhage due to thrombocytopenia resulting from a decreased production as in congenital megakaryocytic hypoplasia, a complex syndrome designated TAR (thrombocytopenia-absent-radii) or in inherited thrombocytopenia, X-linked or recessively transmitted [11, 27, 41]. Replacement therapy with platelet concentrates should also be considered if serious bleeding occurs in severe systemic perinatal infections, designated TORCH (infections due to toxoplasma, rubella, cytomegalovirus, herpes and other infections including syphilis and Gram-negative bacteria) [11, 27].

"Wash-out" thrombocytopenia, following ET may also require replacement when the baby is bleeding.

The use of platelet concentrates (1 unit every 12 to 24 hours) and FFP (10 to 15 ml/kg every 12 to 24 hours) as initial therapy [69] in an infant with confirmed DIC is debated. It has been said that replacement with blood products, without heparinization or without cessation of the consumptive process, simply provides more substrate for the continuation of intravascular clotting [70, 71].

In infants who are destroying platelets rapidly due to alloimmunization and/or passive transfer of maternal autoantibodies, platelet therapy may not be effective in preventing significant haemorrhage. An approach to treatment in these infants may be ET to remove antibodies (see above). Subsequent transfusion of washed maternal platelets has been advocated for alloimmune purpura [10, 11].

Platelet transfusion is generally reserved for infants who bleed or whose platelet count is very low and who are at risk of bleeding because of trauma (surgery, intubation).

A single donor platelet concentrate, usually supplied in a volume of 30 ml in plastic bags, will be sufficient to increase the platelet count by about 100 000/μl in a term infant. In preterm infants a dose of 10 ml of platelet concentrate/kg is usually sufficient [26, 41].

Granulocytes

There are still a number of serious obstacles to the routine use of granulocyte transfusion therapy. Interpretation of clinical studies is complicated by many variables which potentially affect the outcome of infectious episodes in severely ill neutropenic patients.

In neonatal age, inherited chronic neutropenia might benefit from granulocyte transfusion if complications such as infections of the skin and mucosal infection, omphalitis or sepsis are present and antibiotic therapy failed to improve the clinical condition.

Infants with alloimmune neutropenia, in situations of proven sepsis, could be transfused with maternal granulocytes [72].

Recently a programme for granulocyte transfusion has been started in some neonatal intensive care units [73].

Several functional disorders of neutrophils can be observed in the newborn particularly in the course of sepsis when infants with a normal or even an increased number of functionally inactive neutrophils may be comparable to a neutropenic patient. Sepsis particularly by Gram-negative bacteria may often be irresponsive to antibiotics. These considerations have induced the use of granulocyte transfusions in very low birth weight newborns in order to overcome a hazardous condition.

The results seem to be promising, although the dose, the frequency and the length of period over which the transfusions have to be given have not been standardized. In experimental pseudomonas septicaemia in dogs made granulocytopenic, Appelbaum et al. [74] observed a maximum rate of survival after transfusing 2.0×10^8 PMN/kg. However, the extrapolation of laboratory experiments to the clinical situation is always unsafe.

Risks may derive from granulocyte transfusion, as the transmission of CMV or toxoplasma infection is potentially increased, compared to transfusion of other blood components [75, 76]. Moreover, a unique complication of granulocyte transfusion is graft-versus-host reaction (GVH) particularly in immunodepressed patients.

The risk of GVH in neonates is not well known. Irradiation (3000 to 5000 r) of granulocytes before transfusion may prevent GVH [75, 77].

Further studies are necessary to determine precisely the factors in favour of this therapy in septic newborn infants.

Plasma fractions

The derivatives of plasma obtained by chemical processes such as coagulation concentrates, immunoglobulin and albuminoid preparations are not largely used in the neonate.

Human albumin fraction can be utilized as plasma volume expander or instead of plasma for partial ET in the symptomatic polycythaemic newborn (see above).

Salt-poor albumin in 25% solution, has been utilized to increase the transport of "free" (not bound to albumin) bilirubin as an isolated treatment, or more often before ET or in the container of blood to be used for ET [78–80].

The use of albumin seems to make little difference to the amount of bilirubin removed by ET [81] unless the reserve albumin-binding capacity was very low. Moreover, it has been reported that large amounts of caprylate and N-acetyltryptophan are present in some commercial albumin preparations. These substances, added to stabilize the protein during heating for inactivation of hepatitis virus, could compete with bilirubin for albumin-binding sites [82].

Most neonates with mild and moderately severe haemophilia (factor VIII or factor IX levels over 1 to 2% of the normal) bleed little or not at all. Prolonged and/or life-threatening haemorrhage, however, can easily be corrected by the administration of specific concentrates of factor VIII or factor IX in sufficient amounts to raise the factor level to even 50% of the normal. Also, in surgical emergency an inherited deficiency of a clotting factor in the newborn asks for correction. Dosage guidelines according to Hathaway [26] for those infants who show bleeding manifestations are,

$$\text{Units factor VIII} = \text{desired blood level (\% of normal)} \times \text{wt kg} \times 0.5$$
$$\text{Units factor IX} = \text{desired blood level (\% of normal)} \times \text{wt kg} \times 1.0$$

A unit of the factor is the equivalent clotting activity in 1 ml of fresh normal plasma. Congenital deficiency of factors other than VIII or IX are extremely rare and rarely has bleeding been reported in neonatal age.

Prolonged oozing from the umbilical stump has, however, been observed in most cases of factor XIII deficiency. Treatment by FFP has already been mentioned.

FFP and vitamin K must be used instead of prothrombin complex concentrate (thrombogenic) to treat haemorrhage due to severe liver disease such as hepatitis or cirrhosis due to alpha-1-antitrypsin deficiency, intrauterine infections and metabolic disorders (galactosaemia, tyrosinosis).

Human normal immunoglobulin (HNI) does not find indications in the neonate, regularly furnished by transplacental passage of IgG, unless the mother had hypo- or agammaglobulinaemia. A preparation of Ig enriched for IgA and IgM such as IgGAM [83] could be a support in the treatment of neonatal Gram-negative sepsis.

Human specific immunoglobulin preparations (HSI) are utilized for the prevention of particularly severe diseases such as hepatitis B, pertussis, etc.

In conclusion it can be said that in transfusion therapy of the neonate just as at any other age it is necessary to consider the "optimum use of blood" [84] keeping in mind not only the benefit but also the risks of the treatment.

As already mentioned, the hazards are metabolic, haemorrhagic and mechanical and the transmission of infectious diseases. Particularly great is the risk of transmitting hepatitis B or non-A non-B with any of the blood products except albumin or IgG preparations although even with these the risk is not altogether absent. The same is true for frozen red cells.

Table 2

Guidelines for the management of neonatal haematological problems

Choice of blood products	Clinical disorders	Treatment
Whole blood	a) Hyperbilirubinaemia Sepsis, DIC, RDS	a) ET: 160–200 ml/kg
	b) Hypovolaemic shock	b) Transfusion: 10–20 ml/kg
Packed cells	a) Anaemia of different aetiology without shock	a) Transfusion (see formula in the text)
	b) Serious anaemia with heart failure (severe HDN, or, rarely, chronic feto-maternal transfusion)	b) Partial ET (see formula in the text)
Fresh plasma or FFP	a) Sepsis b) Hypovolaemia c) Polycythaemia	a), b): transfusion 10–20 ml/kg c) Partial ET (see text)
Platelets	a) ↓ Production: – congenital forms associated or not with leukemia (rare) – inherited forms, X-linked or recessive	Only when bleeding: a), c): 30 ml of platelet concentrate increase platelet count by $10^5/mm^3$ in a 3 kg neonate
	b) ↑ Destruction: – maternal autoimmune thrombocytopenia – drug-induced thrombocytopenia – alloimmune thrombocytopenia	b) Removal of antibodies by ET – transfusion of compatible platelets (?)
	c) Mixed forms: – infections – due to "wash-out" after ET	
Granulocytes	– Chronic congenital neutropenia – alloimmune neutropenia – functional deficiency: sepsis and other conditions	Recently introduced: number and frequency not well defined*

* On the basis of clinical results, Laurenti et al. suggest for the treatment of neonatal sepsis granulocytes in an amount of $0.5-1 \times 10^9$/kg once or twice daily (Laurenti F. et al. *J. Pediat. 98*, 118 (1981).

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A New Method for Detecting Haemoglobins with Decreased Stability: Tryptic Digestibility Test

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A method based on measuring the tryptic digestibility of haemoglobin has been elaborated for the detection of mutations affecting the conformation of the haemoglobin molecule. By the aid of this technique not only haemoglobins that prove labile by other procedures can be detected, but such mutants can also be discovered which confer no decreased stability as reflected by clinical and conventional laboratory examinations. The method is suitable for tackling questions of theoretical or basic research interest, whilst in a simplified form it can be applied as a routine laboratory test.

Introduction

Several mutations that occur in haemoglobin cause the decreased stability of the molecule. This is shown by the fact that out of the 257 mutants described so far, 52 are regarded more or less as labile haemoglobins [1] but the number of mutations leading to the loosening of the molecular structure is presumably higher.

Decreased stability may be of different degrees depending on the site and nature of the mutation. In most cases labile haemoglobins cause congenital Heinz-body haemolytic anaemia (CHHA). Some mutants lead to clinical symptoms only on the effect of certain drugs and chemicals, during pregnancy or intercurrent infections. The detection of unstable variants, the molecular structure of which is only slightly altered and does not manifest itself in any haemolytic disorder, is a function of the sensitivity of various stability tests.

The stability of a given haemoglobin variant is usually evaluated on the basis of its susceptibility displayed under various conditions such as heat treatment or exposure to apolar media. However, there may occur amino acid substitutions which do not render the molecule more sensitive to either heat or apolar effects, still they loosen the structure. With this thought in mind we have developed a novel and easily applicable procedure, based upon a principle different from those exploited so far, for the study of the intact conformation of the haemoglobin molecule. Our method relies on the difference in digestibility of native and denatured proteins.

Materials and Methods

The experiments were carried out with freshly prepared haemolysates of blood from normal donors and haemoglobinopathic patients. In view of the fact that all patients examined were heterozygotes, the stability tests were performed both on the whole haemolysate before chromatographic separation and on the isolated mutant components.

In experiments *in vitro* a slight denaturation of haemoglobin was effected by acetylphenylhydrazine (APH) treatment. To this end, to the washed red cells of a healthy donor 5 mg/ml APH was added and the suspension was incubated at 37 °C for one hour. Another aliquot of the blood sample was used as untreated control. The stability of haemoglobins prepared from control and APH-treated fractions was compared by measuring the stability exhibited at 50 °C according to Dacie [2], at 65 °C by a modification of previous methods [3, 4], and in an apolar medium [5].

For the investigation of proteolytic digestibility the following procedure was elaborated. A haemoglobin solution of 10 g/l concentration was prepared by an appropriate dilution with 0.2 mol/l phosphate buffer pH 7.6, Trypsin (Serva, TPCK-treated) was added at a 1 : 50 (w/w) ratio to the haemoglobin. After incubation for 1, 2, 4, 7, and 22 hours at 37 °C, samples were taken and the undigested protein was precipitated with 10% (by weight) trichloroacetic acid. The light absorption of supernatant obtained after removal of precipitate by centrifugation was read at 280 nm. Total digestibility was defined as the 280 nm absorbance value obtained after a similar digestion procedure of a denatured aliquot of the same haemoglobin sample previously kept at 100 °C for 5 minutes. Digestibility was expressed in percentage of the latter value.

Results

The study of tryptic digestibility of various haemoglobin mutants has shown that the method is suitable for the detection of such small degrees of instability which escape recognition by other stability tests. The results are demonstrated on the examples of Hb Santa Ana β 88 F4 Leu-Pro, a labile haemoglobin detectable also by conventional methods and on APH-treated haemoglobin as positive controls, by comparing their tryptic digestibility with that of Hb J Buda (α 61 E 10 Lys-Asn), Hb G Pest (α 74 EF3 Asp-Asn), and Hb A, species that proved stable with the use of earlier methods.

The stability data of haemolysates containing Hb Santa Ana in about 10%, Hb J Buda and Hb G Pest in about 25% each, and of APH-treated Hb A, obtained by methods described in the literature, are summarized in Table 1. The analogous data of chromatographically isolated haemoglobins are presented in Table 2.

As seen from the tables, in contradistinction to the pronounced lability of Hb Santa Ana, Hb J Buda and Hb G Pest, both exhibited stabilities similar to that of Hb A when tested by conventional methods, as regards both whole

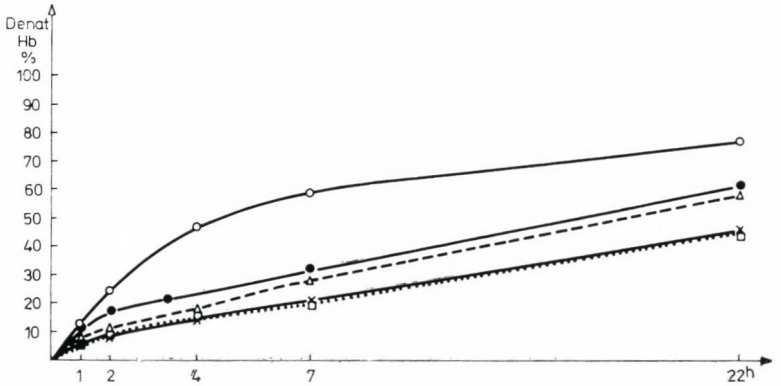

Fig. 1. Tryptic digestion of haemolysates containing Hb A ×—× Normal haemolysate; ○—○ Hb Santa Ana/Hb A; △— — —△ Hb J Buda/Hb A; □ . . . □ Hb G Pest/Hb A; ●— — —● APH treated Hb A

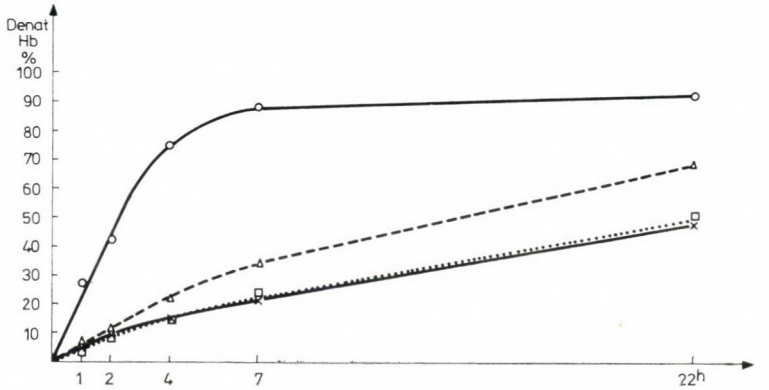

Fig. 2. Tryptic digestion of chromatographically isolated haemoglobin components; ×— — —× Hb A; ○— — —○ Hb Santa Ana; △— — —△ Hb J Buda; □ . . . □ Hb G Pest

haemolysates and isolated haemoglobins. The mild APH-treatment, as expected, caused a slight lability of the incubated samples.

The susceptibility of the samples to tryptic digestion is demonstrated in Figs 1 and 2. It is seen from the curves that some species which proved unstable by conventional screening procedures were found labile in our test system. Furthermore, out of Hb J Buda and Hb G Pest, which appear stable in the usual lability tests, the structure of Hb J Buda proved looser and hence more susceptible to proteolysis than either Hb A or Hb G Pest.

The slight instability of Hb J Buda has already been forecasted by the changes that occurred during storage of the haemolysate of a Hb J Buda/Hb G Pest double heterozygote patient, but this could not be corroborated by the methods then available. It is seen from the electrophoretic patterns of Fig. 3 that

Table 1

Analysis of haemolysates containing HbA*

Method	Denatured haemoglobin, per cent				
	Hb Santa Ana/Hb A	Hb J Buda/Hb A	Hb G Pest/Hb A	APH treated Hb A	Hb A
50 °C 2 h [2]	7.3	1.8	1.6	6.8	1.7
65 °C 5 min [3, 4]	12.3	2.8	3.0	14.7	3.2
Isopropanol test [5]	21.1	1.1	1.2	25.2	1.2

* After mild APH-treatment the sample still contained undamaged HbA.

Table 2

Stability data of chromatographically isolated haemoglobin mutants

Method	Denatured haemoglobin, per cent			
	Hb Santa Ana	Hb J Buda	Hb G Pest	Hb A
50 °C 2 h [2]	21.4	1.8	1.7	1.7
65 °C 5 min [3, 4]	55.1	3.3	3.3	3.2
Isopropanol test [5]	64.2	1.1	0.9	1.2

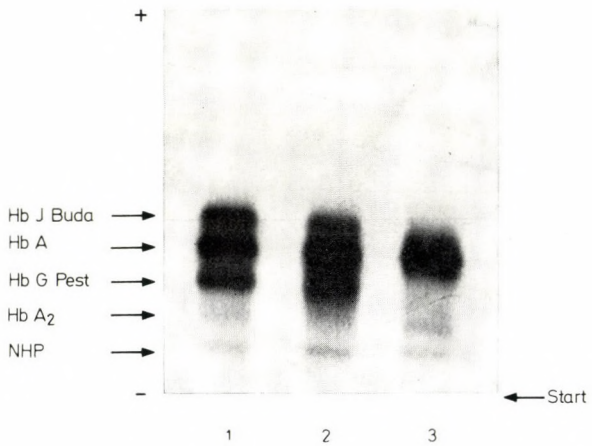

Fig. 3. Starch-gel electrophoretic patterns at pH 8.6, of the haemolysate of a Hb J Buda/Hb G Pest double heterozygote. 1, immediately after withdrawal; 2, after 2 months' storage at −20 °C; 3, normal control

during the course of storage at −20 °C for 1 to 3 months the amount of Hb J Buda slightly decreased as compared with the other two components. Thus the mild lability of Hb J Buda, which could already been suspected from denaturation during storage, could be verified by means of studying tryptic digestibility.

The moderate lability of Hb J Buda was demonstrated in the haemolysate of family members of the double heterozygote who were heterozygotes only for this variant. The fact that lability was detectable already in the whole haemolysate containing haemoglobin A (although naturally somewhat masked by the large amount of Hb A present) suggests that for the screening-like test of stability, isolation of the haemoglobin variant is not indispensable.

Loosening of the conformation of Hb J Buda is easily detectable when the isolated component is being analyzed. Just like with Hb Santa Ana, higher values were obtained with Hb J Buda throughout the entire digestion procedure, already in the whole haemolysate and in a more pronounced manner when the isolated haemoglobins were subjected to analysis. Therefore, it is not by all means necessary to follow up the entire digestion process, but it suffices to measure the initial value and that obtained after 7 or 22 hours of digestion. With this modification the method may be used as a simple routine laboratory test for stability.

Discussion

Mutations that lead to instability of the haemoglobin molecule can be classified into various groups. The range includes mutations affecting amino acids involved in binding haem, the replacement of hydrophobic residues located in the interior of the molecule by hydrophilic ones, or the incorporation of a new amino acid that is too small or too large, to be able to establish the appropriate intramolecular contacts, or its altered charge interferes with the latter. The new amino acid may also alter interactions between subunits, or between haem and globin chains, which if accompanied by a high degree of dissociation can effect the denaturation of free chains. Finally, if the new amino acid is proline and is substituted into a position where the former residue was part of an α-helix, its imino structure may abolish the helix structure thereby rendering the molecule labile.

Haemoglobin derivatives of decreased stability may be produced also as the result of various oxidative effects. For the modelling of this non-genetic disorder did we apply the acetylphenylhydrazine treatment, which leads through methaemoglobin to the formation of choleglobin and haemichrome [6], which is one of the possible pathways of the denaturation of labile haemoglobins.

The classical test method for stability is the study of heat sensitivity [2]. Since this classical method is of limited sensitivity, several modifications of it have been described [3, 4, 7].

There is only one method that is based upon a different principle: the very sensitive isopropanol test elaborated by Carrel [5], which relies on the weakening of hydrophobic interactions of the protein under the influence of an apolar medium. Furthermore, it has been shown that some of the haemoglobins that proved labile in light of the above methods bind the haem group less strongly or is precipitated on SH-group blocking [8, 9]. The haemoglobin mutants in which

the amino acid replacement affects these interactions (cf. above) can be detected by methods based upon the given specific property [10, 11].

A good example of lability due to impaired α-helical structure brought about by the substitution of proline as a new amino acid is provided by Hb Santa Ana, also studied by us, in which proline entering at position 4 in helix F causes such a high degree of instability that is readily detectable by the classical methods.

The same does not hold for Hb J Buda, in which at position E 10 Lys is substituted by Asn. The E 10 Lys residue is located on the surface of the molecule and gives one out of the 80 intramolecular contacts between haem and globin [12, 13]. In spite of this anomaly, Hb J Buda displays the same stability as Hb A when examined by the procedures described in the literature. The instability observed by us according to the criteria of digestibility and storage, still suggests that Asn at position E 10 is unable to make the contact secured by Lys in Hb A. If at the homologous position of the β-chain Lys is replaced by Glu (Hb I Toulouse [β-66 E 10 Lys-Glu]), a haemoglobin disease ensues with clinical symptoms.

It thus seems that our observation that during storage Hb J Buda is being denatured faster than Hb A (or Hb G Pest coexisting with it in the same patient) corroborates the conclusion drawn from tryptic digestibility, i.e., that the conformation of Hb J Buda is loosened.

The enhancement of tryptic digestibility of proteins on denaturation or dissociation in the case of various oligomeric proteins is a well-known phenomenon [15]; the increase of digestibility of APH-treated samples falls into the same category. There is a similar rise in tryptic susceptibility of Hb A treated with p-chloromercurybenzoate, which is presumably the consequence of oligomeric dissociation [16]. In the case of haemoglobin mutants, structural changes of such a degree are obviously improbable, but smaller structural distortions very probably occur more frequently than detected by conventional laboratory techniques. Our procedure, which has long been recognized as a tool in probing native protein conformations [17], has proved useful and suitable for the purpose, i.e. for detecting small conformational alterations in the haemoglobin molecule that would remain unnoticed by other conventional methods.

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Fractionation of Plasma Proteins with PEG

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Plasma proteins were fractionated with polyethylene glycol (PEG-4000) and the effects of pH, ionic strength and temperature were examined. Fractionation with 15% PEG was the most suitable for obtaining a precipitate containing mainly IgG, and a supernatant containing chiefly albumin. These fractions may then be purified further.

Introduction

A basic principle in modern haematologic therapy is the use of blood derivatives. Albumin is the plasma protein used in highest quantities, this is followed in order by cryoprecipitate, factor VIII concentrate and PCC (prothrombin complex concentrate), i.e. proteins used in the therapy of haemophiliacs and, finally, IgG preparations. With the development of the technology of plasma protein preparation, an increased use of transferrin, anti-thrombin III and other plasma proteins is to be expected. In Hungary, 80–100 000 litres of plasma are necessary yearly for supplying a sufficient amount of plasma derivatives. Therefore, it was attempted to find large-scale fractionation methods for the economic production of as many sorts of protein as possible.

Cohn's cold ethanol techniques are widely used [1] for the large-scale production of plasma proteins. With slight modifications this has been the dominant technology since World War II. Other fractionation methods [2] were not found suitable for large-scale technical application. The greatest disadvantage of the Cohn method is the use of a temperature below 0 °C for eliminating protein denaturation and even at such temperatures partial denaturation sometimes occurs. A purified product can only be obtained by multiple precipitation, resulting in a 70 to 80% yield for albumin. For eliminating these disadvantages, several modifications have been introduced [3, 4]; these, however, have only reduced, but not solved the problems.

Polson et al. were the first to use polyethylene glycol (PEG) for the fractionation of plasma, and produced fibrinogen and IgG [5]. They studied the correlation between protein solubility, pH, ionic strength, and temperature and postulated that at a given pH there is a linear correlation between the log concentrations of protein and PEG. According to Juckes [6] and Laurent [7] this correlation can be expressed as follows:

$$\log S = X - aC + fS \qquad (I)$$

where

S = protein concentration,
C = PEG concentration,
X = constant,
a = constant, characteristic of the protein-PEG interaction,
f = constant, characteristic of the protein-protein interaction.

If the protein concentration is low, the protein-protein interaction and fS are also low.

Thus, fS is negligible as compared to X − aC. In cases of low protein concentration therefore the protein solubility (at a given pH) depends only on the PEG concentration.

If the protein concentration is high, the protein-protein solubility depends not only of the PEG concentration but also on the initial protein concentration (fS is not negligible).

We have therefore investigated the correlation between PEG concentration and protein solubility in plasma, and on the basis of the results it was attempted to elaborate an optimum fractionation system for producing good quality protein products.

Materials and Methods

Plasma was obtained by centrifuging 400 ml donor blood drawn into 110 ml ACD solution, at 1200 g for 20 min. Plasma was stored at −30 °C when not used immediately.

Precipitation of protein was performed with 50% PEG-4000 (Fluka) solution in distilled water. The pH was adjusted with mol/l NaOH and mol/l HCl, respectively. The protein precipitate was dissolved in distilled water or in 1/15 mol/l Na_2HPO_4 + 1/15 mol/l KH_2PO_4 solution pH 8.4.

The protein content of the solutions was estimated by the biuret method. "Versatol" plasma was used for plotting the calibration curve. The quantitative composition of protein fractions was determined by cellulose acetate membrane (Schleicher and Schüll) electrophoresis. The purity of the end product was tested by agar immunoelectrophoresis besides membrane electrophoresis. The electrophoretograms were evaluated with the Zeiss ERI 10 densitometer.

The solubility curves were determined as follows: 2, 4, 6 . . . 24% PEG was mixed with 5 cm^3 plasma at the given pH, by addition of 50% PEG solution.

After standing for 30 min the precipitated solutions were centrifuged at 3000 *g* (Janetzky T 32-c) for 40 min. Then the supernatant was decanted and the precipitate was dissolved in the original volume of distilled water. The protein composition of the single fractions was calculated from the total protein content and the quantitative assay of the electrophoretograms. Sterility, pyrogenicity and toxicity tests were carried out according to the Hungarian Pharmacopoeia (Ph. Hg. VI).

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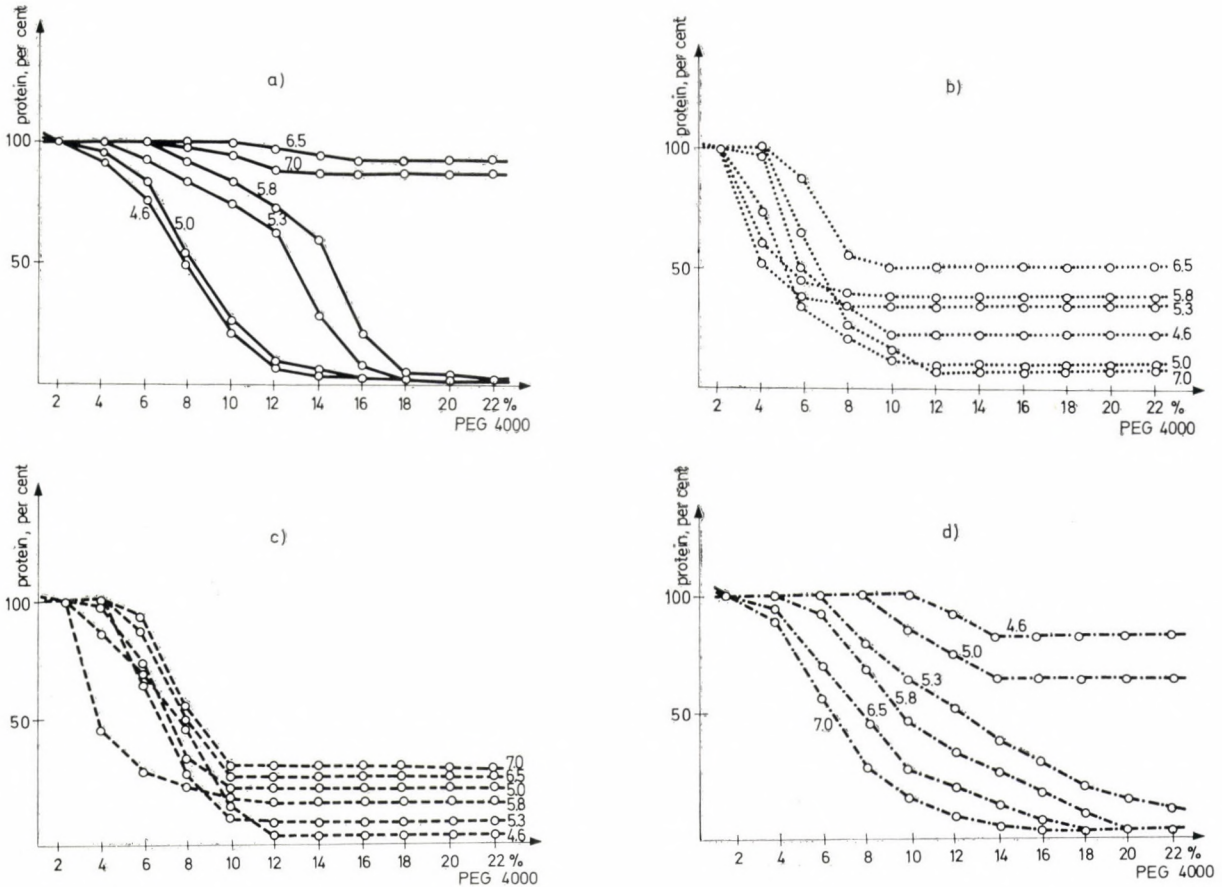

Fig. 1. Solubility of plasma proteins as a function of the PEG concentration at various pH-s. a) albumin; b) α-globulins; c) β-globulins; d) γ-globulins

Results

Figure 1 demonstrates the protein solubility at various pH-s as a function of PEG concentration. As the PEG concentration increases, the protein solubility decreases. Generally, the nearer the pH of the solution to the isoelectric point of the given protein, the less PEG is necessary for precipitating the proteins. If the pH differs substantially from the isoelectric point, the proteins cannot be totally precipitated at a PEG concentration as high as 24%. The curves indicate that the plasma proteins can be separated into several fractions by changing the PEG concentration and the pH. Therefore, we performed our further experiments with 100 ml plasma according to the process demonstrated in Fig. 2. Changing the pH and the PEG concentration, the fractions obtained were:

1. fibrinogen,
2. IgG,
3. albumin.

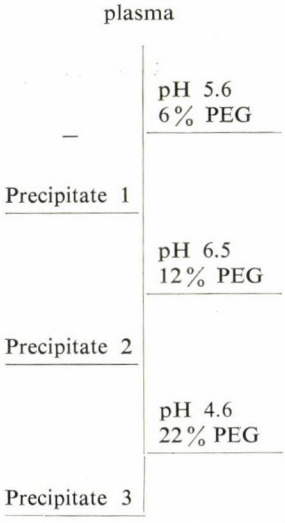

Fig. 2. Three-step fractionation

Table I displays the distribution of albumin and IgG in the three-step fractionating process. IgG is unambiguously concentrated in precipitate 2. Albumin is distributed between precipitates 2 and 3. Therefore, the method was modified to increase the albumin yield. Precipitate 1 contained fibrinogen in the majority. The clinical use of fibrinogen obtained from large quantities of pooled plasma is not advisable due to the high risk of hepatitis B transmission. Thus, thereafter we applied a two-step method. The total amount of IgG can be precipitated with 15% PEG at pH 6.5. Besides the effect of PEG concentration and pH, we examined the effect of temperature and ionic strength on the composition of the precipitates.

Table 1

Distribution of albumin and IgG in the single fractions, per cent

	Albumin	IgG
Precipitate 1	14	0
Precipitate 2	40	100
Precipitate 3	46	0

Table 2

Effect of ionic strength on the distribution of the proteins between precipitate and supernatant (pH 6.5, 15% PEG)

Ionic strength	Protein part found in the precipitate (n = 20)	
	Albumin	IgG
0.01	0.11 ± 0.01	1.0
0.15	0.20 ± 0.05	0.98 ± 0.02
2	0.14 ± 0.02	0.85 ± 0.05

Table 2 demonstrates the effect of ionic strength on the distribution of the proteins between precipitate and supernatant. The albumin content of the precipitate separated from plasma showed no correlation with the ionic strength, but the IgG yield decreased when the ionic strength increased.

Table 3

Effect of temperature on the distribution of proteins between precipitate and supernatant (pH 6.4, 15% PEG)

Temperature	Protein part found in the precipitate (n = 20)	
	Albumin	IgG
+ 4 °C	0.20	0.98
+20 °C	0.30	1.0

Table 3 displays the effect of temperature, showing the albumin and IgG content of the precipitate at +4 °C and +20 °C. The albumin content of the precipitate increased with the increase of temperature.

It was assumed that the increased viscosity of the solution at +4 °C would increase the local concentration of PEG. In this way, with the high local concentration of PEG the coprecipitation of albumin would increase. In fact, a very slow (3 hours) addition of 15% PEG to the plasma at +20 °C and vigorous mixing reduced the albumin content to 10% in the precipitate.

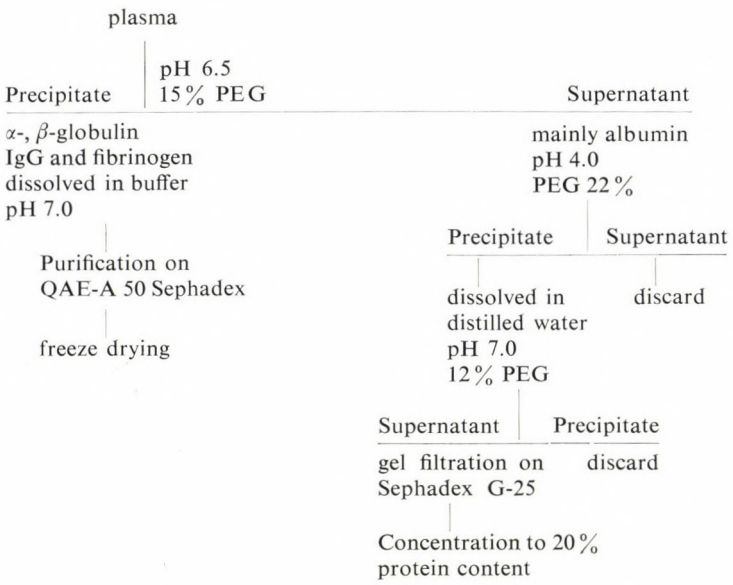

Fig. 3. Plasma fractionation scheme

On the basis of the findings, we elaborated the process shown in Fig. 3. IgG was precipitated with 15% PEG; the precipitate contained approximately 60% IgG. Ion exchange chromatography as described by Houstra [8] was found suitable for purifying IgG.

Albumin was precipitated from the supernatant with 22% PEG at pH 4.6. After dissolving the precipitate, the contaminating alpha and beta globulins were precipitated with 12% PEG at pH 7.0. The albumin purity of the supernatant was over 98%. For removing the PEG, the albumin was precipitated, the remaining PEG was removed on a Sephadex G-25 column. The elute was freeze-dried, dissolved in 20% albumin and filtered. Figure 4 shows the degree of purity of the albumin produced in this way.

The albumin and IgG produced were neither pyrogenic nor toxic.

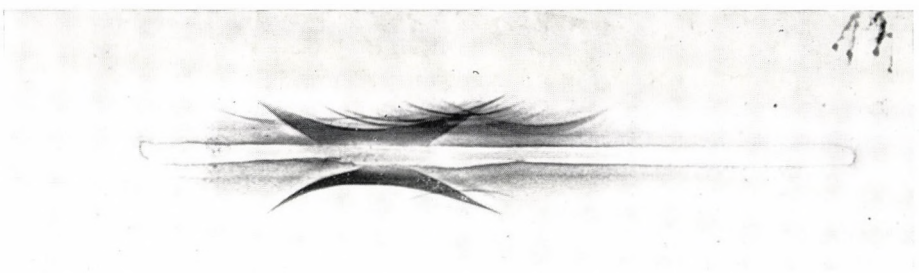

Fig. 4. Immunoelectrophoresis of albumin

Conclusions

Based on the results it was concluded that crude protein fractions can be gained by using PEG-4000; the fractions can afterwards be purified. Separation with 15% PEG at pH 6.5 was the best, offering the following advantages:

1. A yield higher than that with the Cohn method. The IgG yield was approximately 90%, the albumin yield 80%.

2. The whole process can be performed at room temperature, avoiding the inconvenience of working in a cold room, or using expensive temperature controlled equipments.

3. The risk of denaturation is slight, PEG being one of the mildest "precipitating" substances.

Both Polson et al. [9] and Laurent and Ogston [7] concluded that PEG displaces proteins from aqueous solutions; this is due to the fact that the coiled PEG molecule is capable of binding large quantities of water. Thus, similarly as the salting-out process, the activity of the solvent decreases, leading to precipitation of proteins in the order of their solubility. In the presence of PEG, the effect of pH on protein solubility does not change, thus solubility is minimum at the isoelectric point. Up to now the effect of PEG on protein solubility was examined exclusively in single purified protein solution [10] or in highly diluted solutions [5]. Under these circumstances, near to the isoelectric point the Laurent–Juckes equation seems to be valid. In concentrated solutions like plasma which contain several proteins, the relationship between PEG and protein concentration is more complicated. The minimum solubility at the isoelectric point is observed only with albumin and gamma-globulin, but not with protein mixtures such as alpha and beta-globulins. For more detailed data, further experiments and more knowledge of the processes are necessary.

It is thus possible to produce by PEG fractionation plasma proteins for therapeutic use. Increasing the number of fractions, the purity of the single proteins increases, but the yield decreases. So it is advantageous to produce less fractions with a higher yield and to purify them by procedures, e.g. chromatography [11].

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The Effect of Na_3-Citrate on the Solubility of Cryoprecipitate

(Citrate Effect on Cryoprecipitate)

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Cryoprecipitate stored frozen could be redissolved only if it contained more than 10 mmol/l Na_3-citrate. It is shown that in the insoluble residue less than 10 mmol/l Na_3-citrate is fibrinogen. In saline containing 10 to 20 mmol/l Na_3-citrate after thawing, the cryoprecipitate was dissolved entirely.

Introduction

In the middle 1960-s Pool and Shannon described a simple and well reproducible method for concentrating factor VIII [1]. A precipitate rich in factor VIII and fibrinogen [2] was obtained from plasma separated from blood collected in ACD or CPD, frozen at $-30\,^{\circ}C$ to $-80\,^{\circ}C$ [3] and then thawed at $+4\,^{\circ}C$. This precipitate is either stored frozen and reconstituted in a small amount of supernatant plasma or solvent before use, or redissolved immediately and stored in the lyophilized state [4]. In Hungary the precipitate is immediately reconstituted, final preparations of 4–6 bloods are pooled and stored below $-20\,^{\circ}C$ until use. The preparation is thawed directly before transfusion in a $37\,^{\circ}C$ water bath [5].

It is important that no insoluble precipitate should be formed in the course of thawing and refreezing, because that would reduce its agent content and make it unfit for therapeutic use.

In the present work it was attempted to determine the minimum Na_3-citrate concentration necessary for protecting the preparation.

Materials and Methods

Preparation of cryoprecipitate

Whole blood collected in bottles containing ACD or heparin (3–4 IU/ml blood) was centrifuged within 4 to 6 hours at 1330 *g* at $+4\,^{\circ}C$ for 30 min. Plasma of 4–6 blood units was pooled and redistributed in aliquots to obtain equal starting AHG concentrations. Fresh plasma was frozen at $-30\,^{\circ}C$ in the deep-freezer, then thawed at $+4\,^{\circ}C$ in a cold room ($+2 \pm 2\,^{\circ}C$) during a night. Cryo-

precipitate was separated from the supernatant plasma by centrifugation at 1330 *g* at +4 °C for 20 min [6]. The precipitate was dissolved immediately in 1/10 volume of the starting plasma and stored at −30 °C.

Adsorption to $Al(OH)_3$

Blood coagulation factors II, VII, IX and X were removed with $Al(OH)_3$ gel. The solution was treated with 20% $Al(OH)_3$ gel (9 : 1) at room temperature. After 5 min stirring, the solution was centrifuged at 4000 *g* for 20 min [7].

Precipitation of fibrinogen with polyethylene glycol (PEG)

Twenty-four per cent PEG solution was added to 4.5% final concentration at pH 6.5, the mixture was stirred for 20 min and then centrifuged at 4000 *g* for 20 min [8].

Factor VIII assay

A modified method of partial thromboplastin time assay was used [9]. 0.2 ml kaolin phospholipid suspension, 0.1 ml factor VIII-deficient plasma and 0.1 ml diluted sample were incubated at 37 °C for 3 min, and then coagulation was initiated by the addition of 0.1 ml 0.025 mol/l $CaCl_2$. Mixed lyophilized plasma of 30–40 normal donors was used for factor VIII reference curve, which was compared to the reference lyophilized plasma sample of the National Institute for Biological Control, London.

Two parallel dilutions of each sample were evaluated. The mean of four assays performed by a Schnitger–Gross coagulometer are given.

Determination of fibrinogen

One millilitre 0.55 $CaCl_2$ and 1 ml thrombin solution coagulating normal plasma in 10 sec were added to 1 ml test sample. Immediately after the addition of thrombin, a glass rod was immersed in the test tube. After standing 10 min at room temperature the clot adhering to the glass rod was pulled out and the excess fluid was removed from it by gentle pressure.

The glass rod was immersed for 5 min in 10 ml saline, and then in 10 ml distilled water for 5 min. Subsequently, the glass rod was put in 3 ml n NaOH and then in a water bath for 2 to 3 min until the fibrin had dissolved.

Total protein determination

Protein was determined by the biuret method [10].

Electrophoresis on cellulose-acetate membrane

Electrophoresis was performed on 11 × 2 cm cellulose acetate membrane strips (Sartorius membrane filter). The strips with the samples were soaked in veronal buffer pH 6.8, then run in the chromatographic chamber at 0.4 mA/strip for 80 min. The strips were stained with amido black and then treated with a

1 : 1 : 1 mixture of methanol, 10% acetic acid and distilled water until entirely clear. Subsequently the strips were placed on slides and treated with a 3 : 7 mixture of isopropanol and dioxane until entirely transparent. After drying, the slides were scanned with an ERI 65M densitometer.

Results

Effect of Na_3-citrate solvent on the solubility of cryoprecipitate stored frozen

Na_3-citrate-free preparations were made from blood drawn into heparin, as a minimal amount of citrate may remain in the cryoprecipitate if made from plasma separated from ACD blood.

Cryoprecipitate prepared from heparin-plasma was dissolved in 0.15 mol/l NaCl. The effect of citrate was measured by the addition of 0.15 mol/l NaCl + + 0.021 mol/l Na_3-citrate to the preparation [11].

The protein content of the preparations was measured by cellulose acetate membrane electrophoresis directly before reconstitution and after storage in the frozen state (Fig. 1). Samples reconstituted in normal saline did not dissolve

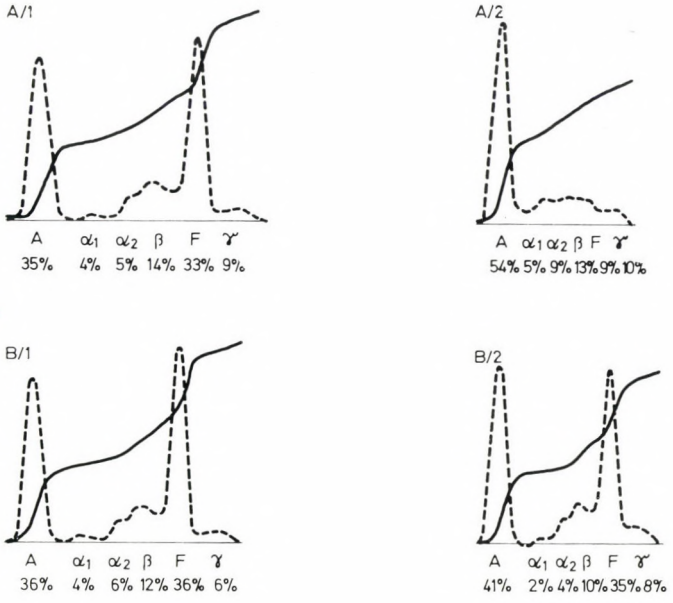

Fig. 1. Protein composition of cryoprecipitate obtained from blood drawn into heparin. A. 0.15 mol/l NaCl and B. 0.15 mol/l NaCl + 0.021 mol/l Na_3-citrate solvent. 1. Before freezing; 2. After thawing

Table 1

Effect of Na_3-citrate concentration of the solvent on the dissolution of cryoprecipitate and on factor VIII activity after thawing

Composition of solvent	Total protein decrease		Fibrinogen decrease		Factor VIII decrease	
	mg/ml	per cent	mg/ml	per cent	U/ml	per cent
1. 0.15 mol/l NaCl 0.021 mol/l Na_3-citrate	0	0	0	0	0.2	7
2. 0.15 mol/l NaCl 0.010 mol/l Na_3-citrate	0	0	0.28	4.5	0.35	13
3. 0.15 mol/l NaCl 0.005 mol/l Na_3-citrate	1.25	14	0.98	18	1.2	48
4. 0.15 mol/l NaCl 0.0025 mol/l Na_3-citrate	1.25	14	0.98	18	1.2	48
5. 0.15 mol/l NaCl	2.1	21	1.9	38.5	2.25	79

Mean of two assays

entirely, and after refreezing and thawing an insoluble precipitate was found. Samples containing citrate dissolved totally. Figure 1 shows that the fibrinogen content of the citrate-free sample decreased, while that of the citrate-containing sample did not. Therefore, the residue is either fibrin or fibrinogen.

Effect of Na_3-citrate concentration

Cryoprecipitate samples prepared from blood collected in ACD were dissolved in normal saline containing 0 to 0.021 mol/l Na_3-citrate.

Total protein, fibrinogen content and factor VIII activity of each sample was determined directly after reconstitution of the cryoprecipitate, and after freezing at $-30\,^{\circ}C$ and thawing. Table 1 displays the decrease of total protein, fibrinogen and factor VIII activity.

In preparations containing 0.021 mol/l or 0.010 mol/l Na_3-citrate, the total protein, fibrinogen content and factor VIII activity did not decrease significantly. On the other hand, samples containing less than 10 mmol/l citrate did not dissolve, and their total protein and fibrinogen content and factor VIII activity decreased. The change was most marked in the citrate-free samples.

Effect of citrate factors

Next, it was studied whether citrate had a neutralizing effect on Ca^{++} in the course of dissolution, i.e. if it prevented the active process of coagulation.

Table 2

Effect of Na_3-citrate concentration of the solvent on the dissolution of cryoprecipitate and factor VIII activity after thawing

Experiment	Composition of solvent	Total protein decrease		Fibrinogen decrease		Factor VIII decrease		Number of tests
		mg/ml	per cent	mg/ml	per cent	U/ml	per cent	
Untreated cryoprecipitate	0.15 mol/l NaCl	3.2 ± 2	21 ± 3.6	3 ± 1.4	44.2 ± 6.8	1.9 ± 0.4	78.7 ± 3.3	4
	0.15 mol/l NaCl 0.021 mol/l Na_3-citrate	0	0	0	0	0.2	7.7 ± 1.5	4
Cryoprecipitate treated with 20% $Al(OH)_3$ at 9 : 1 ratio	0.15 mol/l NaCl	3.8	25	2 ± 0.1	49.5 ± 0.5	1.3 ± 0.05	81.5 ± 6.4	2
	0.15 mol/l NaCl 0.021 mol/l Na_3-citrate	0	0	0	0	0.1 ± 0.1	8 ± 8	3
Cryoprecipitate treated with PEG at 4.5% final concentration	0.15 mol/l NaCl	0	0	Did not contain fibrinogen		0.1 ± 0.1	10.0 ± 10.3	3
	0.15 mol/l NaCl 0.021 mol/l Na_3-citrate	0	0	Did not contain fibrinogen		0.15 ± 0.05	16 ± 4	3

Blood coagulation factors participating in the coagulation process were removed from the reconstituted cryoprecipitate. Part of this cryoprecipitate was dissolved in 0.15 mol/l NaCl solution, another part in 0.15 mol/l NaCl + 0.021 mol/l Na_3-citrate, then both were distributed into three parts:

1. not treated,
2. II, VII, IX, X factors removed by $Al(OH)_3$ adsorption,
3. fibrinogen extracted by PEG (polyethylene glycol).

Total protein and fibrinogen content and factor VIII activity were measured in each sample directly after reconstitution and after storage in the frozen state.

Changes in protein content were estimated by cellulose acetate membrane electrophoresis (Table 2).

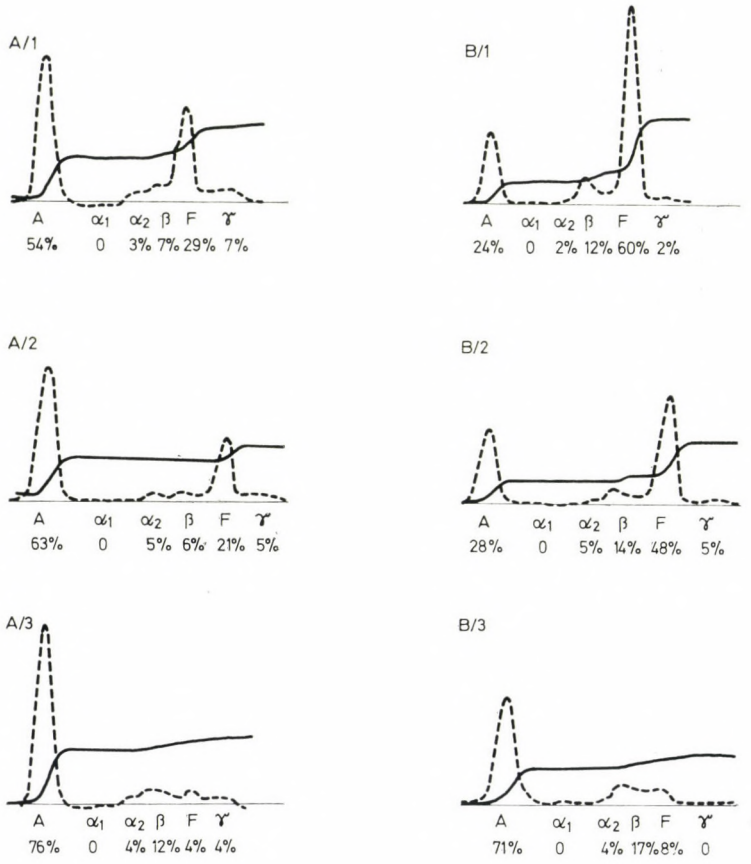

Fig. 2. Protein composition of cryoprecipitate dissolved in A. 0.15 mol/l NaCl, B. 0.15 mol/l NaCl + 0.021 mol/l Na_3-citrate solvent. 1. untreated; 2. treated with $Al(OH)_3$ gel; 3. treated with PEG

Cryoprecipitate dissolved in citrate-free normal saline and treated with $Al(OH)_3$, and thawed after storage in the frozen state contained an insoluble residue. The total protein and fibrinogen content, and factor VIII activity decreased proportionally to the amount of residue. Fibrinogen was removed from the samples with PEG at 4.5% final concentration. No residue had formed in samples treated with PEG.

No residue was formed in samples containing 21 mmol/l citrate either. The small decrease in factor VIII activity observed in samples containing citrate was assumably due to the freezing and thawing procedure. The decrease of the fibrinogen content of citrate-free samples could be followed by electrophoresis (Fig. 2).

Discussion

Preparation of cryoprecipitate is based on the experience that in plasma frozen to $-30\,^{\circ}C$ or lower, then thawed at $+4\,^{\circ}C$, 3% of the plasma proteins do not dissolve, but a precipitate rich in fibrinogen and containing factor VIII is formed. When this precipitate is reconstituted, both these components dissolve.

Experiments show that in cryoprecipitate obtained from blood drawn into heparin, the Na_3-citrate content of the solvent influences the dissolution of fibrinogen after storage in the frozen state. Using plasma separated from blood collected into ACD, we observed that if the final product contained at least 10 mmol/l Na_3-citrate, no residue was formed after freezing and thawing.

Cryoprecipitate contains a high amount of labile fibrinogen [12, 13, 14]; Treatment with $Al(OH)_3$ and PEG shows that the decrease of fibrinogen and factor VIII activity are not a result of an active coagulation process, but a problem of dissolution. During the freezing procedure the fibrinogen loses its hydration layer and when thawed, it is presumably unable to redissolve. The insoluble residue encloses the factor VIII complex, and therefore, factor VIII activity decreases.

Due to the "salting in" effect of Na_3-citrate, a hydration layer develops around the hydrophobic parts of the protein, stabilizing the molecular conformation [15]. Na_3-citrate is used as a stabilizer in the factor VIII purification procedure [16]; the mode of action is unknown [17]. The effect of citrate and other stabilizers is under investigation.

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T Acute Lymphoblastic Leukaemia with Breast Tumour
A Brief Report

Anna POROS, Susan R. HOLLÁN, Katalin NATONEK,
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(Received September 29, 1980)

Acute lymphoblastic leukaemia of L-2 type was diagnosed in an 18-year-old female with breast tumour. Her peripheral leukaemic cells reacted with anti-human peripheral T cell serum and part of the cells expressed SRBC receptor indicating the T origin of her leukaemia. She had a tumoral mass in her breast consisting of the same leukaemic cells. Combination chemotherapy was applied and she went into complete haematological remission with simultaneous disappearance of the breast tumour.

Introduction

The lymphoblasts of around 25% of patients with acute lymphoid leukaemia (ALL) carry one or more T cell markers and such ALL was reported to be a distinct variant [2, 4, 8]. Recent reports on a correlation between T cell markers and individual clinical features of patients with T ALL suggest that T ALL is not a uniform disease [2, 4, 7]. Here we present a T ALL with leukaemic breast infiltration.

Case report

An 18-year-old female with complaints of fatigue and fever was admitted to our Department on January 2, 1979. Physical examination revealed hepatosplenomegaly and moderate lymphadenomegaly in the submandibular region. In her right breast a fixed, indurated lobular tumour elongated in shape (3 × 6 cm) was palpable. Chest X-ray films did not demonstrate a mediastinal mass. The haemoglobin was 11.7 g/dl, the platelet count 60×10^9/l and the WBC 129×10^9/l. Both in peripheral and bone marrow smears 90% of the cells were lymphoblasts of L-2 type [1]. Around 20% of the lymphoblasts had a hand mirror configuration both by light and electron microscopy (Fig. 1). Cytochemical characteristics of the blasts: PAS, Sudan B, peroxidase, acid-phosphatase, NASD chloroacetate esterase and muramidase reaction were negative and DNAse did not digest the nuclei of two thirds of the cells. Smears obtained by needle biopsy from the tumour in the breast contained cells with the feature of lymphoblasts seen in bone marrow and peripheral blood smears. Thus, acute lymphoblastic leukaemia with leukaemic infiltration in the breast was diagnosed.

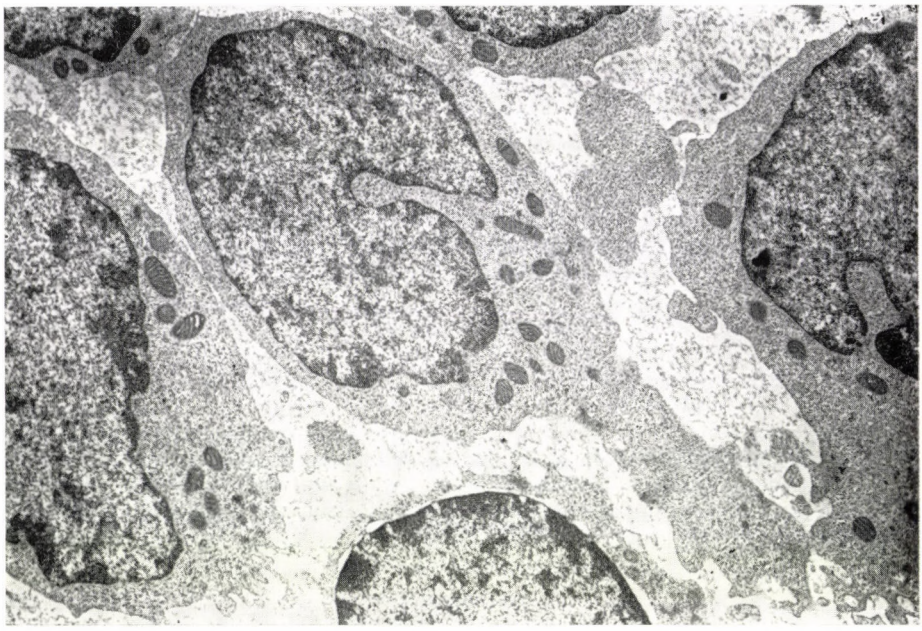

Fig. 1. Blast cells of patient's bone marrow. A typical hand mirror cell can be seen in the centre of the picture × 7450

Twenty per cent of the peripheral blood cells separated by Ficoll-Isopaque gradient, rosetted with sheep red blood cells (SRBC) and papain treatment of SRBC increased the rosette-forming cells to 36%. One per cent of the cells was stained with FITC-labelled polyvalent anti-human Ig (Hyland). All cells reacted in complement-dependent cytotoxicity assay with rabbit anti-human peripheral T cell serum prepared in our Institute [6]. In contrast, the cells did not react at all with the anti-B lymphocyte serum kindly donated by W. Bodmer.

Combination chemotherapy (L-2 protocol [5]) was given together with RBC, thrombocyte and granulocyte concentrates. After three weeks' induction therapy she went into partial remission, but the node in the breast was still palpable. For familial reasons the therapy was delayed three weeks which lead to a severe relapse with fever and enlargement of the breast tumour, and of liver, spleen and lymph nodes. In spite of this, consolidation chemotherapy resulted in a complete haematological remission with disappearance of the breast tumour. Thereafter she was given maintenance therapy. After a remission of seven months, she developed acute hepatitis B from which she did not recover. Her leukaemia relapsed and she died of cerebral haemorrhage.

Discussion

Early reports suggested that bad prognosis, short duration of remission, presence of mediastinal mass and acid-phosphatase-positive lymphoblasts are characteristics of ALL of T cell origin [2, 3, 7, 8]. Accumulating data on patients with T ALL have, however, indicated recently its marked heterogeneity [4, 7, 9]. The additional use of xenogeneic anti-T serum recognizing receptor distinct from SRBC receptor suggested the existence of an intermediate subgroup within T ALL [2, 5, 9]. A negative acid-phosphatase reaction was also described in some T ALL [2, 3, 4, 7].

The leukaemic blasts of our patient showed the morphology of lymphoblasts and cytochemical reactions characteristic of myeloblasts and the acid-phosphatase reaction were negative. All her peripheral blasts reacted with the anti-T serum suggesting their T origin. The expression of SRBC receptor was low or absent in a considerable part of the lymphoblasts. Interestingly, the leukaemic tumour mass was found not in the mediastinum but in the right breast. Such localization of a solid tumour-like leukaemic infiltration has not yet been reported in patients with T ALL. Since no anamnestic data were available whether the breast tumour preceded the leukaemic blastosis, or the lymphoblastoma was accompanied by leukaemia, a so-called lymphoma leukaemica cannot be excluded.

Although we are aware that the data of a single patient are never sufficient for drawing conclusions, we have presented this case because it may contribute some additional information to clinical subgrouping within T ALL.

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Abstracts

Maltosyl isothiocyanate: An affinity label for the glucose transporter of the human eryhtrocyte membrane. 1. Inhibition of glucose transport. R. E. Mullins and R. G. Langdon (Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia). *Biochemistry 19*, 1199 (1980).

Maltosyl isothiocyanate (MITC) has been synthesized from maltose with an overall yield of 88%. It has been found to be a potent irreversible inhibitor of zero trans influx of glucose with human erythrocytes. Kinetic analysis of glucose transport after treatment of erythrocytes with MITC revealed that V_T was diminished while K_T was unchanged. Transportable sugars and competitive inhibitors of monosaccharide transport protected against MITC inhibition, while carbohydrates which do not interact with the transporter gave no protection. Covalent inhibitors of anion transport were without effect on glucose transport. MITC fulfilled the kinetic requirements for an affinity label of the glucose transporter of human erythrocytes.

G. Gárdos

Maltosyl isothiocyanate: An affinity label for the glucose transporter of the human erythrocyte membrane. 2. Identification of the transporter. R. E. Mullins and R. G. Langdon (Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia). *Biochemistry 19*, 1205 (1980).

Maltosyl isothiocyanate (MITC), a potent irreversible inhibitor of glucose transport in human erythrocytes has been found to react almost exclusively with band 3 of the human erythrocyte membrane. The incorporation of [^{14}C]MITC into band 3 was found to be antagonized by transportable sugars or competitive inhibitors of transport. On the basis of [^{14}C]MITC incorporation into band 3 and MITC inhibition of transport, it is estimated that there are 3×10^5 glucose transporters present in the erythrocyte membrane. It was found that [^{14}C]MITC-labeled band 3 could be converted into ^{14}C-labeled band 4.5 during the Triton X-100 extraction procedure described by Kasahara and Hinkle. On the basis of the evidence presented here and in the preceding paper, it is suggested that in the native erythrocyte membrane a component of band 3 is the glucose transport protein and that during purification with nonionic detergents the transport protein may be enzymatically degraded with some retention of activity.

G. Gárdos

Inhibition of human erythrocyte Ca^{2+}-transport ATPase by phenothiazines and butyrophenones. K. Gietzen, A. Mansard, H. Bader (Abteilung Pharmakologie und Toxikologie, Universität Ulm, Ulm, BRD). *Biochem. Biophys. Res. Comm. 94*, 674 (1980).

Antipsychotic agents such as the phenothiazines — trifluoperazine, fluphenazine and chlorpromazine — and the butyrophenones — penfluridol, fluspirilene and primozide — which were shown to interact with calmodulin in a Ca^{2+}-dependent fashion, competitively inhibit Ca^{2+}-transport ATPase of human erythrocyte membranes. Activation of the ATPase by calmodulin is half-maximally antagonized by these agents in the concentra-

tion range of 2.6—22 μM whereas inhibition of the basal ATPase (in the absence of calmodulin) is achieved at about 10-fold higher concentrations.

G. Gárdos

Light-scattering measurements of hemoglobin binding to the erythrocyte membrane. Evidence for transmembrane effects related to a disulfonic stilbene binding to band 3. J. M. Salhany, K. A. Cordes and E. D. Gaines (VA Medical Center and the Department of Internal Medicine and Biomedicinal Chemistry and The Cardiovascular Center, University of Nebraska Medical Center, Omaha, Nebraska). *Biochemistry 19*, 1447 (1980).

Hemoglobin binding to isolated human erythrocyte membranes was studied by using light scattering. The light-scattering change induced upon binding gave saturation curves which were quantitatively comparable to those obtained by centrifugation measurements under stoichiometric conditions. Binding isotherms were constructed as a function of both hemoglobin and membrane concentrations. Two classes of binding sites were identified. A high-affinity class, consisting of $\sim 1 \times 10^6$ hemoglobin tetramer sites per cell, was shown to be coincident with the glyceraldehyde-3-phosphate dehydrogenase binding site on the cytoplasmic portion of band 3, the membrane protein involved in anion transport. A second class of $\sim 4 \times 10^6$ sites/cell had a very much lower affinity and are presumably located elsewhere on the inner surface of the membrane. Following these characterizations, DIDS [4,4'-bis(isothiocyano)-2,2'-stilbenedisulfonate] binding to externally exposed portions of band 3 is shown to alter hemoglobin binding by changing the isotherms from noncooperative to apparent negative cooperative at low membrane concentrations. These results suggest that DIDS binding to band 3, presumably at the anion transport site, results in transmembrane conformational changes which affect the affinity of protein binding sites on the cytoplasmic side of the membrane.

Ilma Szász

Human erythrocyte ankyrin. Purification and properties. V. Bennett, P. J. Stenbuck (Department of Molecular Biology, The Wellcome Research Laboratories, Research Triangle Park, North Carolina). *J. Biol. Chem. 255*, 2540 (1980).

Human erythrocyte ankyrin (band 2.1) is a polypeptide tightly associated with band 3 on the cytoplasmic surface of erythrocyte ghosts, and has been identified previously as the high affinity membrane attachment site for spectrin. This protein was solubilized in the absence of detergent, and purified in milligram quantities by DEAE-chromatography, and preparative rate zonal sedimentation on sucrose gradients. The protein migrated as a single band of M_r = 215 000 on sodium dodecyl sulfate electrophoresis, with and without reduction, and comprised 96% of the Coomassie blue-staining material. Ankyrin exhibited an apparent Stokes radius of 58 Å by gel filtration, a $s_{20,w}$ of 6.9 S by sedimentation on sucrose gradients, a partial specific volume of 0.73 cm^3/g based on amino acid composition, and an estimated frictional ratio of 1.46. Charge shift electrophoresis indicated that ankyrin contains a substantial amount of hydrophobic domain. Controlled proteolysis of ankyrin produced discrete fragments, consistent with domains in the molecule of M_r = 18 000, 27 000, 72 000, and 95 000. Ankyrin contains the M_r = 72 000 fragment identified previously as containing the membrane attachment site for spectrin. ^{125}I-labeled ankyrin was at least 98% immunoprecipitated by monospecific anti-M_r = 72 000 fragment IgG. Furthermore, ankyrin was 2- to 3-fold more active on a molar basis than M_r = 72 000 fragment in competition for binding of labeled ankyrin or fragment by anti-fragment IgG. Ankyrin formed a complex with spectrin in solution with a stoichiometry of 1 mol of ankyrin/mol of spectrin heterodimer and this association was displaced by the M_r = 72 000 fragment. ^{125}I-labeled ankyrin bound to immobilized spectrin and was displaced by 50% with 30 to 40 nm unlabeled ankyrin or M_r = 72 000 fragment. Ankyrin, but not the M_r = 72 000 fragment, formed a specific complex with soluble band 3-band 4.2.

Ilma Szász

Dissimilar modes of expression of β- and γ-actin in normal and leukemic human T lymphocytes. J. Leavitt, A. Leavitt and A. M. Attallah (Department of Health, Education, and Welfare, Public Health Service, Food and Drug Administration, Bureau of Biologics, Division of Virology and the Division of Pathology, Bethesda, Maryland). *J. Biol. Chem. 255*, 4984 (1980).

Actin polypeptides are the most abundant protein components of replicating normal human T lymphocytes, comprising 16% of the total cellular protein. Unlike other replicating cell types which synthesize equal proportions of β- and γ-actin polypeptides, T cells synthesize β-actin as the predominant species. By contrast, leukemic T cells (the Molt-4 cell line) synthesize nearly equal proportions of β- and γ-actin. In addition, novel forms of β-actin in normal T cells and of β- and γ-actin in Molt-4 cells appear to be present, perhaps resulting from a post-translational modification process. Otherwise, both divergent T cell types express a relatively common spectrum of polypeptide species, thereby suggesting that regulation of actin biosynthesis may be involved in the neoplastic transformation process.

Ilma Szász

Evidence for two insulin receptor populations on human erythrocytes. V. Herzberg, J. M. Boughter, S. Carlisle, D. E. Hill (Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas). *Nature 286*, 279 (1980).

Human erythrocytes specifically bind ^{125}I-insulin in a manner similar to cells in which insulin exerts a physiological response. In addition erythrocytes are of practical value for correlating *in vitro* insulin binding with *in vivo* carbohydrate intolerance. The competitive binding of labelled and unlabelled insulin to erythrocyte receptors is typically curvilinear when plotted according to Scatchard. The curvilinear nature of the Scatchard plot describing insulin binding to membrane receptors, although originally attributed to heterogeneous sites, has been more recently interpreted as negative cooperativity between homogeneous sites. Evidence reported here, however, suggests that there are two populations of insulin receptors on erythrocytes. Specific concentrations of concanavalin A (Con A), a lectin which mimics insulin activity, are shown here to inhibit one population of receptors leaving another population unaffected.

B. Sarkadi

Distribution of chloroquine in normal, pronase-treated and malaria-infected red cells. Y. Yuthavong (Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand). *Life Sciences 26*, 1899 (1980).

Pronase treatment of mouse red cells in the presence of chloroquine leads to greatly enhanced accumulation of the drug, which after freeze-thaw or hypotonic lysis is found to be located mainly in the membrane fraction. Much lower proportions of the drug are found in the membrane fraction prepared from *Plasmodium berghei*-infected red cells, which also have a high capacity for chloroquine accumulation. Pronase treatment of infected cells result only in a slight enhancement of total accumulation. The membrane-bound fraction of the drug is, however, increased while the fraction in the lysate is decreased. Membranes prepared from hypotonic lysis of normal or *P. berghei*-infected cells have similar capacites for chloroquine binding. These results show that the distribution of chloroquine in pronase-treated and malaria-infected cells are different and that pronase treatment of both normal and infected cells followed by lysis leads to availability of potential membrane binding sites.

B. Sarkadi

Inhibition of the red cell calcium pump by quercetin. A. Wüthrich and H. J. Schatzmann (Department of Veterinary Pharmacology, University of Bern, Bern, Switzerland). *Cell Calcium 1*, 21 (1980).

Quercetin inhibits active Ca^{2+} transport into inside-out vesicles (IOVs) of human red cell membranes with an apparent K_I of 6 μM. In the same preparation K_I for inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase was found to be 4 μM. The apparent affinity of disrupted red cell membranes for quercetin

is 5 times less than that of IOVs. Quercetin crosses the red cell membrane rapidly at pH 7.4. Quercetin is not competitive with ATP or Ca^{2+}. The action of quercetin is not specific for the Ca^{2+} transport system. The compound is equally active in inhibiting the $(Na^+ + K^+)$-ATPase of human red cell membranes. The Mg^{2+}-ATPase requires higher quercetin concentrations for inhibition ($K_I = 240$ μM) and is stimulated by 10—20% at quercetin concentrations below 50 μM.

B. Sarkadi

The erythrocyte membranes in β-thalassemia. Lower sialic acid levels in glycophorin. I. Kahane, E. Ben-Chetrit, A. Shifter and E. A. Rachmilewitz (Biomembrane Research Laboratory, The Hebrew University Hadassah Medical School, Jerusalem and The Hematological Services, Hadassah University Hospital, Mt. Scopus, Jerusalem, Israel). *Biochim. Biophys. Acta 596*, 10 (1980).

The sialic acids content of glycophorin of thalassemic erythrocyte membranes is about 25% lower than in glycophorin of normal erythrocyte membranes. Glycophorin extracted from old thalassemic erythrocytes separated by density centrifugation, has about half the sialic acids content found in glycophorin extracted from young thalassemic erythrocytes. Possible sialidase activity was sought in the plasma and erythrocyte membranes of thalassemic erythrocytes. No increased sialidase activity was detected in the plasma of the patients as compared to that of normal donors. Thus, other sites for sialidase activity, or other possibilities have to be explored to account for the increased sialic acid hydrolysis of glycophorin of the thalassemic erythrocytes.

B. Sarkadi

MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

News Item

The Joint Meeting of the 18th Congress of the International Society of Haematology and the 16th Congress of the International Society of Blood Transfusion was held in Montreal from 16th to 22nd August 1980. It was attended by more than 4000 haematologists from many countries of the world.

During the closing ceremony, Prof. M. M. Wintrobe, President of the International Society of Haematology (1978–1980) transferred this office to Prof. S. R. Hollán. She will remain president till 1982 and will be responsible for the organization of the next Congress (1st–7th August 1982) in Budapest.

The next Congress of the European–African Section of the International Society of Haematology will take place in Athens (30th August–4th September 1981) and Prof. P. Fessas will organize this meeting.

Printed in Hungary

Akadémiai Nyomda, Budapest

E. Gluckman, L. Papon, J. Hors, A. Devergie, M. Busson, J. Gony, J. Dausset

HLA markers in patients suffering from aplastic anaemia. Haematologia *14*, 165–172 (1981).

135 patients, suffering from aplastic anaemia (AA) and their families were genotyped for HLA. The antigen and haplotype frequencies were compared to an HLA genotyped control panel composed of 209 normal couples and their healthy offsprings, and to another series of 2286 normal individuals. An excess of HLA-A2 was observed in the patients: 61% versus 42% ($pc < 0.001$) (relative risk: 2) and versus 48.5% ($p < 0.01$) in two control series, respectively. When considering the HLA-A, B antigens shared in common by the parents of the AA patients, an excess of HLA-A2 was observed: 32% as compared to 17% shared by normal couples ($p < 0.001$). An excess of homozygous HLA-A2 was noted in the AA patients (14%) in comparison to the normal controls (4%) ($p < 0.001$). The mechanism of this association is discussed as well as the hypothesis of a gene involved in haematopoiesis which might interact within the HLA-A region.

E. Carapella De Luca, M. Stegagno

Blood transfusion therapy in the newborn. Haematologia *14*, 173–190 (1981).

This review deals with the various indications, the choice of blood products and the main aspects of their administration for transfusing neonates. Some special problems peculiar to neonatal age, that both neonatologists and blood transfusion services have to take into account, are emphasized. Exchange transfusion is the procedure most frequently used in blood transfusion therapy of neonatal hyperbilirubinaemia of various aetiology, severe anaemia and hyperviscosity due to polycythaemia. The procedure also represents a rational therapeutic approach in the bleeding thrombocytopenic newborn. More recently exchange transfusion has been utilized in the management of DIC, RDS and sepsis. Besides its advantages, metabolic, haemorrhagic and cardiac hazards of this "massive transfusion" are considered. Just as at any other age, the red cell preparation is the blood component most frequently utilized in the transfusion therapy of the neonate, considering not only the treatment of anaemia without hypovolaemic shock, but also the cases of iatrogenic blood loss, a common problem in the high risk neonatal intensive care unit. As transfusion of small increments of blood may often be required for the sick neonate and premature infant, different methods to cope with such conditions are discussed.

J. G. Szelényi, M. Horányi, S. R. Hollán

A new method for detecting haemoglobins with decreased stability: Tryptic digestibility test. Haematologia *14*, 191–197 (1981).

A method based on measuring the tryptic digestibility of haemoglobin has been elaborated for the detection of mutations affecting the conformation of the haemoglobin molecule. By the aid of this technique not only haemoglobins that prove labile by other procedures can be detected, but such mutants can also be discovered which confer no decreased stability as reflected by clinical and conventional laboratory examinations. The method is suitable for tackling questions for theoretical or basic research interest, whilst in a simplified form it can be applied as a routine laboratory test.

F. Haskó, R. Vassilyeva

Fractionation of plasma proteins with PEG. Haematologia *14*, 199–206 (1981).

Plasma proteins were fractionated with polyethylene glycol (PEG-4000) and the effects of pH, ionic strength and temperature were examined. Fractionation with 15% PEG was the most suitable for obtaining a precipitate containing mainly IgG, and a supernatant containing chiefly albumin. These fractions may then be purified further.

E. Faludi, V. Harsányi

The effect of Na_3-citrate on the solubility of cryoprecipitate. (Citrate effect on cryoprecipitate.) Haematologia *14*, 207–214 (1981).

Cryoprecipitate stored and frozen could be redissolved only if it contained more than 10 mmol/l Na_3-citrate. It is shown that in the insoluble residue less than 10 mmol/l Na_3-citrate is fibrinogen. In saline containing 10 to 20 mmol/l Na_3-citrate after thawing, the cryoprecipitate was dissolved entirely.

A. Poros, S. R. Hollán, K. Natonek, G. Lelkes, É. Gyódi

T acute lymphoblastic leukaemia with breast tumour. Haematologia *14*, 215–218 (1981).

Acute lymphoblastic leukaemia of L-2 type was diagnosed in an 18-year-old female with breast tumour. Her peripheral leukaemic cells reacted with anti-human peripheral T serum and part of the cells expressed SRBC receptor indicating the T origin of her leukaemia. She had a tumoral mass in her breast consisting of the same leukaemic cells. Combination chemotherapy was applied and she went into complete haematological remission with simultaneous disappearance of the breast tumour.

INSTRUCTIONS TO CONTRIBUTORS

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

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

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

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

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International Quarterly of Haematology

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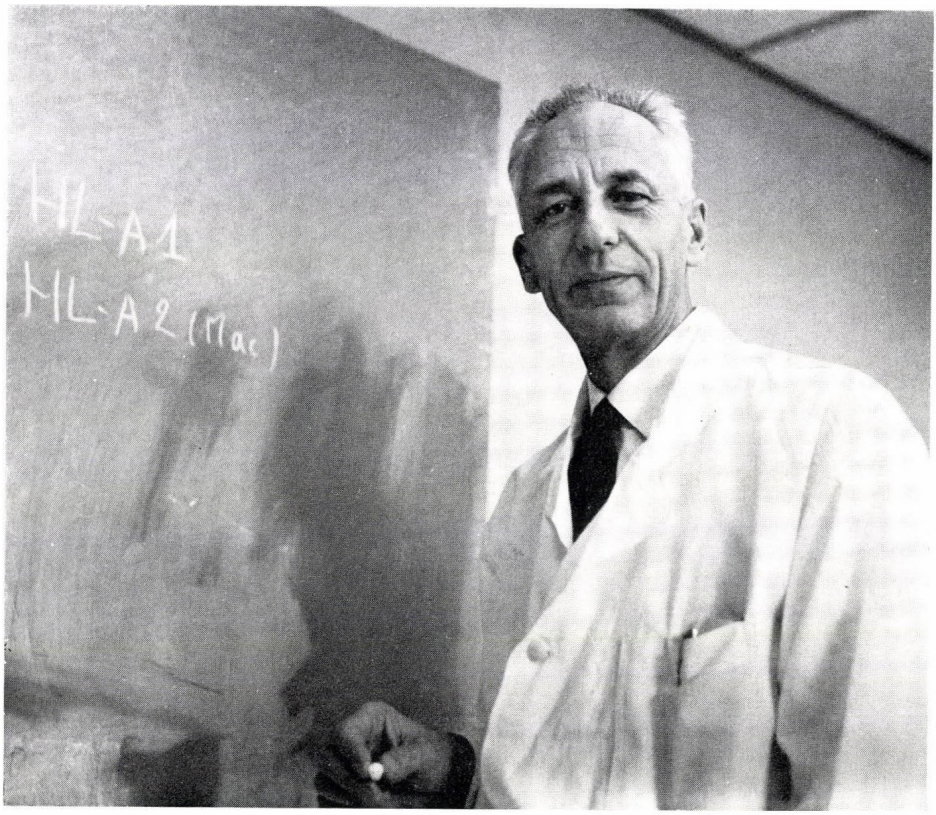
Nobel Prize Laureate
1980



Jean Dausset

An Appreciation

The 1980 Nobel Prize for physiology and medicine has been awarded jointly to George D. Snell, Baruj Benacerraf and Jean Dausset for their work on genetically determined structures on the cell surface regulating immunological reactions.

On behalf of the Editorial Board of *Haematologia* I take pride in honouring our dear Friend and member of our Editorial Board, Jean Dausset on this occasion.

Jean Dausset is not only an outstanding immunogeneticist, but his education and research career is also deeply rooted in haematology and blood transfusion. In the mid 1950s, he focussed his attention on research of antibodies agglutinating the leukocytes in leukopenic patients, and antibodies agglutinating or lysing platelets in thromobocytopenic patients. He soon realized that these were in fact alloantibodies also present in the sera of polytransfused patients and pregnant women. His genius led him to recognize that he had encountered a previously unknown type of genetic polymorphism of human leukocytes which might be of great importance in transplantation. He spared no effort to collect and qualify sera to characterize the new alloantigens of the system. On the basis of family analyses he was able to show that the variation was determined by a single genetic system, localized to a single chromosome.

He described the first human leukocyte antigen and named it "Mac". This was the first antigen of the major human histocompatibility complex (the HLA region), and is now known as HLA-A2. Together with many outstanding scientists in HLA research he was most active in defining the HLA-A, B and C antigens and in analyzing the structure of HLA gene regions. He pioneered in focussing attention to the association of HLA antigens with disease and in applying HLA testing to find the most compatible donor-recipient combinations for transplantation.

One of his most important contributions was to point to the basic biological role of the HLA system in immunological, evolutionary and cell differentiation processes in the cooperation between different cells, and in autorecognition by all cell types. The wealth of results from many outstanding laboratories in this field of research has revealed that the major histocompatibility complex provides a uniquely sensitive system for detecting cells with changed membranes and for killing alienated cells.

It is extremely rare to witness such immediate clinical application of fundamental discoveries. I take great pleasure in congratulating Jean Dausset on this very unique privilege in a scientist's life. He deserves this privilege not only for his outstanding scientific achievements but also for his fine intellectual and noble human qualities which have earned him the admiration and affection of his many friends and colleagues throughout the world.

Susan R. Hollán
Editor-in-Chief

Biography

Name: Jean Baptiste Gabriel Joachim *DAUSSET*

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Education		*Year of Graduation*	*Degree*
College:	Lycée Michelet	1939	A.B.
Medical School:	University of Paris	1945	M.D.
	Harvard Medical School	1948	
Internship and Residency			
	Paris Municipal Hospitals (Hôtel-Dieu, St. Louis, Broussais, St. Antoine) Internal Medicine and Haematology	1946–1950	

Hospital Appointments

Institution	*Title*	*Year*
National Blood Transfusion Centre	Director of Laboratories	1946–1963
Paris Municipal Hospitals	Biologiste des Hôpitaux	1963–1978
Institute for Research into Diseases of the Blood	Co-Chairman	1963–1968
Institut Nationale de la Santé et de la Recherche Médicale	Director of Research Unit "Immunogénétique de la Transplantation Humaine"	1968–present
CNRS	Co-Director, Oncology and Immunohaematology Laboratory	1978–present

Teaching Appointments

University of Paris, Faculty of Medicine	Associate Professor	1958–1968
	Professor of Immunohaematology	1963–1977
College de France	Professor of Experimental Medicine	1977–present

Honorary Teaching Appointments

New York University	Visiting Professor	1970–1971
University of Brussels	Professor Honoris Causa	1977
University of Geneva	Professor Honoris Causa	1977

Membership of Academies

Honorary foreign member of the Belgian Royal Academy of Medicine	1969–present
Académie des Sciences de l'Institut de France	1977–present
Académie de Médecine	1977–present

American Academy of Arts and Sciences; elected foreign honorary member	1979
Yugoslav Academy of Arts and Sciences; elected honorary member	1979
Hungarian Academy of Sciences; elected honorary member	1981
American Academy of Sciences	1981

Advisory Groups

Scientific Advisory Councillor of the Minister of Education	1946–1950
Member, National Advisory Research Council	1968–1969
Conseil Scientifique, Pasteur Institute (Vice-President)	1975–1979
WHO expert in Immunology	1974–present

Societies

Société Française d'Immunologie	elected President	1978–1981
Société Française d'Hématologie	President	1971
Transplantation Society	Secretary	1966–1970
	Vice-President	1976–1978
Société Française de Transplantation	elected President	1978–
British Society of Immunology	Member	1965
Société Française de Transfusion	Member	1964

International Meetings

International Tissue Transplantation Conference of the New York Academy of Sciences	Co-Chairman	1966
First Congress of the Transplantation Society	Co-Chairman	1967
Fifth Workshop and Conference on Histocompatibility	Secretary	1972
Sixth Workshop and Conference on Histocompatibility	Chairman	1975
Fourth International Congress of Immunology	President	1980

Awards and Prizes

Grand Prix des Sciences chimiques et naturelles (Académie des Sciences)	1967
Médaille d'Argent du Centre National de la Recherche Scientifique	1967
Grande Prix Scientifique de la Ville de Paris	1968
Prix Cognacq-Jay (Académie des Sciences)	1969
Stratton Lecture Award	1970
Landsteiner Award AABB San Francisco	1970
Gairdner Foundation Prize	1977
Koch Foundation Prize	1978
Wolf Foundation Prize	1978
Nobel Prize	1980

Decorations

Legion of Honour, Officier	1969
Ordre National du Mérite, Grand Officier	1977
Palmes Académiques, Officier	1958
Médaille Coloniale (Tunisia Campaign)	1945
Dutch Red Cross	1970

Military Career

Captain, Medical Corps, French Army Reserve		
Second World War:	French Campaign	1940–1941
	Tunisia Campaign	1944
	Normandy Campaign	1945

Medical Literature

Immuno-Haematology, Biological and Clinical Aspects, Vol. 1, 719 pages, Flammarion, Paris	1956
Human Transplantation, edited in collaboration with F. T. Rapaport, 1 728 pages. Grune andStratton, New York	1968
Histocompatibility Testing, edited in collaboration with J. Colombani, Munksgaard, Copenhagen	1972
Histocompatibility, in collaboration with G. Snell and S. Nathanson, Academic Press, New York	1976
HLA and Diseases, edited in collaboration with A. Sveejgaard, Munksgaard, Copenhagen	1977

About 300 scientific papers.

Haematologia 14 (3), pp. 231–245 (1981)

The Porphyrias — A Review

J. WALDENSTRÖM

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(Received November 20, 1980)

The development of porphyrias is discussed, based on the author's personal experience. Already in 1956, metabolic blocks were presented as a possible explanation for the different porphyrias. This has been confirmed by recent research on disturbances of enzyme function. These studies are discussed at some length.

*

Introduction

In 1911, the German author Hans Günther wrote a monograph on what he called "Hämatoporphyrie". The basis of this group of diseases should be common constitutional defect called "Porphyrismus". The two porphyrias were haematoporphyria congenita and haematoporphyria acuta. The presence of chronic cases was also discussed. In 1925, Günther wrote another monographic discussion of these diseases and treated in great detail the congenital haematoporphyria that was later regarded as Günther's disease [1].

The word porphyria means a genetically determined condition with a disturbance of porphyrin and/or pyrrole metabolism. Porphyrinuria is purely symptomatic and develops in all organisms under the influence of certain substances such as lead. The porphyrins have a very central position in the living organisms. Their structure with many alternating double bonds gives them not only a very strong colour with characteristic spectral properties but also a strong fluorescence in UV light. It is remarkable that the red of the blood in the animal kingdom as well as the green in the leaves of the plants should be caused by porphyrins. When studies of patients with porphyria led to the discovery of porphobilinogen as a precursor, botanists were able to work out the synthesis of chlorophyll that passes through the same chain of metabolites. It has also been found that vitamin B_{12} with its central position in DNA metabolism contains a ring of four pyrroles – the so-called corrin ring – that is in many ways similar to the porphyrin nucleus. This vitamin also contains a metal atom bound to the ring. Cobalt in this case, whereas haeme contains iron, and chlorophyll, magnesium. The porphyrin ring must have unique properties that may be important in several connections.

I became interested in the porphyrias in the early thirties and published, in 1934, some observations on eleven persons with what we now call acute intermittent porphyria [2].

When I started work on this disease there were only five cases known from Sweden. In that paper I made the remark that "the lack of positive evidence makes it necessary to leave the question of the hereditary constitutional liability to acute porphyria to the future". We later found on systematic questioning that this disease is dominantly hereditary and that there are many families in whom several sibs have died with exactly the same symptoms. Reliable information regarding heredity in a certain patient can only be obtained if there are positive data about diseases and causes of death in all family members.

Some years later, in 1937, our experience was collected in a monograph on the subject [3]. I could then give the case histories of 100 Swedish porphyriacs and described family members, who had never had any clinical signs but still excreted typical pyrrole substances in their urine. I regarded them as latent porphyrics and it could be shown that some of them later developed many manifest symptoms of the disease.

Our studies have been continued during the four decades together with Birgitta Haeger-Aronsen and others. The diagnostic possibilities were much improved after the introduction of quantitative methods for the determination of delta-aminolaevulinic acid (ALA) and porphobilinogen (PBG). The disease is now generally called acute intermittent porphyria (AIP). Even if it is clear that this is a chronic metabolic disturbance with primary excretion of large quantities of a pyrrole substance (PBG) and its precursor (ALA), the old name porphyria must be kept, although it is illogical.

Acute intermittent porphyria, AIP, has been known for a long time and was discussed in a monograph by Günther already in 1925. In 1937, the present author established the fact that PBG is a good marker of the trait. Much later, in 1956, Granick and van den Schrieck demonstrated the presence of ALA in the urine of acute porphyrics [4]. In 1957, Haeger-Aronsen discovered ALA in the urine from lead-poisoned persons [5] and the connection between this substance with colics, neurological symptoms, urinary excretion of coproporphyrin also in non-porphyrics, was thus established. The fact that ALA might be the toxic substance in the severe porphyria attack was thus made plausible.

In the meantime, a large number of patients with a history of acute porphyria was published by Dean from South Africa [6]. All these patients had the typical signs of "Swedish" acute porphyria, but at the same time they showed light sensitivity. We have never seen any cutaneous symptoms in our patients in Sweden and we believed that the diseases were different.

In the United States, Cecil Watson studied a very large material of patients with typical symptoms and some of them also showed light sensitivity. It therefore became clear that both types, "South African" and "Swedish", are current in the United States. Watson discussed the possibility that Swedish porphyrics did not develop any sign of light sensitivity because of the poor sunshine in Sweden [7]. These problems were the subject of much discussion and the South African porphyria was given many names before it had finally been settled that the pattern of porphyrin excretion with the faeces was really different in the two so-called

"types" of porphyria. Barnes, Dean, and Eales and our group compared our results and it became clear that patients in South Africa always excreted considerable quantities of porphyrin in the stools [8, 9]. Their faeces showed bright red fluorescence in UV light. This porphyrin was mostly protoporphyrin and was not found in the faeces from the Swedish families. Much later some families with typical South African porphyria were found in Sweden and the South African clinicians also detected a few family members with the Swedish type in their country. The name porphyria variegata, PV, was coined for the disease in South Africa. To my mind the most important finding in this work was the firm establishment of the fact that patients suffered from either one or the other of these diseases when they belonged to the same family. Both the clinical and the biochemical picture remained true to type. One difficulty in this early work was that patients who were carriers of the variegata trait, often did not excrete any increase in ALA and PBG between the acute attacks. Carriers could therefore only be detected by analysis of the urine.

In 1939, the present author published a paper with a clinical analysis of a number of patients who suffered from severe neurological symptoms of acute porphyria [10]. The histories showed that practically all patients had been treated with barbiturates or other types of sleeping pill. This was probably the first description of a genetically determined sensitivity to certain drugs. Pharmacogenetics was much developed during the war when primaquine sensitivity became well studied in the American army (see later).

In 1957, the author discussed porphyria and drugs at a symposium [11], on the basis of therapeutic data regarding 100 patients with acute porphyria. Twenty-two of these persons had died from acute porphyria. It is possible that only one had never been treated with barbiturates; this patient died in coma that may perhaps have been caused by inappropriate ADH. The very remarkable improvement in prognosis when we have learnt to avoid barbiturates is demonstrated in a table summarizing 219 Swedish cases of AIP. We found in the decade 1927 to 1937, 19 members of the big Swedish family with AIP had died from the disease. During the next decade, when sedatives had been avoided, only two died.

From our work together with A. Engel in Boden and B. Haeger in Malmö it became clear that the concentration of AIP found in the northernmost part of Sweden was very marked. This was caused by the occurrence of the disease in some very big families in this sparsely populated part of the country. The biggest family, originating from a couple married in 1672, now contains some carriers of the trait. The geography of the porphyrias has been treated by the author [12]; it was demonstrated that the trait was dominant. At the same time, South African workers were able to observe the same heredity in the offspring of the early Dutch settlers in the Cape province of South Africa. As a matter of fact the very large majority of patients with acute porphyria in this part of the world are descendants of one couple also married in 1672.

The rule that the different porphyrias remained true to type has been very

important for the delineation of new inherited diseases. As a matter of fact, a metabolic disturbance caused by a gene occurring in one family is probably one of the best examples of a really homogeneous cause of a disease. This does not mean that AIP in members of different families is necessarily caused by the same point mutation in the AIP determining gene. The same obviously holds true for South African porphyria. It is even probable that different point mutations in the same gene may cause more or less severe symptoms. This is sometimes called penetrance – an obscure expression. I have earlier made the parallel with haemophilia. We have haemophilia A, B, and C, and at least in A and B there are families where all afflicted members have severe, medium or slight symptoms. Some of these problems will be discussed later in this paper.

Several studies have been performed in order to decide the occurrence of the two main acute porphyrias in different parts of the world. It is clear that acute abdominal porphyria is common in Sweden and in South Africa. The two conditions show certain differences, however, and it is well known that they both occur with about the same frequency in England, Germany, the Netherlands in Europe as well as in the USA. Among the Scandinavian countries, Finland has been carefully investigated by Mustajoki and Koskelo [13]. During nine years, 107 patients with AIP and 45 with the South African type were found. These 152 patients belong to 42 different families. The two different diseases with symptoms of acute porphyria are both connected with increased excretion of ALA and PBG in the urine during the attack.

There is, however, another type of acute porphyria with excretion of coproporphyrin in the stools and sometimes also in the urine. The disease was first described by Watson et al. [14] and later by Berger and Goldberg in 1955 [15]. Between attacks there is no excretion of ALA and PBG in the urine. The disease often starts in childhood. This is extremely rare in the other acute porphyrias. Also in this inborn error of metabolism, called hereditary coproporphyria, HCP, the symptoms remain true to type. The disease is quite rare but occurs in many countries both in Europe and in America. We have seen only one rather big family in Sweden with one manifest and 13 latent cases. This seems to be typical of the condition [16]. Recent developments regarding this porphyria will be discussed later. It seems that my case XI in the first publication and the same (case I) in the monograph (1937) belong to this group.

It is clear that the so-called congenital form of porphyria, CP (Günther's disease) causes very severe skin symptoms. It has never been found in Sweden.

In the early literature, cases were published where the patients did not have any sign of acute porphyria but developed symptoms of light sensitivity rather late in life. Günther had discussed the problem whether there was what he called a "chronic haematoporphyria" with light sensitivity [1]. A considerable confusion was caused by the fact that South African porphyria was not recognized as a clinical entity at that time. It was typical that the light sensitivity usually developed late in these patients and the condition was therefore given the name porphyria cutanea tarda, PCT, by the author in 1937, and this has been the designation used

ever since [3]. The condition occurs chiefly in elderly males and the influence of alcoholism is evident. There are also signs of liver involvement but severe cirrhosis with ascites is rather rare [17].

In a series of important studies regarding iron metabolism in PCT, Lundvall et al. performed liver biopsies on patients and their sibs and were able to demonstrate marked haemosiderosis and strong fluorescence in UV light in the liver from some of the clinically healthy sibs [16].

The last important addition to the group of different porphyrias has been called erythropoietic protoporphyria, EPP. This condition was first described in Germany in 1954 [19]. The clinical picture was clearly defined by British authors in 1961, and they gave the name to the disease because of the presence of protoporphyrin in the red cells [20]. The trait is hereditary (see below).

The complete list of the known porphyrias is found in Tables 1 and 2.

Table 1

Disease	Symptoms			Urine		Faeces	Treatment
	Skin	Abdomen	Neurology	ALA	PBG	P	
Acute intermittent porphyria	−	++	++	++	++	−	Haem, negative feedback
Porphyria variegata	+	++	++	(+)	(+)	+	Haem, negative feedback
Copro-porphyria	+	+	+	(+)	(+)	+	Haem, negative feedback
Proto-porphyria	+	−	−	−	−	+	Carotene (exchange transfusion)
Porphyria cutanea tarda	++	−	−	− (Urine porphyrin ++)	−	++	Chloroquine (avoid Fe, alcohol, oestrogens)
Porphyria Güntheri	+++	−	−	− (Urine porphyrin ++)	−	++	−

Abbrevations: ALA = delta-amino-laevulic acid
PBG = porphobilinogen
P = porphyrin

Table 2

Drugs	Disease	Heredity	Animals	Skin
++	Acute intermittent porphyria	Dominant	Human	−
++	Porphyria variegata	Dominant	Human	+
+	Coproporphyria	Dominant	Human, bovine	(+)
− Fe?	Protoporphyria	Dominant	Human	+
Fe, alcohol, oestrogen	Porphyria cutanea tarda	+ ?	Human	++
−	Porphyria Güntheri	Recessive	Human, bovine (porcine) (sciurus) (asio)	+++

Present-Day Taxonomy of the Porphyrias

There is no question about the fact that medical diagnostics are often built on very artificial foundations. Our concepts of many diseases are not always more than pictures existing in our own minds. On the other hand, the establishment of pigeon-holes for the classification of sick persons is absolutely necessary and may be very sound, if our systems are revived, and improved when new facts are discovered.

It is rather surprising that the first type of so-called haematoporphyrinuria should have remained unchanged since Günther described it in 1911. It is characterized by extreme light sensitivity, sometimes leading to severe mutilation of nose, ears, and fingers, maximal excretion of uroporphyrin in a bright red urine and also much porphyrin in the stools. The condition is present from birth, the diapers are stained red, and the teeth are brown with a strong red fluorescence in UV light. Other bones too are brown from the uptake of uroporphyrin. This condition has been called porphyria congenita, PC, Mb. Günther, and is definitely inherited as a recessive trait [2]. As a matter of fact this is the only example of recessive inheritance among the porphyrias. It is certainly one of the rarest inherited diseases in the world. In 1965 R. Schmid revised all published cases and only accepted 34 [22]. The other patients certainly had PCT. This latter disease is still sometimes mistaken for congenital porphyria.

In 1954, Schmid, Schwartz and Watson divided the porphyrias into hepatic and erythropoietic forms and they found that PC was erythropoietic as the normoblasts in the bone marrow show strong uroporphyrin fluorescence in UV light [23]. Almost all organs are full of different porphyrins as was shown in the monograph by Borst and Königsdörffer describing the post-mortem findings in the famous patient Petry [24].

I have preferred the simpler clinical designation, cutaneous porphyria and acute abdominal porphyria, according to the dominant symptoms. There is also

another more sophisticated reason for not talking about organs such as liver or bone marrow. The enzymatic defects are present in all cells of the body. This has been beautifully demonstrated by the use of red cells, fibroblasts, lymphocytes and liver cells for the investigation of defective enzyme systems. The mutant gene is of course present in all cells of the body. A recent example of the difficulties connected with liver *versus* bone marrow is the fact that the so-called protoporphyria erythropoietica has had to be rebaptized to erythrohepatica. This makes the division more or less meaningless and I shall continue to talk about cutaneous and acute abdominal prophyrias without skin symptoms.

Genetics and Mechanisms

Günther already discussed the group of cases that he called chronic as opposed to acute, but the word chronic does not mean very much, as all porphyrias are inherited chronic conditions, some of them with acute attacks. I therefore thought that we should divide the porphyria patients with marked skin symptoms into congenital and late (porphyria cutanea tarda). The symptoms never occur early. Most cases seem to be sporadic and it is evident that exogenous factors are of great importance. Together with Birgitta Haeger-Aronsen we noted a few persons in the families, who also had disturbances of porphyrin metabolism and we published our observation of concordant monozygotic twins [25].

In 1956 and 1957 the author published two papers treating the porphyrias as inborn errors of metabolism [26]. In 1959 I discussed the same subject under the title Genetics and Medicine at the Ciba Foundation Symposium on Significant Trends in Medical Research [27, see also 12].

In the early fifties a work by Beadle and Tatum already indicated that the common link between the different inborn errors of metabolism was a metabolic block caused by deficient function of one important enzyme in the metabolic chain. Such blocks could lead to increased concentrations of metabolites immediately before the block and possibly to defects in the formation of normal metabolites behind the block. When the Shemin cycle was established as the synthetic highway to haeme, the porphyrias were established as building-blocks and as disturbances of synthesis, respectively. In 1956, the present author published in a paper a schematic figure demonstrating the possible connections between disease patterns and metabolic blocks [29]. Recent enzymatic work has confirmed these ideas in detail (see Fig. 1). It is perhaps surprising that this inherited metabolic defect should almost never give rise to symptoms before puberty.

Starting from the fact that 50% of the children with one parent suffering from acute porphyria must receive the trait, Birgitta Haeger-Aronsen and the author collected urine samples from a number of such children. It was then found that the values for ALA and PBG in the youngest group were practically normal. In the intermediate age group some urines contained pathological amounts of PBG, but normal ALA, whereas in the group nearest to puberty about half had

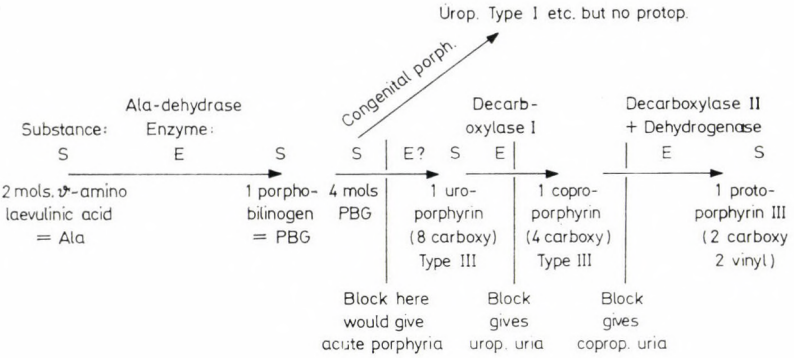

Fig. 1. A tentative scheme proposed by the author [29] to explain different types of porphyria [By the courtesy of *Acta genet. (Basel)* 1956]

a supranormal content of PBG [25]. This seems to confirm the hypothesis that the block was situated immediately before uroporphyrin.

In 1970 Strand, Felsher, Redeker and Marver found that in AIP the conversion of PBG to uroporphyrin was low but the activity of ALA was high [30].

In 1972 Urs Meyer et al. could demonstrate that the capacity to form uroporphyrin in a medium containing PBG was low, when cells from patients with AIP were cultured [31]. This was the first proof of an enzyme being defective in this disease. The results have been confirmed by a number of different authors, and determination of uroporphyrinogen III synthetase is now widely used for the diagnosis of the trait.

At the meeting of the American Association for Clinical Research in Washington in the spring of 1980, R. J. Desnick from Mount Sinai Hospital presented his work on the characterization of a seemingly homogeneous such synthetase from human erythrocytes [32]. The enzyme was purified 42,000 times. On isoelectric focussing, five different fractions were observed, but it was established that all types were reduced by 50% in AIP. Most promising were the attempts to produce an antibody against the enzyme, and it seemed as if several patients with AIP had a protein that reacted with the antibody, but was inactive as an enzyme. This would of course fit in well with the assumption that there is a point mutation in a structural gene that is responsible for the formation of a defective enzyme.

It is interesting to compare the haemoglobinopathies, where we know exactly the site of the point mutation, and the hereditary enzymopathies. We know that changes of nucleotides in the same place in the DNA chain may give rise to completely different clinical pictures. Sickle cell haemoglobin and haemoglobin C are such examples. We also know that changes in one nucleotide at different sites in the chain may give similar clinical results.

It seems therefore probable that several exchanges of nucleotides in the gene coding for the uroporphyrinogen synthetase may cause the disease that we

call AIP. Such changes in the enzyme structure may have different effects on the activity and it is possible that what we call penetrance may be explained by more or less severe disturbances of enzyme structure and function. Biochemically, these are all different mutations. This shows how futile it is to calculate mutation frequencies, *sensu strictiori*.

Interestingly, all the different porphyrias seem to be explained by deficiencies in enzyme function causing blocks, if we regard deficient cosynthetase activity as a block. These results have been obtained in tissue cultures of cells from patients and animals and by studying their effects on isotopically labelled substances. In recent years work on different porphyrias in animals has become of great importance for such enzymatic work. Bloomer in Minneapolis has seen clinically typical protoporphyria in cows [33]. Normal animals have a much higher normal enzymatic activity than normal man. The heterozygotes have a low activity that is about half the normal, whereas the homozygotes are clinically affected. Such findings indicate that the words dominant and recessive are somewhat ambiguous, whereas single and double doses of the pathological allele seems to be a better description.

In man, both Elder et al. [34], and Grandchamp and Nordmann [35] were able to demonstrate that fibroblasts from patients suffering from hereditary coproporphyria show a low activity of what is now called coproporphyrinogen oxidase. This causes a block at the level where coprogen loses two carboxyl groups and is also oxidized to protogen, with the formation of two vinyl groups. The process takes place inside the mitochondria, whereas the other processes occur in the cytoplasm.

Pharmacogenetics

It has already been mentioned that the porphyrias may offer interesting examples of inherited sensitivity to certain drugs. The fact that all inborn errors of metabolism are caused by some structural malformation of certain proteins (enzymes and similar active substances) explains the finding that a number of drug reactions are inherited. When the metabolism of a certain drug is disturbed, new and possibly toxic metabolites may occur or the excretion of the compound itself may be inhibited. Classical examples of this kind are the so-called slow acetylators. These persons have a deficiency in acetylating enzymes and this causes accumulation of certain drugs that are acetylated in a normal person. Hydralazin lupus is a classical example.

The metabolic chain in haeme synthesis represents a number of steps where metabolic blocks may appear. At least five such strategic points are known as explanations of six different porphyrias. It may be interesting to speculate upon the fact that there are other early steps in the chain, where a block may account for defects in haeme formation. If the transition from ALA to PBG is disturbed, this should make the synthesis of haeme defective and would therefore cause

anaemia. It does not seem improbable that enzymologists in the future will explain certain inherited aplastic anaemias by this mechanism. It is also interesting to speculate on the anaemia in chronic plumbism. Recent work has shown that patients with tyrosinosis develop metabolites that may inhibit the condensation of ALA to PBG. This is of course a metabolic block but not an enzymatic block as in the classical errors of metabolism. There are therefore other possibilities of blocking [36].

At the present moment the first block in the chain is represented by the enzyme that condenses four molecules of PBG into uroporphyrinogen III. Waldenström and Vahlquist were able to show that such a condensation occurs also in the absence of an enzyme [37]. Solutions of PBG that are acidified and boiled develop a strong red colour due partly to the formation of uroporphyrin, partly to the development of porphobilin that is certainly a polymer of the monopyrrole.

It has been shown that the enzyme in this step (uroporphyrinogen I synthetase) is deficient in patients with acute porphyria [30, 31]. It is therefore only logical that both blood and urine contain increased amounts of the two precursors ALA and PBG. The group in Granick's laboratory at the Rockefeller Institute led by Kappas was able to show that barbiturates, for instance phenobarbital, induced increased synthesis of ALA synthetase, leading to a higher production of ALA and secondarily of PBG [38]. When the block occurs after PBG, the contents of the two precursors will rise in the body. Many data are in favour of the assumption that ALA poisoning is responsible for the clinical symptoms in the acute porphyric attack.

The problem of enzyme induction is one of the most enigmatic in biochemistry, but it is a well-known fact that barbiturates may induce a number of hepatic enzymes beside the enzyme we are discussing. This effect may be used for therapeutic purposes, for instance in the so-called Crigler–Najjar syndrome. In this condition the conjugation of bilirubin to form glucuronides is disturbed because the specific transferase is defective. Phenobarbital treatment induces enzyme formation and improves the hyperbilirubinaemia. This may protect the children against the so-called kernicterus. It was shown that also salicylamide, administered during the experiment, was not normally conjugated to glucuronide in the child before treatment with phenobarbital had been instituted [39]. I am quoting this example in order to show that the process of enzyme induction by barbiturate in the liver is not in any way unique for ALA synthetase.

The logical way of testing whether certain drugs are detrimental to porphyrics would of course be to make studies of their enzyme-inducing capacity. If they have no such effect, they ought to be harmless. As it is, the list of possibly harmful drugs in patients with acute porphyria is very long, and I think it is safe to state that most synthetic drugs should be avoided. Opium alkaloids and similar synthetic products such as pethidine, have no influence on porphyrin metabolism and may be used — with the usual obligatory precautions — during the acute stage.

The most common porphyria in clinical medicine is porphyria cutanea

tarda, PCT, a disease important from the pharmacogenetic point of view. As already stated, the enzymatic defect is not too well known and heredity has not been established except for a limited number of cases [see 40 and 41]. On the other hand, it is clear that certain substances are quite detrimental for patients carrying this trait. The disease occurs in four types of persons, 1) alcoholics [17], 2) women in fertile age [40], 3) conditions of overload with iron [41], 4) prostatic carcinoma [42].

For many years we have maintained that alcohol is the most important factor in the development of PCT. On the other hand, we have seen patients who were probably teetotallers and still developed the disease. It is therefore quite probable that the importance of alcohol was somewhat overestimated. The very marked overrepresentation of males and the fact that many patients are well after stopping alcohol intake is a strong argument for abstention in patients with PCT.

Another group of elderly males with PCT has been discovered in recent years. Patients with prostatic carcinoma who have been treated with oestrogens develop PCT in a small percentage. The trait is obviously quite rare, but the connection between this form of therapy and PCT cannot be denied [42]. The correctness of this assumption is strengthened by the fact that PCT has developed in non-alcoholic young women who are using contraceptive pills. It is tempting to assume that also here the oestrogen is responsible [43].

The fact that acute porphyria with symptoms is seen about twice as often in women as in men is still a mistery. It is also a fact that an attack hardly ever occurs before puberty. The possibility that the presence of oestrogens could explain these two observations may well be discussed, but is difficult to prove. The effect of oestrogens in the acute porphyrias has been much debated. The common belief is that contraceptive pills should be avoided. Some authors, including ourselves, have seen remarkable improvement in the chronic symptoms of some AIP patients after starting contraceptive pills. This problem is well worth further analysis.

Let us go back to PCT. Iron overload was quoted as one possible factor. Especially in the work of Lundvall and collaborators it has clearly been proved that hepatic iron stores are very large in PCT patients and improvement of this hepatosiderosis by large and repeated blood-lettings alone may cure the disease [18]. It is possible that single persons have developed PCT after very massive medication with iron. We have seen one patient, who was probably not an alcoholic. She suffered from chronic acquired haemolytic anaemia and developed typical PCT [44]. It is well known that hepatic iron stores increase in hyperhaemolytic conditions. Some similar observations have been published. My explanation has been that the increase in hepatic iron has induced a previously latent PCT.

One of the most fascinating examples of paraneoplasia is the fact that certain liver tumours may induce PCT even in the absence of liver cirrhosis. I have discussed this problem in some detail in a recent study of paraneoplasia [45]. It seems that the tumour cells might lack decarboxylases that should have

favoured the transformation from uro to copro to proto. Metabolic analysis of such very rare cases would certainly be rewarding. One elderly Dutch lady was cured from her PCT after removal of the tumour [46]. The causality is therefore established and the tumour contained large amounts of porphyrins, whereas the normal liver was free.

Another interesting and, from a practical point of view, important effect of a certain drug on patients with PCT is worth noting. South African authors found that a patient with PCT had to be treated with chloroquine for a collagen disease. He had a flare-up of his porphyrin symptoms and developed a swollen tender liver and fever [47]. The drug was therefore added to the list of prohibited compounds. Several years later, other authors developed the idea that perhaps small doses of chloroquine may be used for the treatment of PCT [48]. In fact this is at the present moment certainly the best treatment we have beside the prohibition of certain detrimental factors and blood-letting [49].

The last porphyria in the chain to haem has the metabolic block located after protoporphyrin [50]. The deficient enzyme is obviously catalyzing the addition of iron and is thus a ferrochelatase. I prefer to call this disease simply protoporphyria. It was originally regarded as erythropoietic but is certainly also hepatic. In this condition, liver function may severely be disturbed and a number of patients have died from liver failure [51]. The liver is full of protoporphyrin and there is certainly a disturbance in the enterohepatic passage of protoporphyrin. Cholestyramine may be very effective and fasting is certainly detrimental. The patient should carefully be observed as regards symptoms of liver failure and cholestyramine should be given prophylactically.

Therapeutic Attempts

From what has already been said, it seems as if the abstention from harmful factors would be the most important way of improving prognosis.

In my monograph from 1937 and in papers in Swedish during the following years I described the symptomatology and warned against using synthetic drugs, above all barbiturates. It was clear that most patients were diagnosed in surgical and gynaecological departments and increasing knowledge about the disease and the dangers of certain treatments soon improved conditions. It is rather remarkable that a disease, where we had no positive treatment, only avoidance of iatrogenic injuries, should improve so much as regards prognosis.

In later years some new data have influenced our therapeutic thinking. Tschudy has studied effects of diets, high or low in carbohydrates, and recommended a high intake of glucose [53]. These ideas are founded on careful experiments. I have always been surprised by the fact that patients with an acute severe attack are so sensitive to psychotherapy if they are transferred to a hospital that gives them positive information and encouragement. In their previous anxious state they were practically all starving and began to eat when their anxiety had

been overcome. It is also interesting that another porphyria, protoporphyria, should easily be decompensated by fasting and improved on giving carbohydrates.

The latest and most striking treatment of acute porphyrias is built on the idea of a negative feed-back from the last product in the metabolic chain. If haeme is given intravenously in large – but not too large [54] – doses, the production of ALA and PBG is limited by effects on the rate-limiting enzyme ALA synthetase. This has now been tested in many patients with accompanying clinical and biochemical improvement. It is a rather heroic treatment but should be used in severely ill patients. Prophylaxis is of course most important.

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Diagnosis and Treatment of the Immune Hemolytic Anemias*

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Immune hemolytic anemia can be treated by blood transfusion, steroids, cytotoxic drugs, plasmapheresis, and splenectomy. However, the benefits of therapy are dependent upon the relationship of treatment to the etiology of disease. Thus, effective therapy requires sufficient diagnostic precision to distinguish between allogeneic and autologous antibodies, recognize the etiologic role of immunogenic drugs, and define the immunoglobulin classes of both cold and warm reactive autoantibodies along with their complement interactions.

*

Introduction

Immune hemolytic anemia is a very heterogeneous group of disorders each of which is characterized by a decreased number of circulating erythrocytes due to antibody mediated hemolysis *in vivo*. True intravascular immune hemolysis is not seen very often, and most of the immune destruction of red cells that occurs *in vivo* is cellular, principally due to the phagocytic activity of macrophages. These cells can bind, ingest, and lyse red cells that carry either large numbers of IgG_1 or IgG_3 antibody molecules or a small number of such antibodies in the presence of cell-bound C3b [1, 2, 3].

Immune hemolytic anemias are readily classified into four groups: (1) caused by alloantibodies, (2) associated with cold-reactive autologous antibodies, (3) associated with warm autologous antibodies, and (4) drug induced. There is considerable heterogeneity in each of these categories, and this is especially noteworthy for both cold agglutinin disease and warm autoimmune hemolytic anemia.

There are five modalities of therapy for the immune hemolytic anemias. These are blood transfusions, plasmapheresis, steroids, cytotoxic drugs, and splenectomy. However, the choice of therapy is strongly dependent on the etiological character of the disease so that accurate diagnosis becomes essential before appropriate therapy for immune hemolytic anemia can be selected.

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A. Alloantibody Induced Hemolytic Anemia

1. *Erythroblastosis fetalis*

The simplest and best understood form of immune hemolytic anemia is erythroblastosis fetalis due to maternal Rh antibodies. These antibodies, being IgG, cross the placenta and shorten the survival of fetal red cells. Today, although we do not understand the mechanism of protection, disease due to anti-Rh1 (D) is largely prevented by administering Rh antibodies to non-immunized postpartum Rh negative mothers who are at risk of becoming actively immunized to Rh [4]. When mothers are already immunized, the involved fetus or newborn is treated with transfusions of compatible blood. In the fetus, successful intrauterine transfusions supply enough erythrocytes to prevent early fetal death and allow the delivery of a reasonably mature newborn [5]. Exchange transfusions [6] and phototherapy [7] are used in the neonatal period. These may also be life-saving. The transfusions combat severe hemolytic anemia and both procedures control hyperbilirubinemia to prevent permanent brain damage [6].

Another form of erythroblastosis, ABO disease, continues to affect about 1 in 200 live births, principally the non-O babies of some type O mothers [8]. Unlike Rh antigens which are expressed only on erythrocytes, A and B antigens occur in almost all tissues [9]. As a result, maternal ABO antibodies are equilibrated between all fetal A or B antigenic sites, and the fetal red cells are spared from total attack by all of the incompatible maternal antibody. Thus, still-births are rare and hemolytic anemia in the neonatal period is self-limited (usually <14 days in duration) despite pronounced spherocytosis. Intrauterine transfusions are not needed for ABO erythroblastosis, but exchange transfusion therapy may be necessary in the neonatal period to prevent permanent brain damage from severe hyperbilirubinemia. Usually, however, neonatal icterus is readily controllable with phototherapy [7].

2. *Hemolytic anemia from transfused incompatible anti-A and anti-B*

As in the fetus and newborn, hemolytic anemia in older patients also can be caused by an excessive amount of passively acquired, incompatible alloantibodies. This situation is almost entirely restricted to anti-A and anti-B [10], and occurs when non-O patients are given pooled plasma, platelets, or cryoprecipitates. The introduced antibodies cause active hemolytic anemia accompanied by spherocytosis, with a hematological picture very similar to what is observed in ABO erythroblastosis fetalis. Treatment of this problem is symptomatic: avoid, if possible, additional ABO antibodies, and transfuse only with type O red blood cells.

3. *Delayed hemolytic reactions to transfused incompatible red blood cells*

The development of hemolysis at a time removed from the time of transfusion of red blood cells is a significant problem and may present a diagnostic dilemma. The findings may range from a less than expected rise and/or maintenance of rise of hematocrit to well-recognized symptoms of a hemolytic transfusion reaction (fever, chills, hemoglobinuria). Vascular collapse may occur. These clinical findings reflect the result of an anamnestic immune response in a patient already primarily immunized to a specific red blood cell antigen. By definition, the antibody level prior to transfusion is below the level of detectability. Nevertheless, it is possible to discern some of these antibodies prior to transfusion by reducing the ionic concentration of the reaction mixture [10a] and by increasing the washing ratio of serum to cells used in the test [10b]. The direct antiglobulin test may become positive temporarily, with mixed field agglutination indicating the presence of two populations of red blood cells. Because of the passage of time between the transfusion and the onset of laboratory and clinical tests, the diagnosis of autoimmune hemolytic anemia may be entertained. Recognition of the antecedent transfusion, the mixed field agglutination of the direct antiglobulin test, if present, and the detection of a new alloantibody specificity should point to the correct diagnosis.

Therapy, as with all hemolytic transfusion reactions, is supportive reflecting the clinical situation. One point to keep in mind is that, in the face of this reaction, the expected response hematocrit to the transfusion may not occur so that the patient requires additional transfusion much sooner than expected.

B. Autoimmune Hemolytic Anemias

Autoimmune hemolytic anemias are commonly divided into three major types: paroxysmal cold hemoglobinuria (PCH), cold agglutinin disease (CAD), and warm autoimmune hemolytic anemia (WAHA). None of these categories is homogeneous.

1. *Paroxysmal cold hemoglobinuria (PCH)*

PCH can be either acute or chronic, but almost all cases are characterized by IgG antibodies that bind only in the cold to the globoside glycosphingolipid of erythrocytes [11], an antigenic determinant originally called Tj^a [12]. Chronic PCH, often but not invariably of syphilitic origin, is incurable. The disease is manageable when the patient learns how to avoid undue exposure to cold. In contrast, acute postviral PCH, although self-limited, is a very serious and life-threatening condition. Coming on explosively following measles, mumps, chickenpox, or even influenza, the disease can be life-threatening for 10 to 14 days, after which it subsides and disappears, rarely to recur.

There is no specific therapy for PCH. Steroids are of no use, and splenectomy cannot be of value. Blood transfusions may be required in the acute phase. Blood, however, must be used sparingly and must be given at body temperature. Usually Tj(a+) [globoside carrying] packed red cells in small, well-warmed amounts will tide the patient over a critical period. Sometimes, however, only rare Tj(a−) [globoside negative] red cells must be used [13].

2. *Cold agglutinin disease (CAD)*

Cold agglutinin disease (CAD) has no single well-recognized cause. It can be a complication of PPLO pneumonia, cirrhosis, reticuloendothelial neoplasia, or just idiopathic. The specificity of autologous cold agglutinins is usually I, and unusually i, M, N, or H.

a. "*Monoclonal IgM*" *CAD* [14]

The commonest variety of CAD is characterized by high titer IgM cold autoagglutinins that carry only kappa light chains, occasionally lambda light chains, but not both. These antibodies bind best near 0 °C and almost all can fix complement. It is doubtful, however, that only IgM plus C3b on red cells can be associated with increased erythrocyte destruction *in vivo* [2]. Another component is needed, and this appears to be a small amount of IgG cold agglutinin [15]. A minor quantity of IgG can readily account for the observed cell destruction *in vivo* via cell-bound IgG in the presence of C3b that was fixed by the IgM antibody. CAD due to such autoantibodies is extremely resistant to therapy. When very severe, it is mildly responsive to steroids, to therapeutic plasmapheresis, and to a combination of steroids and chemotherapy (e.g. chlorambucil) as for Waldenström's macroglobulinemia [16].

b. *Mixed cryoglobulin CAD* [17]

A much less common variety of CAD is associated with mixed cryoglobulins consisting of IgG plus IgM, and sometimes of IgA as well. The IgG component of these cryoprecipitating complexes carries specificity for some red cell antigenic structure such as I, i, or N [15]. The IgM (and IgA) components have anti-IgG specificity [15]. All of these cryoprecipitating complexes are deleterious to red cells if the IgG portion can bind to these cells, and some are extremely hemolytic in the presence of complement. Diagnosis can be made only if the cryoglobulins are observed, and it is very important to look for these proteins because, in our experience, no matter how severe the disease, it is very responsive to therapeutic plasmapheresis. Just six to eight 500 ml units of plasmapheresis per week can be sufficient for significant clinical improvement within two weeks.

c. *Infectious mononucleosis CAD* [18]

A special variety of relatively non-cryoprecipitable IgM-anti-IgG occurs in infectious mononucleosis where cold-binding IgG anti-i antibodies become targets for IgM-anti-IgG agglutinins. These bound complexes can cause very severe CAD in i-positive patients (such as patients with thalassemia trait), but the disease is self-limited, subsiding as the infectious mononucleosis disappears.

d. "*Cryogel*" *CAD* [19]

Uncommonly, IgM cold agglutinins are cryoprecipitable. As the product of a major malignant clone of cells, the jelly-like cryoprecipitated IgM can represent a very large amount of IgM protein. The disease is responsive to therapeutic plasmapheresis but is not as responsive as is mixed cryoglobulin CAD.

3. *Warm autoimmune hemolytic anemia (WAHA)*

The most heterogeneous immune hemolytic anemias are those associated with warm autoantibodies. Although they are often idiopathic, WAHA is commonly secondary to disseminated lupus, chronic lymphatic leukemia, lymphosarcoma, ulcerative colitis, and many other diseases [20]. The antibodies are directed against a variety of erythrocytic antigens [21] such as Wr^b, LW, and U, but the ones most commonly looked for and observed are in the Rh system (Rh29 [not Rh_{null}], Rh17 [not -D-], Rh5 [e], and even Rh1 [D]).

a. *IgA WAHA* [15]

This is the rarest variety of WAHA, being observed in less than 1 in 50 cases. It is of moderate severity and is responsive to steroid therapy. The disease is commonly overlooked because most antiglobulin reagents are deficient in anti-IgA. To recognize these cases, it is necessary to have a capability to test with specific anti-IgA.

b. *Monoclonal IgG WAHA* [15]

Patients with this disease have red cells carrying bound IgG antibodies that possess either kappa or lambda light chains but not both. Only about 1 in 20 cases of WAHA have this picture. The amount of bound IgG per red cells in these cases is enormous, and we have never observed less than 2500 molecules per washed cell. Thus, complement is not needed for red cell destruction, and cell bound complement is rarely observed. These patients respond to prednisone therapy alone, although some require several months of treatment before their response is adequate.

c. "*IgM*" *WAHA* [15]

By far the commonest variety of WAHA, at least 19 out of 20 cases, is a combination of IgG and IgM autoantibodies with the latter fixing complement. Indeed, the severity of disease in these cases appears to parallel the amount of IgM autoantibody. Unfortunately, warm IgM autoagglutinins are not commonly observed when red cells are tested in physiologic saline. These antibodies, however, become potent causes of spontaneous agglutination when a strong rouleaux-inducing agent is present (e.g. K-90 PVP [15]).

Most of these patients respond to high doses of prednisone (up to 100 mg daily), but many require a combination of prednisone and chemotherapy (e.g. chlorambucil or even chlorambucil plus azothioprine or cyclophosphamide). Some patients with a combination of IgG and IgM autoantibodies, however, can be resistant to steroid treatment, even with chemotherapy. Splenectomy will benefit such cases, not by changing the immunologic picture at the red cell surface, but by allowing the immunologically damaged red cells to circulate for a longer time [15].

C. Drug-induced Immune Hemolytic Anemias

1. *Penicillin hemolytic anemia* [22]

Antibodies directed against drugs can cause hemolytic anemia when the drug given to the immunized patient becomes attached to the patient's red cell membranes where it is a target for the anti-drug antibody. Red cell destruction results either from too much IgG antibody on the red cell or from a combination of IgG antibody plus C3b. The classical example of such a disease is penicillin hemolytic anemia, but other erythrocyte binding drugs may also be responsible (e.g. cephalotin). Primary therapy consists of stopping the drug, although blood transfusions and even steroids may be needed for a brief period.

Penicillin hemolytic anemia is often confused with warm autoimmune hemolytic anemia because both are characterized by strongly positive direct IgG antiglobulin tests. They can be differentiated readily, however, by studying antibody eluates from the patient's red cells: autoimmune antibodies react with red cells from normal persons whereas anti-drug antibodies react only with red cells that carry the specific drug.

2. *Immune adherence hemolytic anemia* [23, 24]

Another and different mechanism for immune hemolysis due to antibodies to drugs is immune adherence. Here, the drug given to the patient combines with anti-drug antibody in the plasma and the resultant soluble antigen-antibody complexes fix complement. Such C3b carrying soluble complexes can now bind

to the C3b receptors of platelets to cause thrombocytopenia, to neutrophils to cause neutropenia, or to erythrocytes to cause anemia. The latter is often, but not always, characterized by a positive direct IgG and/or complement antiglobulin test in the acute phase. This disease is also responsive to removal of the offending drug, but the diagnosis can only be suspected if the direct antiglobulin test is negative. Laboratory diagnosis may be extremely difficult during the acute phase of disease even with a positive direct antiglobulin test. Only when the patient's erythrocytes become antiglobulin negative can they be incubated in a mixture of the patient's serum and an appropriate concentration of drug so that they can become antiglobulin positive. False negative results may occur if this test is performed with the red cells of other persons because other cells may not be as sensitive for immune adherence as the red cells of the patient. Eluates prepared during the acute phase of disease have no agglutinating antibody activity and are not helpful diagnostically.

3. *Hemolytic anemia due to α-methyl dopa*

Drugs can also cause immune hemolytic anemia as a complication, we believe, of drug toxicity to, for example, the liver. One such drug is α-methyl dopa which causes warm autoimmune hemolytic anemia indistinguishable from idiopathic disease. This kind of hemolytic anemia may respond very slowly, if at all, to removal of the drug. The disease does not appear to be significantly different from non-drug related warm autoimmune hemolytic diseases.

A summary of the applicability of the principal modes of therapy, transfusion, plasmapheresis, steroids, cytotoxic drugs, and splenectomy, is given in Table 1.

Are blood transfusions advisable and safe for patients with immune hemolytic anemias? [25]

Although blood transfusion therapy is critical for the management of erythroblastosis fetalis in either the fetus or the newborn, it is often a risky procedure in the care of autoimmune hemolytic anemia. In the first place, with the exception of PCH, autologous cold and warm agglutinins are difficult to distinguish from alloantibodies and, in fact, may hide them. For this reason, blood banks are reluctant to release blood for these patients because the pretransfusion tests for compatibility are not negative. Indeed, unless effective communication is established between the clinician and the blood bank, some of these patients may be allowed to die just because blood was withheld. No one wants to use donor blood that is serologically incompatible by tests *in vitro*, but small transfusions of such blood may be essential to combat life-threatening anemic cardiopulmonary failure.

Table 1

Treatment of Immune Hemolytic Anemias

Disease	Therapy				
	blood transfusions	plasmapheresis	steroids	cytotoxic drugs	splenectomy
I. Caused by alloantibodies					
A. Erythroblastosis fetalis	yes	no	no	no	no
B. Passive incompatibility (ABO)	yes	no	no	no	no
C. Delayed transfusion reaction	yes	no	no	no	no
II. Cold reactive autologous antibodies					
A. Paroxysmal cold hemoglobinuria (PCH)					
1. Chronic	minimal	no	no	no	no
2. Acute postviral	minimal	no	no	no	no
B. Cold agglutinin disease (CAD)					
1. Monoclonal IgM antibodies	minimal	yes	yes (if disease is severe)	yes	no
2. Mixed cryoglobulin CAD	minimal	yes (very effective)	no	no	no
3. IgG with IgM-anti-IgG (e.g. infectious mononucleosis)	minimal	no	no	no	no
III. Warm autoimmune hemolytic anemia (WAHA)					
A. Monoclonal IgG antibodies	minimal	no	yes	no	rarely
B. IgA antibodies	minimal	no	yes	no	rarely
C. IgM + IgG + C	minimal	no	yes	yes	yes
IV. Drug induced					
A. Anti-drug antibodies					
1. Drug bound to red cell membranes	yes	no	yes	no	no
2. C-carrying immune complexes bound to red cell membranes	yes	no	yes	no	no
B. Drug toxicity, such as α-methyldopa	minimal	no	yes	yes	yes

The problem of differentiating alloantibodies from autoantibodies can be extremely frustrating to the blood bank. It requires very careful comparison of antibodies in serum with antibodies in eluates, and attending clinicians must be tolerant of the amount of time that is required to complete such work. This is a unique situation that demands effective communication between clinician and blood bank so that the patient will receive correct and essential therapy.

Although it is sometimes possible to select compatible blood for a patient with WAHA, the vast majority of these patients are incompatible with almost all blood donors. Efforts to define the specificity of warm autoantibodies are not very cost effective, because warm autologous agglutinins are too frequently anti-Rh29 ("not Rh_{null}"), anti-LW, or anti-Wr^b.

If given the opportunity, there is a tendency on the part of some clinicians to over-transfuse patients with immune hemolytic anemia by attempting to maintain an unnecessarily high circulating level of hemoglobin concentration. These patients should not be given any more blood than is needed to keep them out of cardiopulmonary failure, and this is usually not more than 100 ml of packed red blood cells at a time.

Finally, for the management of very severe PCH or CAD, all blood given to the patient should be warmed to 37 °C to avoid the erythrocytic binding of cold-reactive autologous antibody that will be associated with fixation of complement.

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Intermediate Dose Methotrexate (IDM) in Childhood Acute Lymphocytic Leukaemia in Norway*

Report on a National Treatment Program

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Three courses of intermediate dose methotrexate, 500 mg/m^2 at three weekly intervals have been used as part of sanctuary therapy to 95 out of 112 children with acute lymphocytic leukaemia in Norway in the period August 1975 to December 1979. Eight doses of MTX IT were also used for CNS prophylaxis, the first dose was given on the day of admission. As of July 1, 1980, 75 out of 95 (79%) were still in CCR for a median of 36 months (6–57 months).

*

Introduction

Intermediate dose methotrexate (IDM) with leukovorin rescue as sanctuary treatment following induction therapy in acute lymphocytic leukaemia (ALL) in children was introduced by Wang et al. [1]. In 1975 we started to use a similar treatment protocol in Norway, the only major difference being that CNS-prophylaxis with weekly intrathecal methotrexate (MTX) was given from the very start of antileukaemic therapy instead of after 15 days, and that 8 instillations were given instead of 6. Our first experience with this protocol has been published earlier [2]. The purpose of this report is to present the results achieved with IDM as part of the treatment of ALL in the years from autumn 1975 to 1979.

Materials and Methods

A survey of the patients is presented in Table 1. In 1975, only a few departments were using the IDM-protocol, while since early 1976 all paediatric departments in Norway treating leukaemic children have participated. This report will deal with the 95 cases and 17 other cases of ALL diagnosed in the same period in Norway (Table 1).

* Presented at the 12th SIOP Meeting, October 1980, Budapest.

Abbreviations: MTX IT = Methotrexate intrathecally
CNS = Central nervous system
CCR = Continuous complete remission

Table 1

Patient material

Year of diagnosis	Patients receiving IDM in complete remission			Other cases of ALL (see Table 2)			All cases
	SR	IR	Total	SR	IR	Total	
1975	6	2	8	1	1	2	(10)
1976	15	9	24	5	2	7	31
1977	15	8	23	3	2	5	28
1978	13	6	19	0	1	1	20
1979	13	8	21	1	1	2	23
Total	62	33	95	10	7	17	112

SR = Standard risk
IR = Increased risk

Table 2 shows the reason why the 17 cases had not been included in the IDM-trial. Four of the 17, about 25% are still in remission, and one child received no treatment. Seven cases out of a total of 112 (6%) had no remission and 4 cases died in remission early in the study. One case relapsed after prolonged remission.

Table 2

Patients not receiving IDM in complete remission

Diagnosis year	Not achieving complete remission	Died early of infection, in remission	Treated with other protocols	No treatment (Down syndrome)
1975	2	0	0	0
1976	2	2	3	0
1977	1	2	1	1
1978	1	0	0	0
1979	1	0	1	0
Total	7	4	5	1

Only three of a total of 43 cases diagnosed as ALL in childhood in Norway in the two years 1978 – 1979 were not given IDM, one of the three is still in CCR.

We have now dealt with all cases of ALL in Norway. It is easy to do so in a country with 4 million people. It is possible to trace every single case of leukaemia from diagnosis to death or a recent follow-up.

Our treatment protocol (Fig. 1) resembled that of Wang et al. [1]. Induction therapy consisted of vincristine + steroids followed by L-asparaginase for 10 days. After complete remission had been achieved, and after the use of L-asparaginase, the three infusions of MTX were administered intravenously, at 3 weekly intervals. One third of the dose was given as a bolus and the remaining 2/3 in a 24 hour infusion.

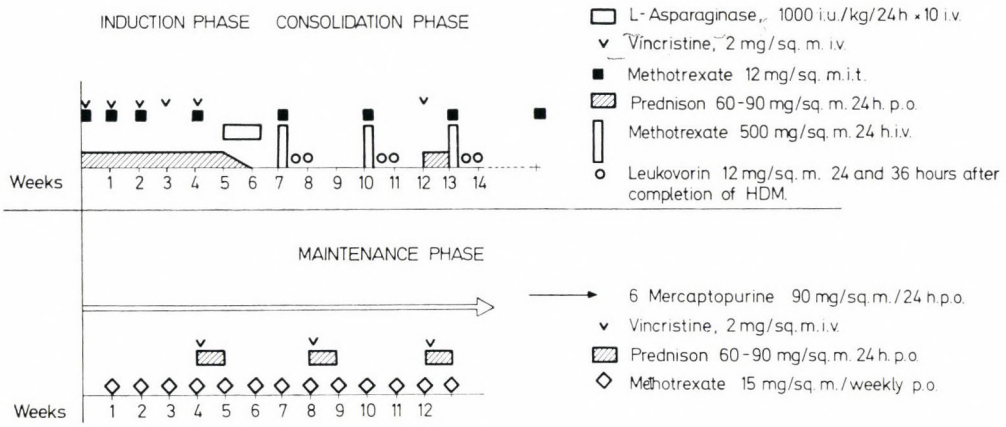

Fig. 1. Treatment protocol for children with ALL

Twenty-four hours following the completion of intravenous MTX, a single 12 mg/m² dose of leukovorin (citrovorum factor) was given. Since October 1976, two doses of leukovorin have been used after 24 and 36 hours, respectively. All the children received MTX intrathecally from the start of induction therapy, on the first day of admission, and all but 6 received 8 doses.

Treatment has been discontinued after 2 to 3 years of CCR. Before discontinuation all patients had normal findings in bone marrow and spinal fluid.

Results

Table 3 shows the survey of the results of therapy in the 95 cases receiving IDM while they were in complete remission. They have been followed up for a median period of 36 months. We have registered a total of 15 relapses before July 1980. Five patients died in CCR. The remaining 75 (79%) were in CCR.

Table 3

Patients receiving IDM in complete remission

Total number	95	
Relapse	15	
Died in CCR	5	
CCR	75	6–57 months; median 36 months.

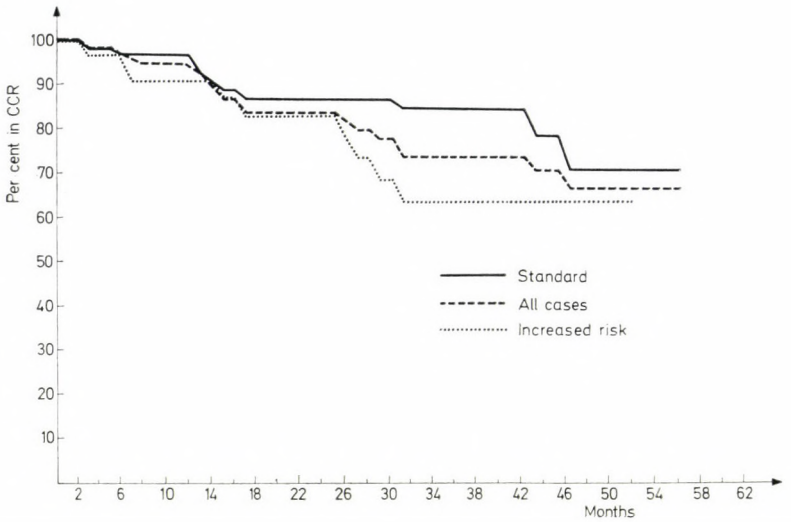

Fig. 2. Length of complete remission in 95 children with ALL in Norway treated with IDM

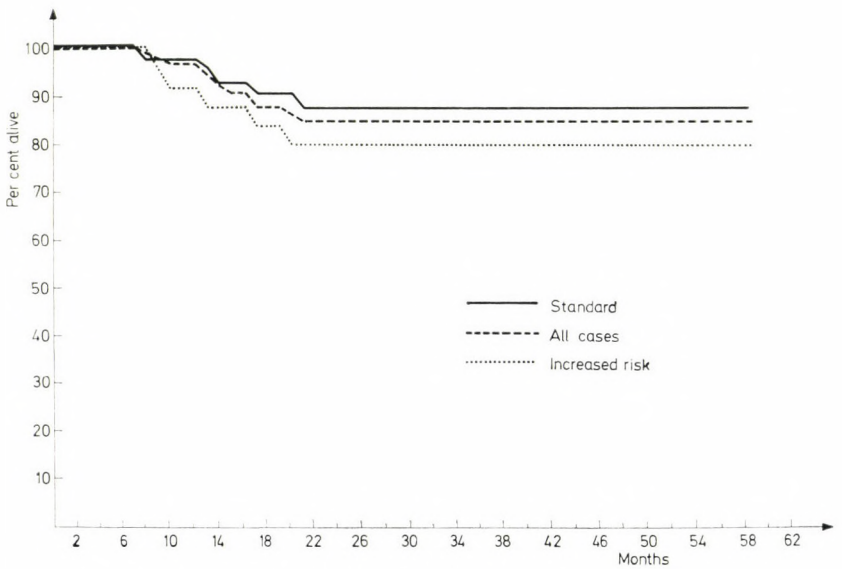

Fig. 3. Survival curves in the 95 children with ALL in Norway treated with IDM

The children have been separated into standard risk (SR) and increased risk (IR) patients. We defined increased risk as a WBC over 30,000/mm and/or age of less than 2 years or older than 10 years. CNS-leukaemia at the time of diagnosis and mediastinal mass were also considered as IR factors.

All five patients who died in CCR, 1 – 2 years after they had received IDM, were in the SR group.

Figure 2 shows the CCR life table for the 95 cases receiving IDM according to the protocol, and the IR and SR groups.

About 2/3 of all cases were still in remission at 57 months according to probability curves.

Figure 3 shows the survival curves. Twelve of the 95 cases had died before July 1, 1980.

Three cases of isolated CNS-leukaemia and 2 cases of combined systemic and CNS-leukaemia (Table 4) have been diagnosed so far. (After this study had

Table 4

Survey of 15 cases of relapse before July 1, 1980.

CNS-leukaemia	3
Systemic + CNS-leukaemia	2
Testicular or other organ leukaemia	0
Systemic	10
Total	15

been completed, 1 systemic, 2 CNS and one combined systemic and CNS-relapses occurred among the patients with initial WBC counts exceeding 100,000/mm^3.)

Not a single case of CNS-leukaemia has been diagnosed by routine spinal puncture in a total of 41 cases before cessation of therapy after 2 – 3 years.

There has been no case of testicular involvement among the 58 boys in the trial, neither among the other 8 cases in the study. We have therefore been reluctant to perform routine testicular biopsies in our cases receiving IDM. Before the IDM period we had many cases of testicular involvement.

Table 5 shows the follow-up on the first 55 of the 95 cases receiving IDM. Thirthy-six cases had discontinued therapy and five others were still in CCR. The cessation rate will probably be 70 – 75 %. Two of the 36 cases had relapsed before July 1980.

Table 5

IDM cases

	No. of cases	
Diagnosed 1975 – 1977	55	
Discontinued therapy before August 1980	36	41 = 75 %
CCR, on therapy	5	

Observation time $\geqq$ 32 months.

The cessation rate for all cases diagnosed as ALL in Norway in 1976 and 1977 will probably be about 10 % below the corresponding cessation rate in the IDM material for the two groups.

Table 6 shows untoward reactions to IDM therapy. Serious reactions were seen in several cases at the beginning of the study, but remarkably few occurred after two doses of leukovorin had been introduced in October 1976.

Table 6

Untoward reactions to IDM

	No. of cases
Pancytopenia + sepsis (?) + stomatitis	2
Convulsions following intrathecal MTX + IDM	1
Cytomegalovirus infection + stomatitis	1
Skin reactions (Stevens–Johnson syndrome in 2) + stomatitis	8
Stomatitis in other cases	18
Total (out of 95 cases)	30

Discussion

This investigation was started as a pilot study in a small Norwegian paediatric department (Tromsø), and was based on a personal communication in 1975 from Sinks at the Roswell Park Memorial Institute. It soon, however, developed into a national program including all paediatric departments in Norway treating children with leukaemia.

Our program includes a more intensive CNS-prophylaxis than the model published by Wang et al. and Freeman et al. [1, 3, 4], and thereby represents something new in the treatment of childhood leukaemia.

From the study published by Freeman et al. [3, 4], and the present study, it is evident that IDM is well tolerated by almost every child in complete remission when they are given adequate rescue therapy with leukovorin. No death from IDM has been observed among a total of 144 cases in complete primary remission in these two studies. Freeman et al. [3, 4] used only one dose of leukovorin as a routine procedure in rescue therapy. We had considerably fewer problems with IDM after we had started using two doses of leukovorin in October 1976.

Our relapse rate, 16%, for all the 95 cases receiving early IDM in primary remission, is low. The (5%) incidence of fatal infections in remission, however, was higher than in several other studies, but comparable with figures from Sweden [5].

The results in the IR group were satisfactory as compared to most other studies. The small number of cases in this group and the rather short period of observation-time should, however, be noted.

The incidence of CNS-relapse in Norway is remarkably low, even when the three cases of CNS or combined CNS + systemic relapse reported after the pre-

sentation of this paper, are included. It is considerably lower than that observed by Freeman et al. [3]. They reported primary CNS-relapse in 14% after 22–68 months of observation. There is a possible explanation for this difference, CNS-prophylaxis was begun at the start of induction therapy in Norway, and we also used routinely 8 intrathecal instillations of MTX compared to the 6 at Roswell Park Memorial Institute [3, 4]. Moe and Finne [6] observed only 2 cases of primary CNS-relapses among 44 cases receiving MTX intrathecally as the only type of CNS-prophylaxis in the period 1971–1974. At least 8 instillations of MTX were given intrathecally.

Not a single case of testicular involvement has been diagnosed in our study so far, and there was no other type of isolated sanctuary relapse. This is true for all 112 cases diagnosed as ALL in childhood in the period when the 95 case sreceived IDM.

At present we therefore see no indication for routine testicular biopsy when using this protocol.

*

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Antibody Detection and Identification by AutoAnalyzer Haemagglutination Systems

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The fundamental aims of antibody detection, the limitations imposed by present manual methodology, and what can be expected through the use of single-channel AutoAnalyzers are described. A combination of three different channels gives adequate power of detection and breadth of coverage for routine needs. Special techniques are also available that are useful in the study of antigen : antibody reactions and auto-immune diseases.

*

Reviews of this subject in 1977 [1] and 1980 [2] described the merits, shortcomings, and *modus operandi* of available automated systems. Here I describe the fundamental aims of antibody detection, the limitations imposed by present manual methodology, and what can be expected if single-channel AutoAnalyzers are used.

Detection of blood group allo- or auto-antibodies by haemagglutination techniques is routinely needed:

(i) In pre-transfusion tests for incompatibility.
(ii) When screening antenatal sera to detect maternal alloimmunization.
(iii) In tests on blood donations to detect those with alloantibodies.

Definitions of 'Clinically-Significant' Alloantibodies

The aim in each of the above instances is to recognize antibodies of 'clinical significance'. Unfortunately, however, the definition of this term is variable (Table 1). Furthermore, these definitions are neither precise nor universally accepted.

Pre-transfusion tests

There is ample evidence in the literature to justify the statement in Table 1 [e.g. 4, 5, 6]. *In vitro* complement-mediated haemolysis of red cells (usually by anti-A or anti-B, but rarely by anti-Le^a, anti-Kidd, anti-Vel, anti-Tj^a, etc.) is always of clinical importance. Specific alloantibodies causing *in vitro* agglutination only at temperatures below 30 °C are not of clinical significance, whereas

Table 1

Definitions of 'clinical significance' of blood-group alloantibodies

Application	Definition
Pre-transfusion tests for incompatibility	Nearly all allo- and auto-antibodies active *in vitro* at 37 °C using a 'prewarm' technique if necessary
Antenatal tests to detect maternal alloimmunization	Rh, Kell, Duffy, Kidd and other IgG alloantibodies active *in vitro* at a nominal 37 °C
Tests on blood donations to detect alloantibodies	Questionable if any are of significance for adult recipients, particularly if issued as red cells.* (See [3] and Table 12).

* = Synonyms: packed cells, concentrated red cell suspension.

if they react *in vitro* at 37 °C, they are – almost always, but not invariably – capable of bringing about increased clearance of incompatible red cells through the reticuloendothelial system [4, 5]. Examples of antibodies that are active *in vitro* at 37 °C but which are *not* of clinical significance are anti-Yk^a, anti-Ch, anti-Rg, anti-Xg^a, anti-Sd^a [unless the donor is Sd(a++)], anti-Kn^a-like, anti-Ge, anti-Lu^b, anti-Cs^a, and anti-Co^a [3, 5]. One cannot, unfortunately, be certain that all examples (apart from anti-Ch and anti-Rg), or which samples, of these specificities will be clinically harmless.

If a bioassay using Cr^{51}-labelled donor cells is done according to the I.C.S.H. recommended procedure [7], it should be possible to estimate whether immediate or delayed ill effects will be apparent should a therapeutic dose of that blood be given. Such tests are particularly useful when all donor units tested are incompatible and the antibody is active *in vitro* above 30 °C but not at 37 °C.

Antenatal tests

IgG 1 and IgG 3 alone can cross the placenta and are of clinical significance. IgG 2, IgG 4, IgM, and IgA cannot cross the placenta in sufficient concentration to cause significant haemolysis of fetal cells. IgG anti-D causes 98% of cases of haemolytic disease of the newborn (HDN) apart from those due to ABO incompatibility. The only other antibody specificities of importance are anti-c, anti-E, and anti-K1. Kidd, Duffy, and Ss antibodies are even less common causes of HDN.

Donor alloantibodies

Here one must consider the possible clinical effect of donor alloantibody on the red cells of either the recipient or of another donor. Mollison [6] points out that "there does not seem to be a single record in the literature of a haemolytic

reaction due to destruction of the recipient's red cells by an incompatible alloantibody other than anti-A and anti-B in the donor's plasma". Two examples of intra-donor incompatibility have been reported which resulted in haemolytic transfusion reactions. In each case the titre (of anti-K1) was 2000 or higher [6].

When both a two-stage enzyme and indirect antiglobulin (IDAT) test were used at 37 °C (the latter on pools of sera), 1801 or 0.4% of 432 202 Canadian voluntary blood donations were found to give a positive reaction. Anti-Rh predominated (69%), followed by anti-Kell (9%), anti-Duffy (2%) and anti-Kidd (0.4%) [8]. Of these 1801 donations, 12% gave positive reactions by IDAT alone.

Table 2

Indirect antiglobulin titres of 715 donor alloantibodies†

Titre	Number of donations	Per cent
20 or < 20	530	74
40 or 80	143	20
160	22	3
> 160	20*	3

* = 17 anti-D, 3 anti-K
† = Unpublished observations, 1977.

In another study, we found that 6% of alloantibodies had IDAT titres of 160 or over; the majority of these were anti-D (Table 2). Thus only 1 in 4000 units contained alloantibodies that might be 'clinically significant' (Table 12). About 25% (Table 2) of all alloantibodies or 1 in 950 of donations can be expected to have titres of 20 or >20 and thus be usable in the production of diagnostic reagents.

Importance of Particular Antibody Specificities

The specificity of antibodies found in a 1971 study [8] of sera from over 90 000 patients requiring transfusion is shown in Table 3.

The important antibodies are those belonging to the Rh, Kell, Duffy, Kidd, and S systems in that order. These are specificities which *must* be detected.

Of particular importance are those antibodies responsible for delayed haemolytic transfusion reactions (DHTR), that is reactions in which the symptoms do not appear until 3–14 days or so after a transfusion of blood that was not apparently incompatible *in vitro*. The antibodies responsible for DHTR belong to the blood-group systems just mentioned, but the order of their prevalence is different. Table 4 shows that one must pay more attention to detection of Kidd antibodies and anti-M than Table 3 would indicate. The particular importance of anti-Jk^a

is emphasized in a recent study [10] in which eight out of 30 single antibodies (27%) responsible for DHTR were anti-Jk^a. Multiple antibodies are frequently found in cases of DHTR.

Table 3

Specificity of alloantibodies found in sera of 92 222 patients before transfusion*

Specificity	Per cent
Anti-D (or anti-D+C, -D+E)	29
Anti-Kell	19
Anti-E	9
Anti-Fy^a	5
Anti-c	4
Anti-Kidd	0.8
Anti-S	0.1
Others active at 37 °C	7
Cold 'harmless' antibodies	26

* = Modified from [8]

Table 4

Specificity of alloantibodies implicated in delayed haemolytic transfusion reactions*

Specificity	Per cent
Anti-E	20
Anti-Jk^a	18
Anti-Fy^a	11
Anti-K	10
Anti-c	9
Anti-C	7
Anti-Jk^b	7
Anti-D	5
Anti-M	4
Others	9

* = Modified from [9]

Relative Powers of Detection of Available Manual and Automated Methods

Table 5 illustrates the extreme sensitivity of a bromelain-PVP AutoAnalyzer channel for the detection of anti-D when compared with the manual indirect antiglobulin test (IDAT). With anti-Kell, however, the range of concentrations detected on the AutoAnalyzer overlaps that detected by IDAT. Table 6 illustrates further the capabilities of a bromelain-PVP channel.

The real test of any technique, however, is when a large number of sera are tested for unexpected alloantibodies in parallel with an established method.

Table 5

Antibody concentrations detectable by different techniques*

Specificity (No. of samples tested)	Method	Concentration detected (ng/ml)	
		Mean	Range
Anti-D (5)	IDAT†	29	12—36
	AA	0.6	0.25—0.9
Anti-Kl (16)	IDAT	27	10—56
	AA	11	5—16

* = Modified from [11]
† = Indirect antiglobulin test; 20 : 1 ratio of serum : red cells
¶ = Single-channel (bromelain-PVP) AutoAnalyzer

Table 6

Summary of data from a study [12] using a bromelain-PVP single-channel AutoAnalyzer to detect alloantibodies demonstrable by manual methods

Antibody specificity	Number of examples		Per cent missed
	Detected	Missed	
Anti-Rh	23	1*	4
Anti-Kell	43	0	0
Anti-Duffy	17	0	0
Anti-Kidd	6	0	0
Anti-S or -s	6	0	0

* = Anti-E missed when R^1R^2 cells were used, but detected by R^2r cells.

Table 7

Summarized data from two studies [13, 14] on the ability of a low-ionic strength-Polybrene single-channel AutoAnalyzer to detect blood-group alloantibodies demonstrable by manual tests

Antibody specificity	Number of examples		Per cent missed
	Detected	Missed	
Anti-Rh	395	6	1.5
Anti-Kell	49	27	36
Anti-Duffy	14	0	0
Anti-Kidd	5	0	0
Anti-S	3	0	0

Table 7 shows that a low-ionic strength-Polybrene (LISP) single-channel AutoAnalyzer will fail to detect some examples of anti-Rh despite its increased sensitivity, and will also fail to detect a substantial number of anti-Kell antibodies.

In manual testing it is a *sine qua non* that more than one test is needed in order to detect all, or nearly all, examples of each antibody specificity. The same is true of single-channel AutoAnalyzers at the present time. This is clearly demonstrated when the data in Tables 6 and 7 are compared. The difference in the proportion of anti-Kell missed is striking.

Power of Detection of Combinations of Different Single-channel AutoAnalyzer Systems

Fortunately, when two or more AutoAnalyzer systems are used in parallel, each tends to complement the other or others. Thus Habibi et al. [14] and Wattar and Govaerts [15] in a study of 807 manually identified alloantibodies detected all examples of the specificities shown in Tables 6 and 7 when different AutoAnalyzer systems were combined and used in parallel. Table 8 illustrates this point.

Table 8

Results of using three [14] or four [15] single-channel AutoAnalyzer systems in parallel to detect manually identified antibodies

Antibody specificity	Number of examples	
	Detected	Missed
Anti-Rh	221	0
Anti-Kell	80	0
Anti-Duffy	15	0
Anti-Kidd	6	0
Anti-S	3	0
Others	482	0

Antibodies Detectable Only on the AutoAnalyzer

The power of detection of both LISP and bromelain-PVP AutoAnalyzer channels is such that specific antibodies may be detected on the AutoAnalyzer which are not demonstrable by manual techniques (machine-only antibodies or MOANs).

Specific MOANs. Only 1–2 molecules of IgG anti-D per red cell are needed on the AutoAnalyzer to support agglutination [16] compared with 100–350 molecules needed to produce visible agglutination by the indirect antiglobulin method [17]. It is probable that specific MOANs have either a low affinity for antigen or a concentration below the threshold of perception of manual tests, or both.

The biological significance of specific MOANs is unclear. A small proportion are almost certainly of "natural occurrence". Others may in the course of time become detectable by manual techniques. An occasional example may cause a delayed haemolytic transfusion reaction [18]. *All specific MOANs should, therefore, be regarded as a warning of possible alloimmunization and the need for special vigilance when future samples are tested.*

"*Non-specific*" *MOANs.* Crockford et al. (cited in [1]) tested on a LISP channel 13 701 sera that had given negative reactions in manual tests. Of these 632 or 4.6% gave positive tracings. When the tests were repeated against a panel of cells, 1.3% gave specific reactions, 1.6% gave reactions of unidentified specificity, and in 1.7% the positive reactions could not be confirmed. The so-called "non-specific" reactions could have been due to cold agglutinins or to thromboagglutinins or leukoagglutinins [14]. Nordhagen and her co-workers in a series of most interesting studies used single-channel AutoAnalyzers to demonstrate the presence of haemagglutinins that react with HLA determinants on the red cells of some but not all individuals who, according to lymphocytotoxicity tests possess the corresponding antigens on their white cells [e.g. 19]; some of these HLA haemagglutinins are capable of destroying a proportion of transfused incompatible red cells.

Selection of AutoAnalyzer channels and their place in routine work

Table 9 summarizes the effectiveness of different channels for the detection of the clinically most important antibodies.

Table 9

Summary of single-channel AutoAnalyzer capabilities for detection of antibodies in the most important blood-group systems

Blood-group system	Remarks
Rh	Much more sensitive than any manual method (Table 5). Occasional manually-demonstrable examples missed (Tables 6, 7). Of antibodies detected on LISP, 32% may be specific MOANs [2].
Kell	Sensitivity about same as manual methods. Bromelain-PVP channel of choice, followed by DIPP. About 6% specific MOANs detected on LISP (2). *Choice of Kk screening cells important owing to variable reactivity.*
Duffy	LISP channel much more sensitive than manual methods. About 16% specific MOANs detected on LISP [2].
Kidd	LISP and bromelain-PVP equally effective. About 43% specific MOANs detected on LISP [2].
Ss	LISP channel of choice.

Little information is available about other IgG antibodies active at 37 °C. We have detected on a LIPP channel [20] single examples of the following specificities: anti-Ge, anti-Do^a, anti-JMH, anti-Tj^a, anti-Jr^a, anti-Yt^a, anti-Lu^b, anti-Kp^b, anti-Yk^a, anti-Kn^a-like, anti-Cs^a, anti-Er^a, anti-Co^b (J. Crockford, personal communication, 1980).

In our experience, the best mixture of channels for general use consists of LIPP [20], DIPP [20], and bromelain-PVP [21] with the addition of curve regeneration [22] to provide a sampling rate of 90/hour.

Pre-transfusion tests

Tests for incompatibility before transfusion have the following purposes:

(i) To determine the ABO and D groups of the recipient.
(ii) To verify the ABO group of each donation (and D group if "Rh negative").
(iii) To determine whether the recipient's RBC are coated with antibody (positive direct antiglobulin test).
(iv) To detect antibody in the recipient's serum that might, or in fact does, react with the donor's RBC.

Wattar and Govaerts [15] use four different single-channels for the last two of the above. Over 200 000 units have been issued on the basis of these results without immediate or delayed ill-effects being noticed (A. Govaerts, personal communications, 1979, 1980). However, most operators of single-channel AutoAnalyzers have limited their use to screening tests against cells of known antigenic composition, and rely on a 5–15 min low-ionic-strength (LISS) IDAT to verify the ABO group and that the donor's red cells are not incompatible.

Prenatal tests

Single-channel AutoAnalyzers are very well suited for the detection and identification of antibodies other than ABO, responsible for HDN. Owing to the number of specific MOANs detected and their apparent lack of significance as a cause of HDN, they should be reported as shown in Table 10. It is our practice to report antibodies active against enzyme-treated cells but not by IDAT as 'weak' unless the two-stage enzyme (EII) titre is >16. Clinical HDN is not normally apparent unless the allo-antibody is detectable by IDAT. Note that Rh immunoglobulin should be administered to women in whom only specific anti-D MOANs have been detected.

Donor alloantibodies

The reasons for testing donations for the presence of alloantibodies may be listed in order of priority:

(i) To provide antibody-free units for children and for preparation of cryoprecipitate or fresh-frozen plasma.

Table 10

Simple classification of *in vitro* findings in antenatal tests

Results obtained by			Action when specific alloantibodies detected	Should Rh IgG be given†
AA	EII*	IDAT		
+	−	−	Report as 'trace'	Yes
+	+	−	Report as 'weak' unless EII titre > 16	No
+	+ or −	+	Report IDAT titre	No

* = EII = two-stage enzyme test (papain-cysteine preferred).
† = if alloantibody is anti-D.

(ii) To detect antibodies suitable for diagnostic reagent production.
(iii) To safeguard the donor through issue of a card showing the antibody specificity.
(iv) To resolve ABO grouping anomalies and identify rare blood groups.
(v) To detect warm alloantibodies of "high titre" that might adversely affect the recipient.

Table 11

Effectiveness of Autogrouper 16-C for the detection of alloantibodies*

Antibody specificity	Number tested	Per cent detected
Anti-Rh	72	85
Anti-Kell	64	86
Anti-Duffy	16	100
Anti-Kidd	8	63
Total	160	85

* = From [23]

The AutoAnalyzer Autogrouper 16-C is far more effective than the BG-15 for the detection of alloantibodies (Table 11). However, it fails to detect about 15% of Rh and Kell antibodies and some of these may have IDAT titres greater than 20 [23]. The simplest procedure to follow is that outlined in Table 12. Rh negative units warrant special scrutiny; units from donors with a history of transfusion or pregnancy, or both, are about eight times more likely to contain alloantibody than units from donors without such a history [3].

If the BG-15 rather than the 16-C is available, all initial antibody screening should be done on single-channel AutoAnalyzers followed by automated identification of specificity. IDAT titres should then be done on 'clinically important' antibodies, i.e. those listed in Table 3.

Table 12

Procedure for testing blood donations for alloantibodies

1. Test all blood donations on Autogrouper 16-C. About 1.3% positive [23].
2. Identify specificity and determine IDAT titre of antibody containing donations.
3. Donations giving negative reactions on Autogrouper 16-C:
   (i) Select Rh negative units and those from donors with history of transfusion and pregnancy (3).
   (ii) Test these units on single-channel AutoAnalyzers (DIPP, LIPP, and bromelain-PVP). Identify specificity on AutoAnalyzer. Determine IDAT titre.
4. Set aside units with IDAT titres of > 16 for reagent production.
5. Issue units (note antibody specificity and titre on unit) as 'Red Cells for Adults Only' unless the titre is > 160.*

* This figure is arbitrary.

Other Applications

Alloantibody identification

When an alloantibody is detected on an AutoAnalyzer channel, it should be identified by being tested against a selected panel of not less than seven samples on that channel. (10 samples are more likely to provide a probability (P) of 1/20 [21]). The same sampling rate, e.g. 90/hour with a Curve Regenerator may be used. Details are given in [21].

Detection and identification of cell-bound antibody. The co-agglutination technique of Lalezari [24] is a particularly valuable tool in problem cases. It may be used as a time-saving substitute for manual fixation-elution. Antibody-coated cells are mixed with selected, uncoated, indicator cells. If the indicator cells have specific sites for the antibody on the coated cells, redistribution of antibody occurs. For reviews, see [25] and [2]; the technique is described in detail in [21].

Temperature-gradient dissociation. This technique [26] can reveal much about the antigen : antibody reaction and has been used in the study of auto-immune diseases. Most of a working day is, however, needed to perform the tests on one sample and to plot the results [21].

Conclusion

As I have pointed out before [2], single-channel AutoAnalyzers cannot be bought as a ready-for-use package as with most other laboratory instruments. Given patience and the right operators [1], the machines have a definite role to play in large-scale routine work and in the study of antigen : antibody reactions.

This role will become increasingly important when microcomputers become an integral part of the system [27].

*

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Activity of Inosine Triphosphate Pyrophosphohydrolase in Fresh and Stored Human Erythrocytes

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The accumulation of inosine triphosphate (ITP) in human erythrocytes incubated with inosine depends on the activity of inosine triphosphate pyrophosphohydrolase (ITPH). High activity of this enzyme is accompanied by a low concentration of ITP and conversely. We showed that ITPH activity decreases with the prolongation of blood preservation time. As a consequence there is a lower accumulation of ITP in fresh erythrocytes incubated in a medium containing high concentrations of inosine, pyruvate and phosphate (IPP) than in red blood cells preserved at 4 °C. Synthesis of ITP in erythrocytes incubated in IPP medium being so intensive, it seems possible that during incubation an intermediate accumulates which decreases ITPH activity.

*

Introduction

Inosine triphosphate pyrophosphohydrolase (ITP-pyrophosphohydrolase: ITPH; EC 3.6.1.19) catalyzes the reaction $ITP + H_2O \rightarrow IMP + PP_i$. This enzyme has been studied only recently when it was found that ITP is present in erythrocytes and in some tissues. The enzyme hydrolyzing ITP was first called inosine triphosphatase (ITPase) [1, 2]. While investigating adenylotranspherase of erythrocytes, Liskopoulou and Alivisatos [1] found that erythrocyte acetone powders have high ITPase activity. In contrast with ATPase which is firmly bound to the cell membrane, the enzyme discovered by them was localized mainly in the stroma-free haemolysate. In 1964 Vanderheiden [3] reported the presence of high concentrations of ITP in human erythrocytes of two siblings. His later investigations showed that the accumulation of ITP in the erythrocytes of some subjects is caused by a deficiency of ITPase [2]. He also demonstrated that human erythrocytes incubated with labelled inosine and orthophosphate synthesize small amounts of ITP [4]. Detailed investigations revealed that ITPase is in fact the ITP-pyrophosphohydrolase requiring Mg^{++} ions and thiol compounds for normal action [5]. The existence of a nucleoside triphosphate pyrophosphohydrolase specific for ITP has recently been demonstrated in the cytosol fraction of various animal tissues and erythrocytes [6–10].

For several years a number of laboratories have investigated the effect of inosine on the synthesis of phosphate compounds in human erythrocytes stored

in acid-citrate-dextrose (ACD) solution at 4 °C. Since the laboratories were mostly interested in the contents of 2,3-diphosphoglycerate and adenosine triphosphate (ATP), none of them studied the synthesis of ITP.

It has been accepted that inosine present in the medium or the hypoxanthine formed from it, is incorporated into IMP [11]. Only in 1974, during investigations into the effect of high concentrations of inosine, pyruvate and phosphate, was it found that outdated human erythrocytes synthesize ITP in amounts close to, or higher than, the physiological concentration of ATP [12-14]. Vanderheiden [15] suggested that the accumulation of such amounts of ITP was due to the outdated erythrocytes used in the experiments and that the weak accumulation of ITP in fresh erythrocytes incubated with inosine depended on the high ITPH activity. He supposed that the activity of this enzyme decreases during blood preservation, thus promoting the accumulation of larger amounts of ITP.

The aim of the present work was to study the ITPH activity in fresh erythrocytes as well as in the red cells of blood stored in ACD solution at 4 °C for two and four weeks.

Material and Methods

Blood was taken from healthy donors into three glass bottles containing ACD solution. Two portions of blood were stored at 4 °C and one was centrifuged, the plasma and buffy coat were removed, and the packed cells were washed with an excess of 0.9% saline solution. Haematocrit and haemoglobin concentrations were determined in washed erythrocytes. Two ml of washed red cells were haemolyzed with a double volume of cold distilled water. Directly before incubation the haemolysate was mixed with an equal amount of 0.1 M dithiotreitol. Incubation of the enzyme with substrate was carried out according to Vanderheiden [2] with the only modification that we used a double volume of the incubation mixture. In the final volume of 4 ml test sample the concentration of the components was: 8 μmol, ITP, 200 μmol, $MgCl_2$, 40 μmol, dithiotreitol in 0.1 M Tris-HCl buffer pH 7.5 and 0.8 ml haemolysate containing 30 to 40 mg haemoglobin. The blank sample was a mixture of the same composition except the haemolysate, which was replaced by 0.8 ml distilled water. Test and blank samples were incubated at 37 °C for 30 min. The reaction was terminated by the addition of 1 ml 20% perchloric acid. The mixtures were cooled and the centrifuged supernatants were quantitatively transferred to other test tubes and neutralized with 40% KOH solution. The precipitated $KClO_4$ was removed by centrifugation. The whole volume of neutralized solution was applied to a column filled with Dowex-1 X 4, 200–400 wet mesh, formate form. Formic acid and ammonium formate gradient were used to elute the products of ITP hydrolysis. 3.5 ml fractions were collected and optical density was measured at 260 nm (spectrophotometer Pye-Unicam SP 500, series 2). Identification of the eluted nucleotides was done on the basis of standard nucleotide separations (IMP,

IDP, ITP) and by comparing the molar ratios of hypoxanthine to phosphate. For quantitative calculations a molar absorption coefficient of 8.1×10^{-3} was used. The amount of ITP was calculated following the chromatographic separations of incubated blank and test samples. ITPH activity was expressed as the amount of μmoles ITP hydrolyzed/g Hb/min.

Results

ITP-pyrophosphohydrolase was examined in blood taken from 20 donors. Figure 1 presents the ion exchange column chromatographic separation analysis of the solutions following incubation with haemolysate as well as the blank samples. Table 1 shows ITPH activity of fresh erythrocytes and cells of blood stored for 14 and 28 days. Initial activity being taken for 100%, after two weeks preservation the enzyme activity decreased by 20%, and after four weeks by 50%. In Table 2 the amounts of IMP, IDP and ITP measured after chromatographic separation of test and blank samples are presented in per cent of total hypoxanthine nucleo-

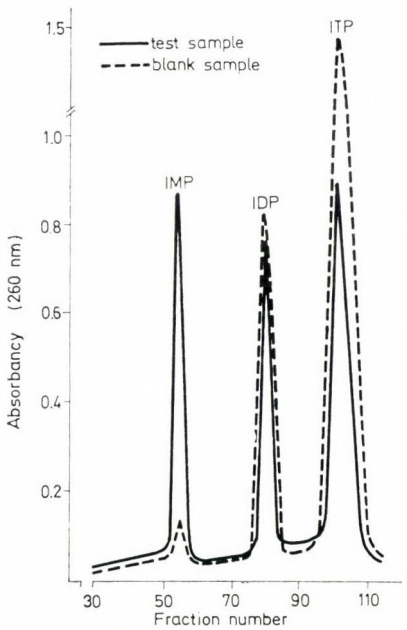

Fig. 1. Ion exchange chromatographic analysis of the solution after incubation with haemolysate (solid line) and of the blank sample (discontinuous line). Note that the blank sample contains small amounts of IMP and greater amounts of IDP. After incubation with haemolysate the amount of IMP increases and, as a consequence of enzymatic hydrolysis, the amount of ITP decreases. The amount of IDP remains almost unchanged in both samples examined

Table 1

ITP-pyrophosphohydrolase activity of freshly obtained human erythrocytes and of cells of blood stored in ACD solution at 4 °C for 2 and 4 weeks. ITPH activity is expressed in μmoles of ITP hydrolyzed by enzyme/g Hb/min

Experiment number	ITP-pyrophosphohydrolase activity		
	Time of blood preservation (days)		
	0	14	28
1	1.680	1.370	0.699
2	2.008	1.464	0.701
3	1.443	1.327	0.792
4	1.881	1.205	1.015
5	1.411	0.827	0.660
6	1.466	1.222	0.542
7	1.825	1.252	0.704
8	1.708	1.376	0.386
9	1.502	1.521	0.714
10	1.470	1.301	0.366
11	1.982	1.515	0.802
12	1.584	1.237	0.887
13	2.204	1.777	1.472
14	1.401	1.079	0.607
15	1.714	1.455	1.063
16	1.419	1.001	0.857
17	2.229	1.974	1.373
18	2.197	1.869	1.434
19	2.414	1.926	1.369
20	2.283	1.910	1.312
Mean	1.791	1.430	0.888
S.D.	± 0.329	± 0.314	± 0.336
Range	1.401–2.414	0.827–1.869	0.366–1.472

Table 2

Amount of IMP, IDP and ITP expressed in per cent of total hypoxanthine nucleotides, in blank samples without haemolysate, and in samples incubated with haemolysate of freshly obtained erythrocytes and of cells stored at 4 °C in ACD solution for 14 and 28 days

Preservation time (days)	n	Hypoxanthine nucleotides		
		IMP	IDP	ITP
blank samples	12	7.7	25.9	66.4
0	20	31.6	27.6	40.8
14	20	29.1	27.1	43.8
28	20	22.5	26.1	51.4

tides. The data indicate that IMP, IDP and ITP constitute 31.6%, 27.6% and 40.8% of the total amount of all three nucleotides, respectively. The amount of IMP eluted from the column decreased systematically throughout particular

periods of blood storage, and ITP concentration increased with prolongation of the preservation time. The IDP contents, calculated in per cent, was practically unchanged in the postincubation solutions from the haemolysates of fresh erythrocytes, the red cells preserved for different periods of time, and of blank samples. As mentioned under "Material and Methods", the incubated samples contained 8 μmoles of ITP. The solutions obtained following incubation were applied quantitatively on the column. Recovery after separation was almost identical and amounted to 72 to 75% in the case of test samples and 80% in blank samples.

Discussion

The results indicate that a prolongation of blood preservation time causes a decrease in ITP-pyrophosphohydrolase activity. Fresh erythrocytes were found to synthesize smaller amounts of ITP than red blood cells from stored blood. The amount of accumulated ITP increased with the prolongation of preservation time. Hence, Vanderheiden's supposition [15] that the amount of ITP accumulated in erythrocytes depends to some extent on the time of blood preservation has been corroborated. Soder et al. [16] found that the extent of ITP accumulation in samples of human erythrocytes is inversely proportional to the activity of ITPH. It has also been shown that red cells of whole blood or 10% suspensions in Krebs–Ringer phosphate medium stored at 4°, 22° or 37 °C for 24 hours accumulate more ITP than fresh erythrocytes [17]. These observations agree with our results, while Henderson et al. [17] stated that ITPH activity in the haemolysate of erythrocytes preserved for 3 to 7 days did not change significantly. Other authors from the same laboratory noticed individual variations in the ability to accumulate ITP [18] and ITPH activity [16]. Recently, Henderson has investigated the relation between ITP accumulation and ITPH activity in 100 individuals [19]. The data obtained completely support Vanderheiden's proposal [2] that low ITPH activity leads to high ITP accumulation and that erythrocytes with high ITPH activity accumulate low amounts of ITP. While incubating red blood cells in a solution containing high concentrations of inosine, pyruvate and phosphate, we found that these cells accumulate significantly higher amounts of ITP than those reported by other authors. The differences seem to be connected with the composition of the incubation mixture and the components used by us probably promoted the synthesis of ITP. It is also likely that conditions of incubation lead to the accumulation of an intermediate which inhibits the hydrolyzing enzyme allowing ITP to accumulate. It was observed for example that fructose-6-phosphate and ketose monophosphates inhibit ITPH [15]. The ITP used in our experiments was partially hydrolyzed, as shown by the permanent presence of IMP and IDP in the particular fluids. There was an equal amount of IDP in the postincubation solution in both the test and the blank samples. This may indicate that ITP-pyrophosphohydrolase was the only enzyme taking part in ITP hydrolysis under our experimental conditions. Recently, ITP-pyrophosphohydrolase of red blood cells

has been purified and its properties were examined [8]. It was shown that the enzyme is highly specific for ITP, does not require thiol compounds for its activity and is inhibited by some bivalent cations. The biological role of this enzyme has not yet been elucidated, especially as far as the red blood cells are concerned. Vanderheiden [7] suggested that ITP synthesized in erythrocytes regulates the level of ATP produced, and helps to maintain a steady-state intracellular level of adenine nucleotides. In the case of ITPH-deficient cells, regulation of intracellular ATP may be controlled by IDPase. Considering the role of ITPH in other tissues, Wang and Morris [10] think that its action prevents the accumulation of ITP, XTP or dITP in cells and the incorporation of these purins bases into RNA and DNA.

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Concentration of 2,3-Diphosphoglycerate and Adenosine Triphosphate in Erythrocytes Following Acute Blood Loss in the Beagle

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The effect of experimental anaemia induced by phlebotomy and the effect of infusion of various fluids on the 2,3-DPG, ATP and total organic phosphate (P_{org}) concentration were examined in the red cells of beagles made anaemic by withdrawal of 30% of the circulating blood. Particular groups of dogs 30 min after phlebotomy were infused with own blood, blood with inosine, pyruvate and phosphate (IPP), Fluigel and Fluigel with IPP. The changes were most distinct in dogs which were not infused with any fluid. After 24 hours the content of 2,3-DPG, ATP and P_{org} increased by 17, 27 and 20%, respectively, in comparison with the results obtained before blood loss. Twenty-four hours following the reinfusion of own blood, a slight increase occurred in the 2,3-DPG and P_{org} content. Infusion of Fluigel caused a decrease of the phosphate compounds concentration after 3 and 6 hours and then the concentration almost returned to normal. Infusion of own blood as well as Fluigel enriched with inosine, pyruvate and phosphate did not lead to any change in 2,3-DPG concentration. The red cells of dogs do not utilize inosine for 2,3-DPG or nucleotide synthesis. The increased cardiac output seems to the the main mechanism for oxygen transport to tissues after an acute haemorrhage.

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Introduction

Acute blood loss decreases the haemoglobin concentration and so a smaller than normal amount of oxygen is transported to the tissues. So far several mechanisms are known to compensate for the decreased amount of oxygen in the tissues. One of them involves a reduced affinity of haemoglobin to oxygen due to increased synthesis of 2,3-diphosphoglycerate (2,3-DPG) in erythrocytes [1]. It is well known that during hypoxia the concentration of 2,3-DPG increases in human erythrocytes. It was also shown that human as well as monkey, rabbit and rat erythrocytes incubated in a medium containing inosine, pyruvate and inorganic phosphate synthesize large amounts of 2,3-DPG, and the content of adenosine triphosphate (ATP) also increases [3, 4, 5]. Similar changes were observed in monkeys infused intravenously with the solution [6].

There is little information on the erythrocyte metabolism of anaemic dogs. The effect of inosine, pyruvate and inorganic phosphate on the synthesis of organic phosphate compounds in erythrocytes of these animals has not been investigated.

The aim of the present work was to determine the concentration of 2,3-DPG, ATP and total organic phosphate in erythrocytes of beagles after an acute blood loss and infusion of fluid containing inosine, pyruvate and inorganic phosphate among others.

Material and Methods

Experiments were done on 17 beagles. After determination of the volume of circulating blood with radioactive-iodine-labelled ^{131}I albumin [7], the animals were subjected to general anaesthesia, and made anaemic by the removal of 30% of the circulating blood by a polyethylene catheter inserted into the femoral vein. The dogs were examined in 5 groups of 3 or 4 dogs each. Group 1: phlebotomy without infusion of any fluid. In the other groups 30 min after phlebotomy the time accepted as an early period of haemorrhagic shock, the following interventions were done; Group 2: reinfusion of own blood; Group 3: infusion of Fluigel, a modified gelatin solution produced by Polfa, Poland; equivalent to Roger–Bellon's Plasmagel; Group 4: infusion of Fluigel containing inosine, pyruvate and inorganic phosphate (IPP solution) at a concentration of 0.1 : 0.1 : 0.066 M in a volume of 7.5 ml/kg body weight [8]; Group 5: infusion of own blood with IPP solution. For infusion we used volumes equal to the lost blood. Blood samples were taken into acid-citrate-dextrose solution (ACD) before (0 hour), and at 3, 6, and 24 hours, after phlebotomy. The blood was immediately chilled, centrifuged, plasma and buffy coat were removed and the red cells were washed twice with an excess of 0.9% NaCl solution. Haemoglobin and haematocrit were determined in each sample of washed erythrocytes. Acid-soluble phosphate compounds were extracted with perchloric acid [9]. The extract obtained was neutralized with 40% KOH solution and the precipitated $KClO_4$ was removed by centrifugation. Extraction and neutralization were performed in an ice bath of 2 to 4°C, and the extracts were stored at −25 °C. Phosphate compounds were separated on glass columns filled with Dowex-1 and eluted in formic acid-ammonium formate gradient [10]. Absorption at 260 nm was measured spectrophotometrically in all fractions. Phosphate analysis was carried out by the Fiske–Subbarow method in Bartlett's modification [11]. Nucleotides and phosphate compounds were identified as described previously [12]. The results were expressed in μmol/g Hb.

Results

Figure 1 presents 2,3-DPG concentration in erythrocytes of dog groups examined before and in particular periods after phlebotomy. The initial 2,3-DPG content in various dogs was in the range of 17.1 to 21.5 μmol/g Hb. In the case of Groups 1 and 2, the concentration of 2,3-DPG did not change significantly after 3 and 6 h after phlebotomy. It increased only after 24 h, by 17%. On the

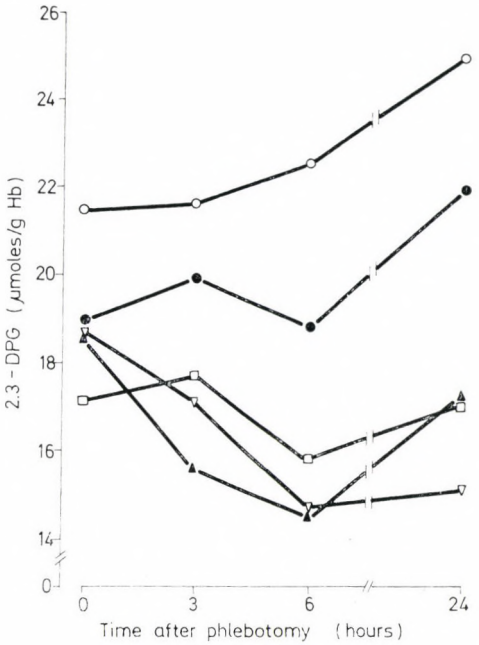

Fig. 1. 2,3-DPG concentration in erythrocytes of beagles before phlebotomy (0 hour) and after phlebotomy and infusion of various fluids. Symbols: ○—○ = group 1 (phlebotomy without infusion of any fluid), ●—● = group 2 (reinfusion of own blood), ▲—▲ = group 3 (infusion of Fluigel), □—□ = group 4 (infusion of Fluigel with inosine, pyruvate and inorganic phosphate), ▽—▽ = group 5 (infusion of own blood with inosine, pyruvate and inorganic phosphate). For details, see Material and Methods

other hand, in Groups 3 and 5, 2,3-DPG concentration decreased after 6 h by 21%. The concentration of this compound in Group 4 was slightly changing throughout the observation period. The ATP level (Fig. 2) was unchanged in the majority of animals, only in Group 1 was a significant increase (27%) observed between 6 and 24 h. In dogs infused with Fluigel a 23% decrease of ATP was noted in the first 3 h after phlebotomy. In the further period a gradual increase occurred until the initial concentration had been restored. Figure 3 shows the changes in total organic phosphorus content; the results obtained were similar to those for the 2,3-DPG concentration.

Discussion

The main role in erythrocyte oxygen transport is played by haemoglobin due to its reversible binding to oxygen. Red blood cells possess an internal mechanism controlling the affinity of haemoglobin to oxygen [2]. Since 1967, when the significant influence of 2,3-DPG on the affinity of haemoglobin to oxygen was

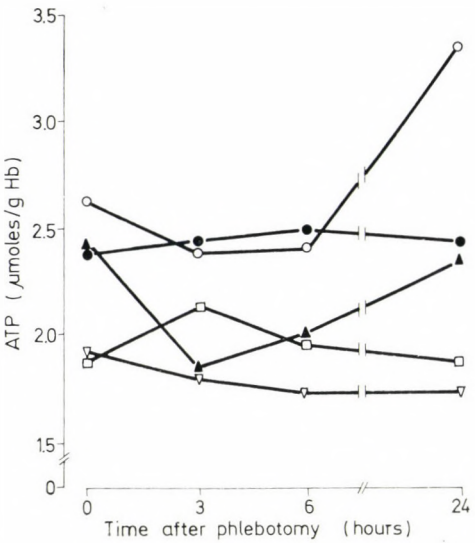

Fig. 2. ATP concentration in erythrocytes of beagles before and after phlebotomy and infusion of various fluids. Symbols as in Fig. 1

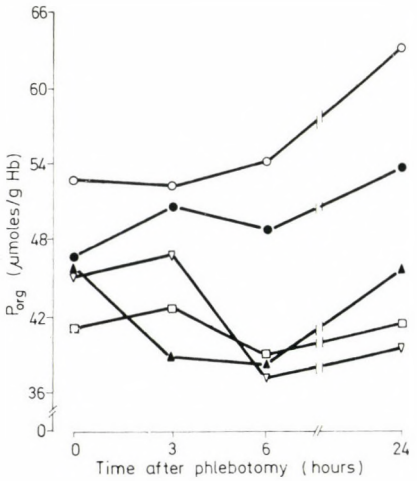

Fig. 3. Total organic phosphate concentration in erythrocytes of beagles before and after phlebotomy and infusion of various fluids. Symbols as in Fig. 1

observed [13, 14], the behaviour of this compound in various stages of hypoxia has become a subject of wider studies. Generally, the organism responds to hypoxia immediately. While investigating the concentration of 2,3-DPG in rat erythrocytes, Duhm [15] found that a two- to threefold oxygen content in the inhaled air caused a significant increase in 2,3-DPG content in a few hours. Naylor et al. [16] found a small increase of the 2,3-DPG content in monkeys 4 h after the beginning of haemorrhagic shock. Despite the acute blood loss, no increased synthesis of 2,3-DPG was observed [17]. This may indicate that dogs possess some other mechanism responsible for the transport of oxygen to tissues. Smith and Agar [17] found that in dogs following phlebotomy to values below 20% of packed cell volume (normal range, 42 to 53%) all glycolytic intermediates except 2,3-DPG, increased significantly. Adenine nucleotides were normal or near normal at the end of the experiment. They also stated [17] that in dogs the increased cardiac output is the most immediate and probably most important response to acute haemorrhage, lasting until the haemoglobin levels have returned to normal. Our results have confirmed this observation. Following blood loss, the haematopoietic activity of bone marrow increased [17, 18] and, as a result, reticulocytes and a young population of red cells appeared in the blood stream. The average age of erythrocytes decreased with the increase of the activity of various enzymes of the glycolytic pathway [18, 19]. The behaviour of hexokinase activity is rather characteristic. In human reticulocytes an increase of its activity was observed whereas in dog reticulocytes the hexokinase activity was lower than in the young population of adult red cells [17]. The highest hexokinase activity was noticed in dogs on days 14 to 16 after phlebotomy, i.e. in the period when the number of reticulocytes decreased almost to normal values [17]. A blood loss of about 20 to 30% of the circulating blood volume requires filling the blood vessels with blood or blood substitutes [20]. The changes observed by us in the content of 2,3-DPG, ATP and total organic phosphates after reinfusion with own blood, as well as following infusion with Fluigel, were insignificant. Rudowski et al. [21] bled dogs until they had lost 20 ml blood/kg of body weight. Fifteen minutes later they were infused with an equal volume of saline enriched with pyruvate, phosphate and chloride (PPC solution). Some dogs were given the same fluid with the addition of adenine. In dogs infused with PPC solution the content of 2,3-DPG after 4 h was identical to that prior to phlebotomy. Only after 24 h was there a slight increase of 2,3-DPG, more than 15% of the initial concentration. On the second and third days, 2,3-DPG concentration was still at the same level. Enrichment of the PPC colution with adenine led to similar results, i.e. 2,3-DPG concentration was slightly increased. The ATP content in dogs infused with both types of fluid was similar as 2,3-DPG. Even 48 h after the infusion of fluid with adenine the ATP content was only 15% higher than initially.

We expected the infusion of IPP solution to cause an increased synthesis of 2,3-DPG and at the same time a higher content of organic phosphate. Such experiments were performed also on monkeys infused intravenously with IPP solution of a similar composition [6]. Three hours after infusion synthesis of 2,3-DPG

increased and after 6 h the increase exceeded the initial value by 34 to 79%. The fact that dog erythrocytes infused with inosine did not synthetize 2,3-DPG is likely to be caused by the lack of purine nucleoside phosphorylase activity [5]. Earlier investigations carried out in this laboratory indicated that human erythrocytes incubated in a medium containing high concentration of inosine, pyruvate and phosphate accumulate large amounts of hypoxanthine nucleotides, independently of the intensive 2,3-DPG synthesis [22, 23]. After the addition of IPP solution with Fluigel and own blood, however, the acid-soluble phosphate compounds of dog erythrocytes contained neither inosine monophosphate (IMP) nor inosine triphosphate. This seems to suggest that the red blood cells are also unable to phosphorylate inosine to IMP. The results obtained by us are in agreement with the earlier observations of other investigators [17, 21]. Like Smith and Agar [17] we found that following acute blood loss in dogs, an increased cardiac output is the main mechanism in preventing oxygen deficiency.

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Intracellular "Labyrinths" in Acute Leukaemia
Do they Indicate the Maturational Ability of Leukaemic Cells?

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The occurrence and ultrastructure of the so-called "labyrinths" were investigated in the bone marrow cells from 13 patients with preleukaemia and acute leukaemia. Except for one patient, labyrinths were found in the myeloid cells from patients with preleukaemia and acute myelogenous leukaemia. Labyrinths could not be detected in lymphoblasts from patients suffering from acute lymphoblastic leukaemia or in plasma cells from a patient with plasma cell leukaemia. In patients with preleukaemia and smoldering leukaemia, labyrinths could be detected in all differentiation forms, from myeloblasts to mature polymorphonuclear granulocytes. Since it is improbable that labyrinths develop in consequence of exogenous effects, their presence in the mature granulocytes points to the *in vivo* maturational ability of leukaemic precursors in certain cases of acute myelogenous leukaemia and preleukaemia.

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It is widely accepted that acute myelogenous leukaemia (AML) arises from a block of maturation of the myeloid stem cells at early stages of differentiation [1, 2]. Though some authors described *in vitro* maturation of leukaemic cells in semisolid cultures, the derivation of these mature cells from normal progenitors cannot be excluded [3]. So it remains a problem whether the seemingly normal polymorphonuclear granulocytes (PMN) found in AML-s derive from normal or leukaemic progenitor cells. So far no leukaemia-specific markers have been found which would indicate the leukaemic character of PMN-s. Bainton et al. [4] described Auer rods in PMN-s of a patient suffering from AML, but these cells contained only azurophilic granules indicating their defective cytoplasmic maturation while their nuclear structure was practically normal.

The so-called labyrinth structures described by Brederoo and Daems [5] in guinea-pig resident peritoneal macrophages were found by others in preleukaemic and leukaemic cells [6–9]. In the present study we examined the occurrence of these structures in the bone marrow cells of 13 patients suffering from acute leukaemia. Our results suggest that the presence of labyrinths may indicate the existence of AML as well as they may probably be considered to represent marker structures of transformed leukaemic cells pointing to the maturational ability of granulocytic precursors in certain cases of AML.

Material and Methods

The ultrastructure of bone marrow cells was studied in 13 acute leukaemic patients. The patients' data are summarized in Table 1.

The following methods were used.

1. Bone marrow aspirates were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 for 45 min, followed by postfixation in 1% osmium tetroxide for 60 min. After dehydration in graded ethanol the samples were embedded in Durcupan ACM.

2. Alcian blue-lanthanum staining was carried out according to Shea [10].

3. Glutaraldehyde-fixed samples were reacted for peroxidase as follows. Cells were incubated for 45 min in 0.1 M cacodylate buffer pH 6.5 containing 0.2% diaminobenzidine tetrahydrochloride and 0.01% H_2O_2. After washing, postfixation and dehydration the marrow fragments were embedded in Spurr's low viscosity medium [11].

Table 1

Summary of cases

No.	Name	Age years	Sex	Diagnosis	Labyrinths	Duration of disease*
1	K. É.	47	F	Preleukaemia → AML	present	18 months †
2	K. F.	67	F	Preleukaemia → AML	present	14 months †
3	Sz. M.	52	F	Preleukaemia	present	18 months
4	R. I.	66	F	Preleukaemia	present	20 months
5	G. T.	32	M	Erythroleukaemia	present	16 days †
6	H. A.	55	F	Erythroleukaemia	present	2 months †
7	O. P.	43	F	AMML	present	4 days †
8	F. A.	53	F	AML	absent	7 days †
9	K. I.	69	F	AUL	absent	1 month †
10	Cs. B.	18	F	ALL	absent	11 months †
11	O. J.	25	F	ALL	absent	8 months †
12	Sz. G.	28	M	ALL	absent	18 months †
13	H. J.	39	M	Plasma cell leukaemia	absent	14 days †

* From the patients' admission.

Abbreviations and symbols: AML = acute myelogenous leukaemia; AMML = acute myelomonocytic leukaemia; ALL = acute lymphoblastic leukaemia; AUL = acute undifferentiated leukaemia; M = male; F = female; † died.

Thin sections were made by a Porter Blum MT-1 ultramicrotome, stained with uranyl acetate and lead citrate, or left unstained, and investigated with a Philips EM 300 electron microscope.

Results

A great number of labyrinth-containing myeloid cells were found in patients with preleukaemia and with smoldering leukaemia. Alcian blue-lanthanum staining revealed continuity between labyrinths and the extracellular space (Figs 1a and 1b). In many cases a close interrelationship was observed between the channel system and the endoplasmic reticulum (Fig. 1b, Fig. 2 inset). Labyrinths could be found from the myeloblasts as far as the mature granulocytes. The smallest labyrinths occurred in the blast cells as a proliferation of the plasma membrane into the cytoplasm (Fig. 2). Larger labyrinths with more sophisticated structure were present in promyelocytes, myelocytes and PMN-s (Figs 3–5). In two cases an unusual large number of labyrinth-containing cells was observed. In one of these cases (K. É., preleukaemia → AML) two distinct PMN populations were seen, one normal and a pathological one showing Pelger-Huët-like anomaly and hypogranularity of the cytoplasm (Figs 4, 5). While labyrinths were found in some of the abnormal PMN-s, apparently normal granulocytes never contained these structures. In the second (preleukaemic) case (Sz. M.) no distinct PMN populations were encountered and in addition to myeloid precursors the labyrinths were also found in some seemingly normal PMN-s (Figs 6a, 6b).

In the two erythroleukaemic patients, large labyrinths were found in the early myeloid precursors. In the first patient (G. T.) poorly differentiated blasts and pseudo-Chediak-Higashi cells contained these structures (Fig. 7). In the second patient (H. A.) they were found in many neutrophilic and basophilic promyelocytes (Fig. 8). Mature PMN-s were not seen in either case.

In a case of acute myelomonocytic leukaemia, labyrinths were seen in a great number of monocytic precursors (Fig. 9). In addition, some immature granulocytic cells also contained them.

Out of the 8 acute non-lymphoid cases only one patient suffering from AML had no labyrinth-containing myeloid cells.

It should be emphasized that labyrinths could be found both before and after chemotherapy. Moreover they were never seen in dividing cells.

In 3 cases of acute lymphoid leukaemia where the cells showed no peroxidase positivity even at the electron microscopic level, labyrinths were not seen inside the lymphoblasts. Similarly, they could not be detected in a case of acute plasma cell leukaemia and acute undifferentiated leukaemia.

Discussion

Labyrinths were originally described by Brederoo and Daems in guinea-pig resident peritoneal macrophages [5]. Similar structures were found by Epstein et al. in 2 patients having giant platelets, thrombocytopenia, nephritis and deafness [12]. Labyrinths were also found in human myeloid precursors in vitamin B_{12} deficiency [13] and in some promyelocytes and myelocytes in preleukaemic and leukaemic patients [6, 8, 9].

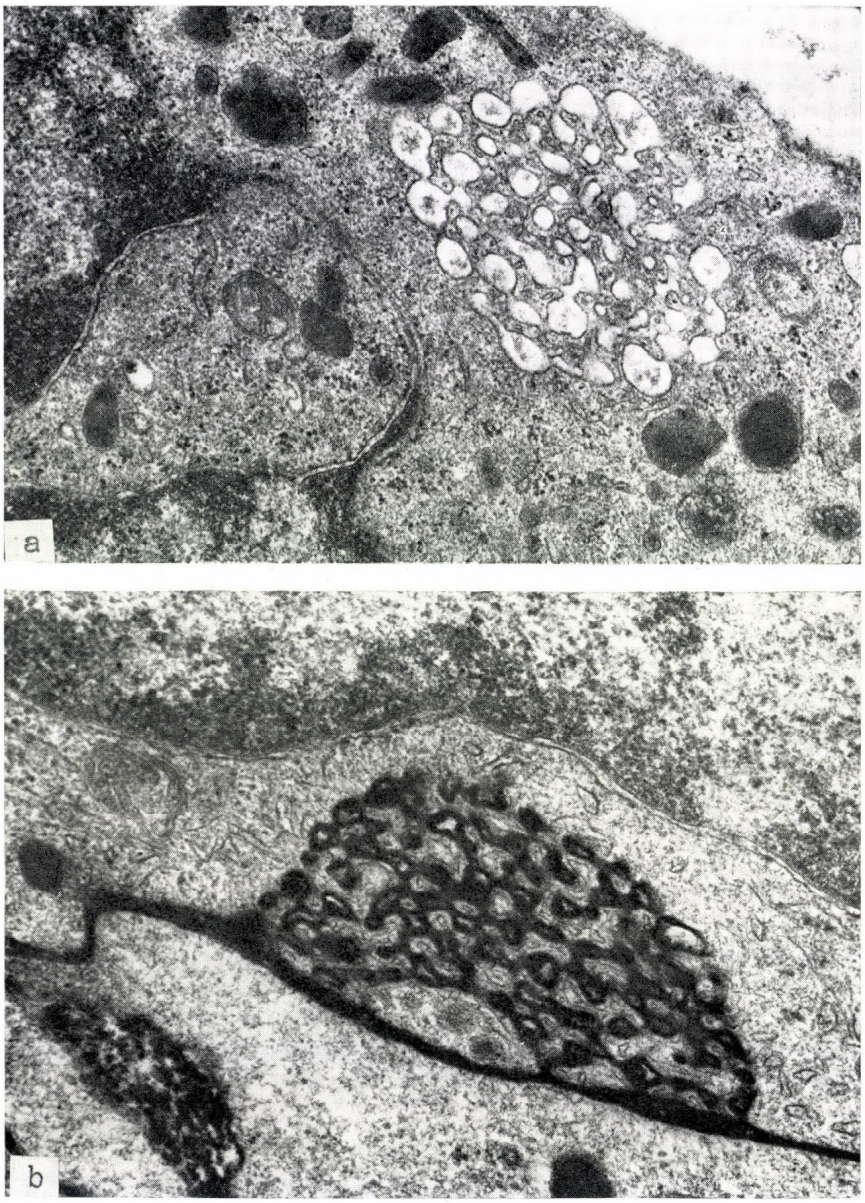

Fig. 1. (a) Characteristic, large labyrinth in a bone marrow myeloid cell from patient 1 with preleukaemia transformed into AML. × 32 000. (b) Alcian blue-lanthanum staining reveals continuity between the channel system and the extracellular space. Note the close relationship between the labyrinth and the endoplasmic reticulum. × 40 000

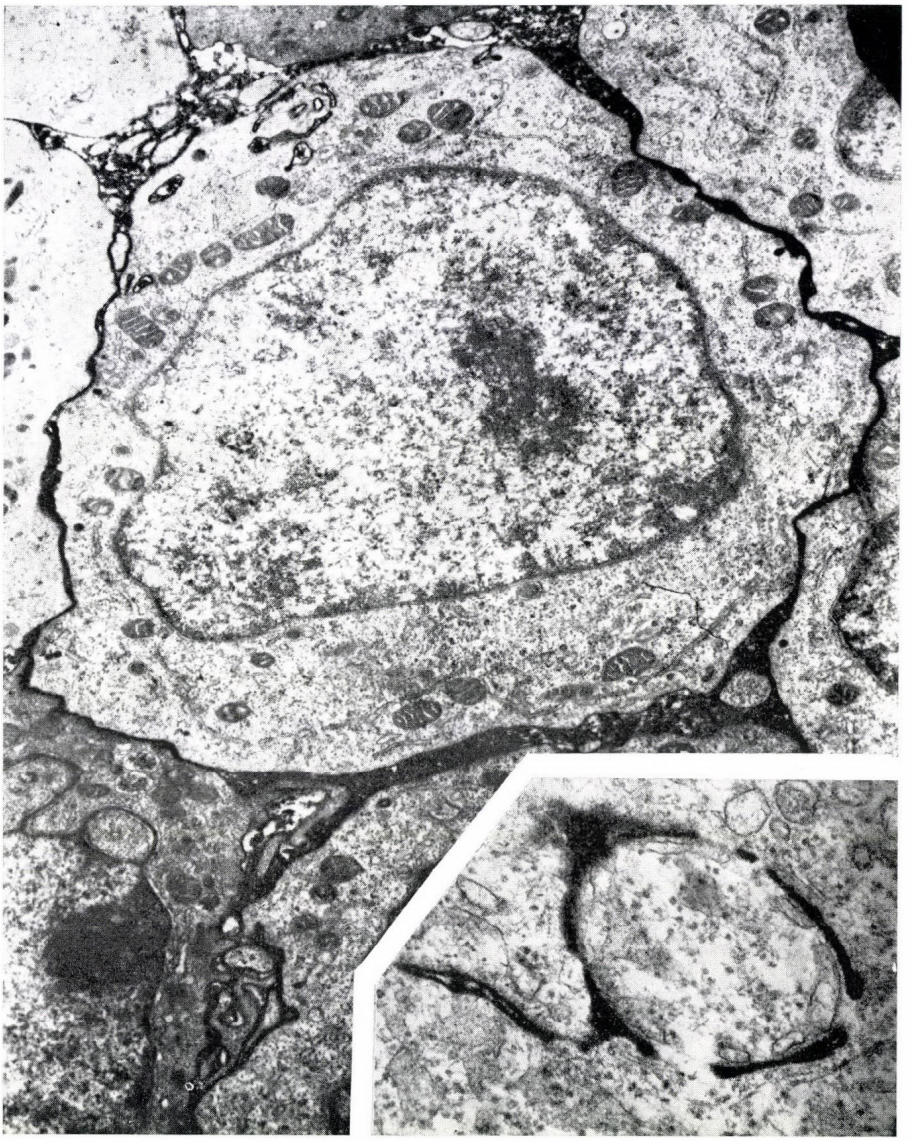

Fig. 2. Myeloblast from patient 1. Note the small labyrinth filled with lanthanum. × 11 200. Inset: Higher magnification of an other small labyrinth which also reveals intimate contact between the membrane system and the endoplasmic reticulum. × 42 000

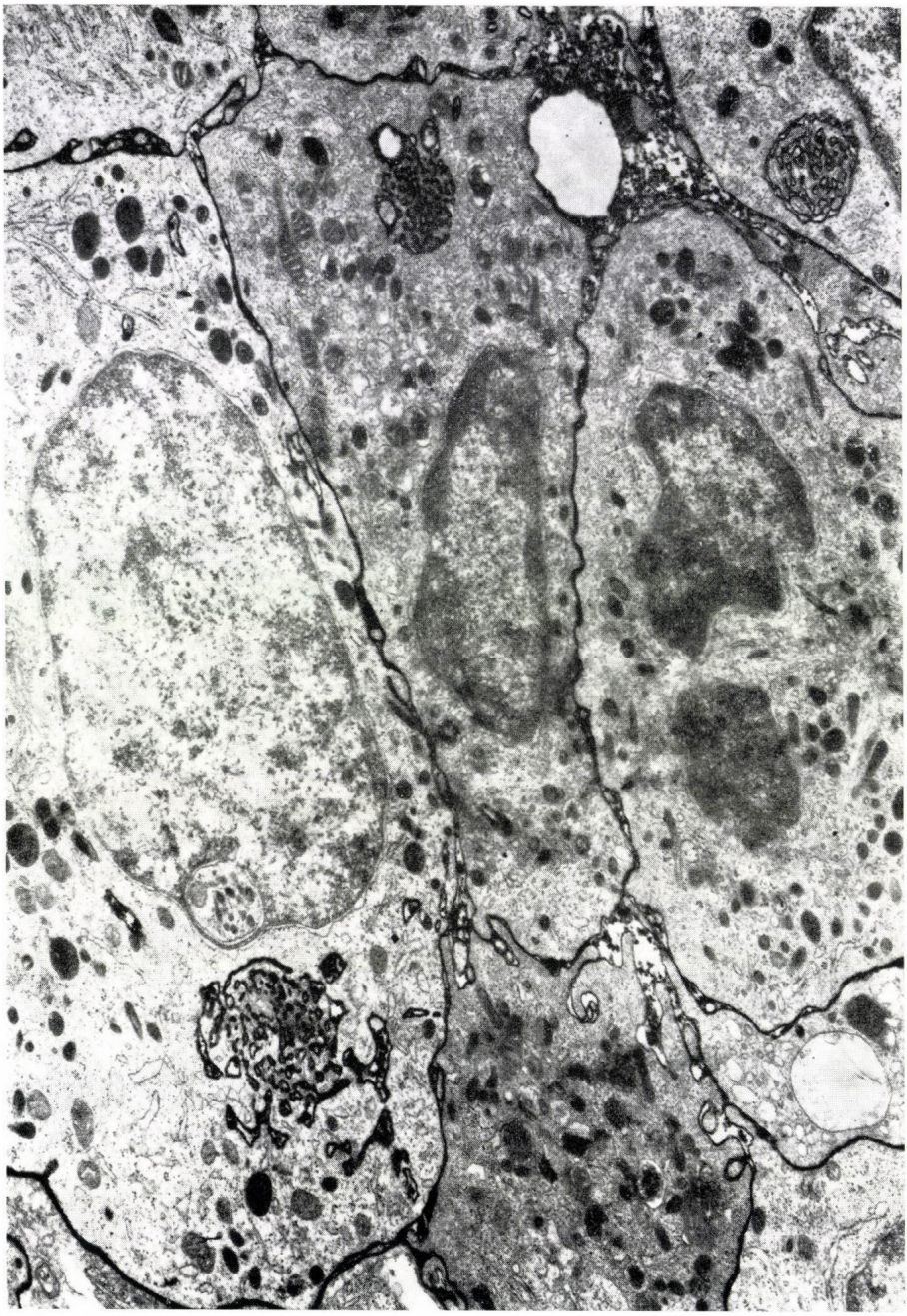

Fig. 3. Labyrinth-containing bone marrow cells with different maturation from patient 1, two promyelocytes (upper right and lower left) and a myelocyte (in the middle). Note the large, lanthanum-filled labyrinth in each cell. × 12 900

Fig. 4. Labyrinth-containing marrow myelocyte and mature PMN from patient 1. Note that the PMN is not normal showing Pelger-Huët-like nuclear anomaly and hypogranularity of the cytoplasm. × 11 200

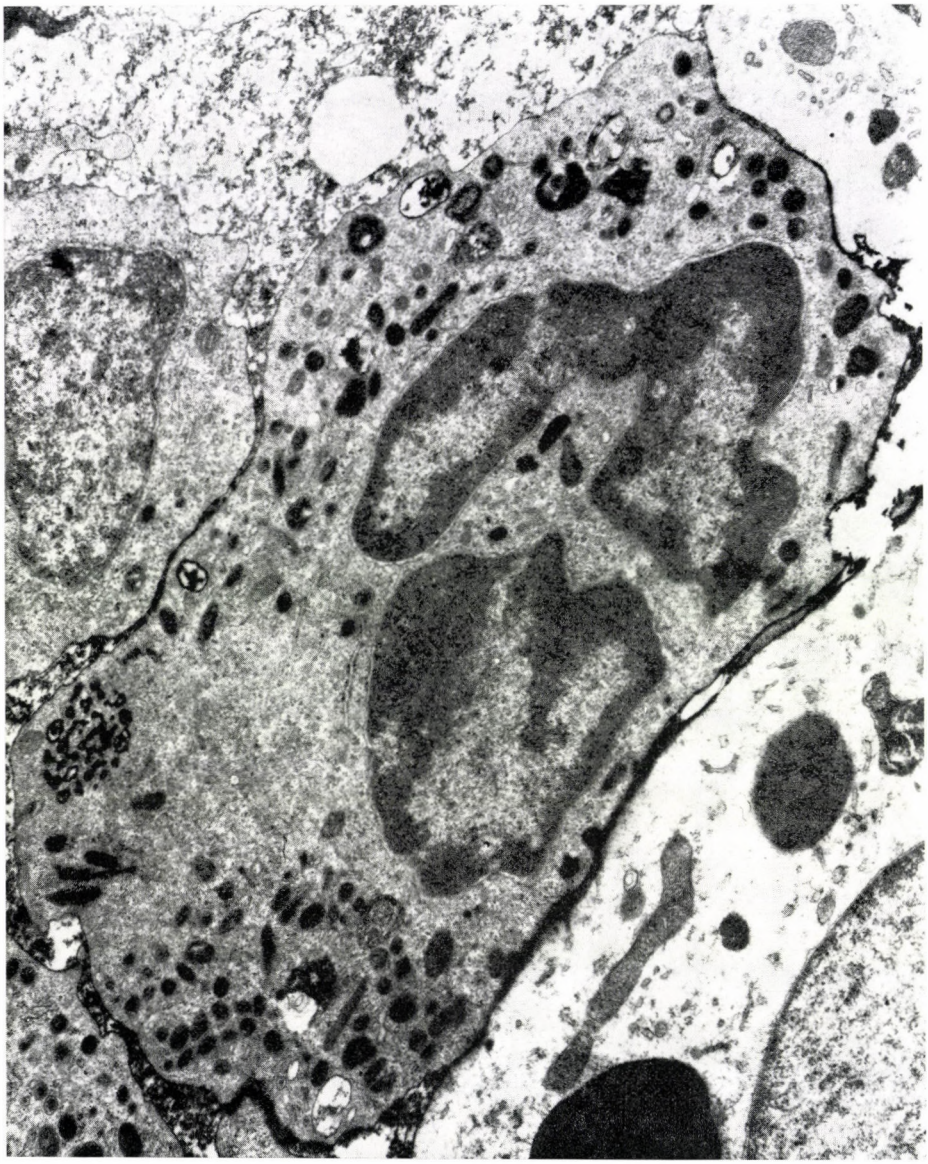

Fig. 5. Higher magnification of a mature marrow PMN showing labyrinth from patient 1. Note the needle-like and abnormally shaped granules. × 14 000

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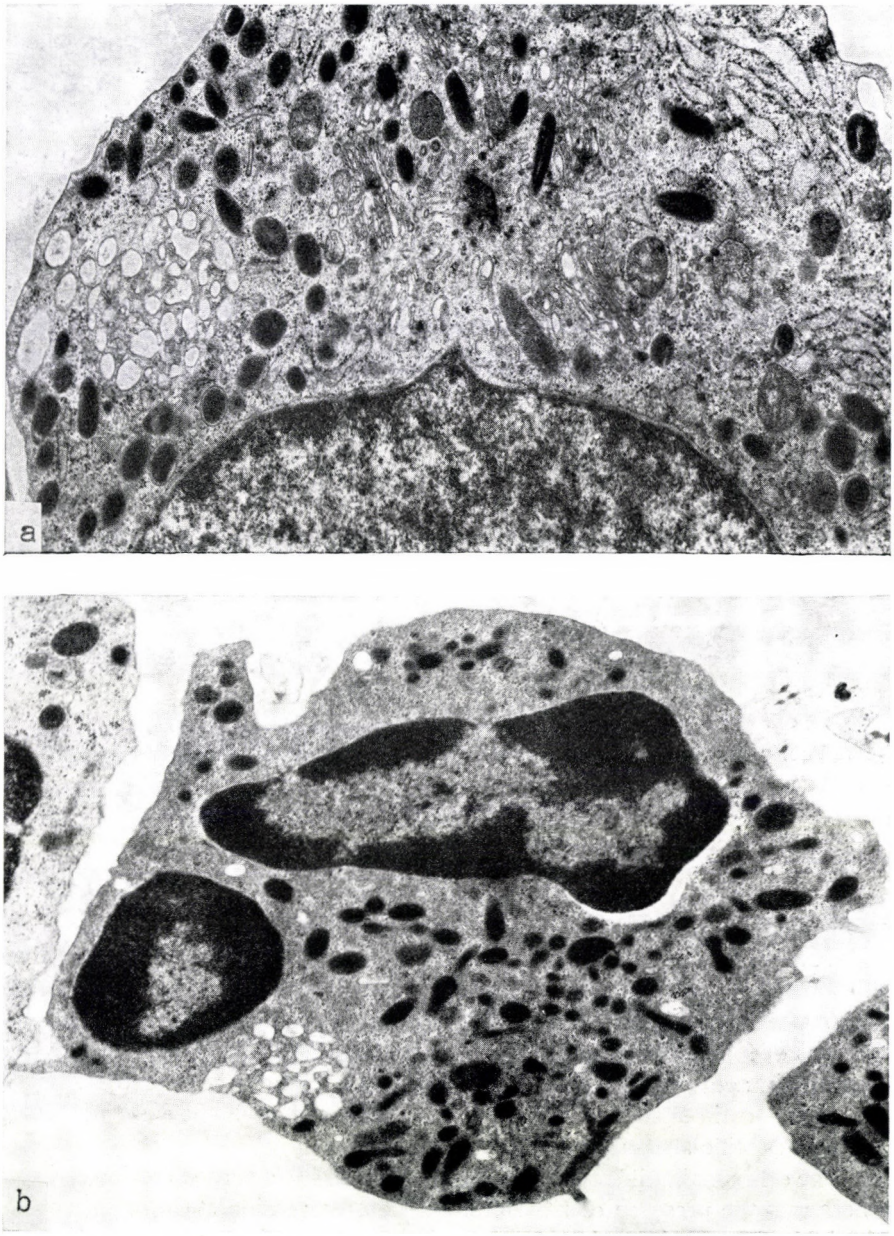

Fig. 6. Labyrinth-containing promyelocyte (a) and mature marrow PMN (b) from patient 3. Note the normal ultrastructure of both cells. × 16 400

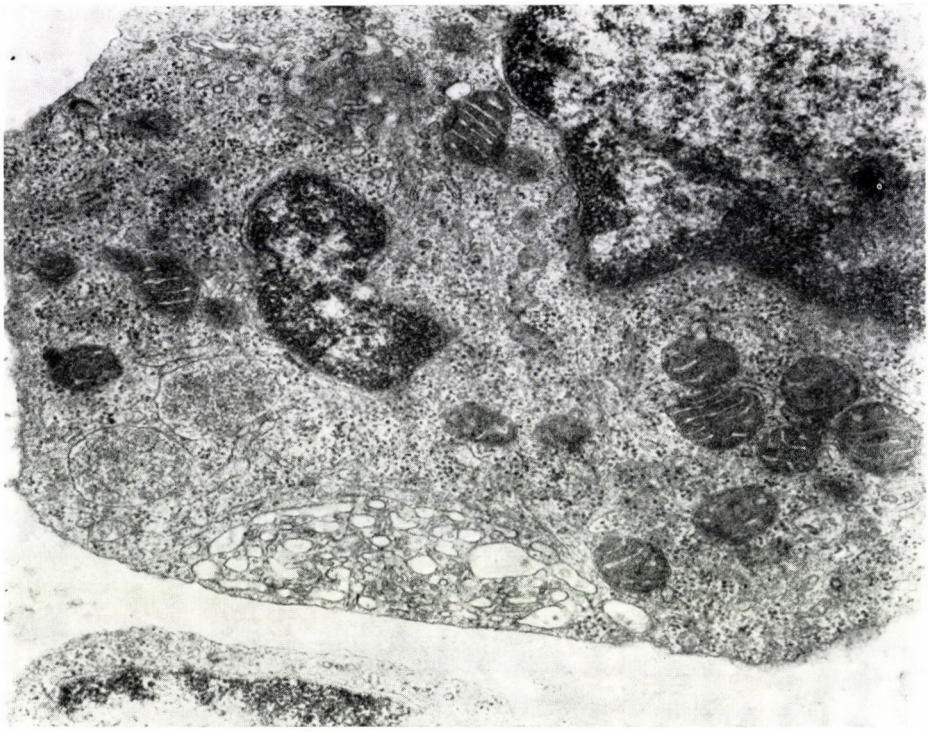

Fig. 7. Poorly differentiated marrow blast cell containing large labyrinth from patient 5. × 25 600

The aim of our investigation was to explore the occurrence of labyrinths in acute leukaemia.

Our results differed from the earlier ones in two respects. First, we found them more frequently in AML than it was stated formerly [6]. Out of 8 AML cases, 7 patients had labyrinths in their granulocytic cells. On the other hand, the blast cells of 3 patients with ALL showed no labyrinths at all, and there were no labyrinths in acute plasma cell leukaemia, either. Therefore, the labyrinths may have a diagnostic significance in doubtful cases, although they are not strictly leukaemia-specific structures.

The other main difference between our findings and those described by other authors was the presence of labyrinths in each maturation stage in the myeloid series in 4 of our patients. In preleukaemia and smoldering leukaemia, labyrinths could be observed in all differentiation forms from myeloblasts to mature PMN-s (Figs 2 – 6). The labyrinths might be due to exogenous factors affecting both the normal and the leukaemic cells at the same time, but we think that the labyrinths belong to cells arising from leukaemic clones, indicating thereby the differentiat-

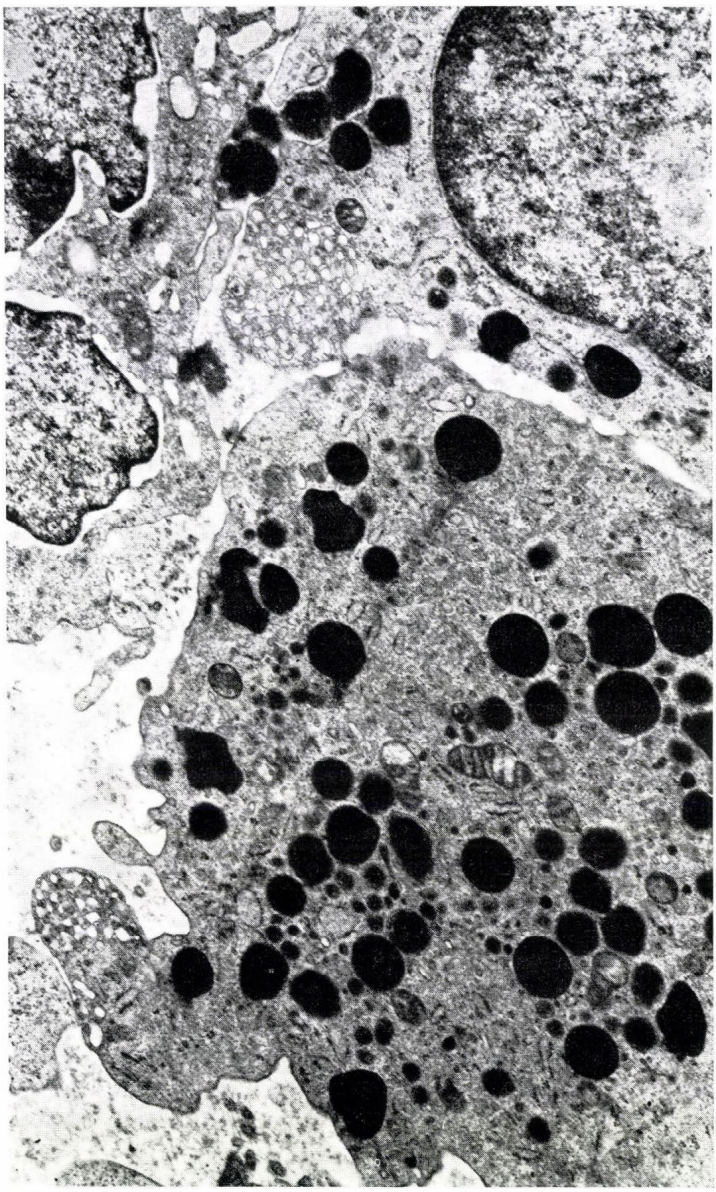

Fig. 8. Two basophilic promyelocytes containing large labyrinths from patient 6. Note the large, sometimes irregularly shaped granules, some of which are being expelled out of the upper cell. × 13 600

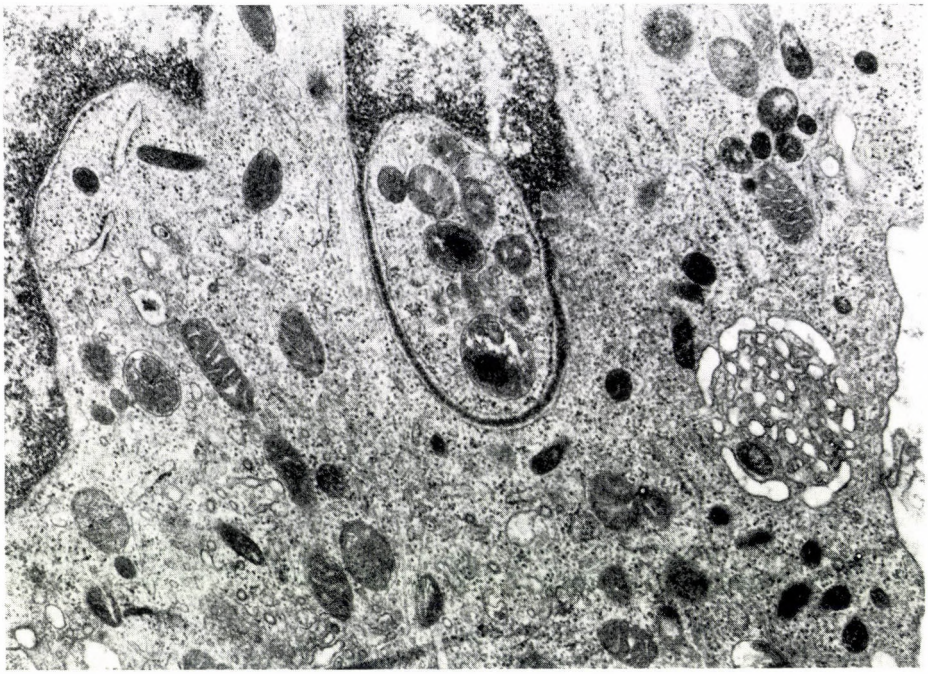

Fig. 9. Part of a marrow promonocyte from patient 7 with AMML. Note the cytoplasmic structures characteristic of monocytic cells (short profiles of endoplasmic reticulum, numerous small granules and vesicles) and a large labyrinth at the right side. × 20 500

ing ability of malignantly transformed stem cells. Two observations suggest this hypothesis. First, in that case of AML where two distinct PMN populations were simultaneously present, only the pathological PMN-s contained labyrinths. Some needle-like and irregularly shaped granules of these pseudo-Pelger cells (Figs 4, 5) were evidently abnormal and similar to those of early precursors containing labyrinth structures. The coexistence of these abnormalities in the same cells and the lack of labyrinths in the apparently normal PMN-s seem to be inconsistent with the exogenous character of the labyrinths and support their clonal nature. Second, in severe, overt leukaemias where no mature cells were seen in the bone marrow, only precursors arising from myeloid stem cells (myeloblasts, promyelocytes, promonocytes) formed labyrinths (Figs 7–9) indicating a possible failure of the common myeloid precursor.

If the labyrinths are really endogenous markers of malignantly transformed cells, this indicates that in certain preleukaemic cases pathological blasts may differentiate into almost normal PMN-s. In some cases of overt leukaemia this differentiation is impaired and apparently pathological, while in the most severe cases no or only minimal differentiation occurs. Therefore, as it has already been

suggested [14–17], the complete maturational inability of leukaemic progenitors becomes questionable at least in certain cases of AML and preleukaemia.

The origin and function of the labyrinths is not known. Since they were observed in untreated patients, too, the exogenous effect of the applied drugs could be excluded. The so-called "emperipolesis" described by Breton-Gorius [8] has been observed in none of our cases. The intimate relationship between the labyrinths and the endoplasmic reticulum may suggest a role in transport between the endoplasmic reticulum and the extracellular space. The absence of labyrinths in dividing forms may reflect their dynamic nature, in other words they probably disappear during mitosis and develop again in the progenies. Detailed investigation of further preleukaemic and leukaemic cases including cytochemical techniques as well as membrane markers are necessary to elucidate the true nature of these structures.

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Iron Stores in Healthy Pregnant Women as Determined by Immunoradiometry of their Serum Ferritin Levels

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Immunoradiometric determination of the serum ferritin level allows to obtain the true value of the volume of iron stores in pregnant women. To complete our earlier examinations, in 752 primigravidae aged 20 to 29 years, the serum ferritin level has been determined in the 3rd, 5th and 8th month of pregnancy. One hundred and fifty-three of the subjects were given iron therapy, while 599 received no iron during pregnancy. The haemoglobin value begins to decrease in early pregnancy, reaches its minimum in month 5 and stays at this level until month 8. In the primigravidae, the mean haemoglobin level decreased to 12 g/dl by the 5th month of pregnancy and persisted at that level until delivery. Serum ferritin and iron values decreased continuously from the beginning until the end of pregnancy. The decrease in serum iron level was essentially less than that in the serum ferritin level. In spite of the 12.1 g/dl haemoglobin value considered normal and of the about normal mean serum iron value, the serum ferritin level was extremely low in 10% of the pregnant women, a proof of depleted iron stores. In about another 10%, haemoglobin values higher than 11 g/dl were found, with serum ferritin levels of 5 ng/ml or even lower. In this latter group, the serum iron level and the saturation index already pointed to iron deficiency in months 5 to 8 of the pregnancy. Our assumption that iron dosage was insufficient in an appreciable part of subjects given iron therapy, was well founded. It is concluded that the overwhelming part of pregnant women is in need of iron therapy from the 3rd month of pregnancy.

*

Introduction

Except for certain pathological conditions involving grave cell damage, the immunoradiometric assay (IRMA) for serum ferritin determination allows precisely to determine the volume of iron stores in the organism.

Ferritin is an iron-storage protein of 450 000 mol. wt. consisting of a protein shell and of an iron-containing core. It occurs in high concentrations in the liver, spleen and bone marrow.

Addison et al. [1] were the first to demonstrate that in iron-deficient patients the serum ferritin level is lower than the normal limit value of 20 to 200 ng/ml and is higher in the case of iron overload. The mean serum ferritin level amounts to 50 ng/ml in healthy males and 30 ng/ml in healthy females [2].

The improved diagnostic approach to iron deficiency, an endemic condition even in developed industrial countries, and to haemosiderosis, a condition responsible for grave organic lesions, necessitated the introduction of serum ferritin

determinations. For that purpose, relying upon the findings of Addison et al. [1] we have developed a kit for the immunoradiometric determination of serum ferritin [3].

Subjects and Methods

The pregnant women of an antenatal clinic in Budapest were included in the study. Their erythrocyte count, haemoglobin (Hb) level and their iron metabolism were examined. To obtain a homogeneous group, only the laboratory findings of the 20 to 29 years old subjects were processed. Determinations were performed in the same stages of pregnancy, in months 3, 5 and 8. As a control, 54 female blood donors in the 20 to 29-year age group, giving blood for the first time in their life, were also examined. Blood was always collected between 8 and 10 a.m. Our study covered the period from June 1st, 1977, to December 31st, 1979.

For data processing, the subjects were divided into two groups.

Group I: 599 subjects not given iron therapy.

Group II: 153 subjects given iron therapy.

Iron therapy was prescribed after determining the results of blood sampling in month 3. The dose of orally administered ferro-iron amounted to 100 to 150 mg daily. Only the record cards and the personal accounts of the pregnant women informed us about the continuity of iron therapy and of the amount of iron they have taken. The data therefore cannot be considered reliable since, as it has turned out, in many cases the subjects neglected to take iron.

Hb in the form of cyanhaemiglobin was determined by Lynson Junior photometer, erythrocyte count was determined by an automatic Picoscale cell counter (produced by Medicor), haematocrit was measured by a microhaematocrit centrifuge (Janetzky). Serum iron and total iron-binding capacity (TIBC) were determined by the method of Ness and Dickerson [4]. The serum ferritin level was determined by radioimmunometry [3].

Results

Data on pregnancy anaemia, the values obtained by conventional methods for the determination of pregnancy iron deficiency and the results of serum ferritin measurements are presented in Fig. 1.

As it clearly appears from Fig. 1, the decrease of the values begins in early pregnancy, reaches its minimum in month 5, and stays at the same level until month 8. This is in good agreement with our first results [5]. The Hb level generally decreased to 1.86 mmol/l (12.0 g/dl) by month 5 and persisted there until delivery.

Serum ferritin and serum iron behaved differently. Their values began to decrease at the beginning of pregnancy and continued to do so until its end.

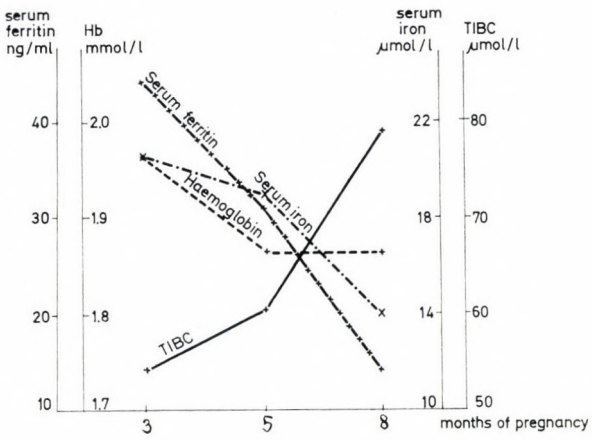

Fig. 1. Haemoglobin, serum iron, TIBC, and serum ferritin level during pregnancy

Serum iron diminished less than did the serum ferritin level during the second trimester but the difference was not significant, whereas in the third trimester the change was significant ($p < 0.001$), especially for serum ferritin. The rise of TIBC values continued throughout the entire pregnancy. Since, however, the hormonal changes occurring during pregnancy, as e.g. the change in the oestrogen level, appreciably affected the transferrin level, the estimation of this parameter in respect of iron metabolism is not realistic. As compared to that in non-pregnant women by month 3 the serum ferritin level slightly decreased. This decrease, however, could not be assessed because of the high standard deviation, but by month 5 it became explicit ($p < 0.01$), then it fell below the normal level in month 8 when it decreased to 14 ng/ml ($p < 0.001$). Extremely low ferritin levels (1 to 2 ng/ml) were often found among 8-month pregnant women, indicating totally depleted iron stores. Accordingly, serum ferritin is the most unambiguous and earliest indicator of changes in iron metabolism in the course of pregnancy.

Next, attempts were made to approach the problem from a different aspect. The subjects with extremely low serum ferritin levels were selected. The Hb, serum iron and transferrin saturation of subjects with serum ferritin levels ranging from 5.1 to 10 ng/ml are shown in Table 1, and those with serum ferritin levels below 5 ng/ml in month 8 in Table 2.

In the 59 subjects in month 8 (see Table 1) the mean Hb level amounted to 1.75 mmol/l (i.e. 12.1 g/dl). This value is still considered normal and judged as the manifestation of pregnancy haemodilution by the majority of obstetricians. Consequently, they neglect the introduction of iron therapy and do not consider it an omission not to have described iron in the 3rd month though the serum ferri-

Table 1

Hb, serum iron, TIBC values and transferrin saturation in pregnant women with 5.1—10 ng/ml serum ferritin level in the 8th month of pregnancy

	month 3 n = 8	month 5 n = 14	month 8 n = 39
Hb mmol/l (g/dl)	1.95 (12.6)	1.84 (11.9)	1.88 (12.1)
Serum-iron μmol/l (μg%)	13.0 (73)	15.5 (87)	14.5 (81)
TIBC μmol/l (μg%)	60.6 (339)	67.3 (376)	79.5 (444)
Transferrin saturation (%)	0.22 (22)	0.23 (23)	0.19 (19)

Table 2

Hb, serum iron, TIBC values and transferrin saturation in pregnant women with 5 ng/ml or lower serum ferritin level in the 8th month of pregnancy

	month 3 n = 0	month 5 n = 7	month 8 n = 58
Hb mmol/l (g/dl)	—	1.89 (12.2)	1.75 (11.3)
Serum-iron μmol/l (μg%)	—	12.8 (72)	10.5 (59)
TIBC μmol/l (μg%)	—	80.3 (449)	82.6 (462)
Transferrin saturation (%)	—	0.16 (16)	0.13 (14)

tin level definitely suggests a grave depletion of iron stores. In addition, the serum iron value is at the lower limit of normal, 14.5 μmol/l (i.e. 81 μg%). The saturation index alone shows that the lower level of normal has been exceeded.

In Table 2 the data of 58 subjects in the 8th month of pregnancy are summarized (see column 3). They had a Hb level of 1.75 mmol/l (11.3 g/dl). This represents for many of those in charge of antenatal care and for many obstetricians a value not absolutely indicating iron treatment, in spite of the complete depletion of iron stores shown by the serum ferritin level.

As a next step, the incidence of iron deficiency in pregnancy was studied together with the question how the iron therapy applied in antenatal clinics reduced the development of pregnancy anaemia.

The results are summarized in Fig. 2. It is evident from Fig. 2, that the ratio of pregnant women found to be iron-deficient according to their serum ferritin levels being below 20 ng/ml, increases rapidly with the progress of pregnancy. As a result of iron therapy started in the 3rd month, the 20% incidence of iron deficiency in month 5 roughly agrees with the 17% observed in the 3rd month and is but slightly higher than the 13% value of the normal controls. In contrast, without iron therapy, the incidence of iron deficiency in month 3 was 35% and

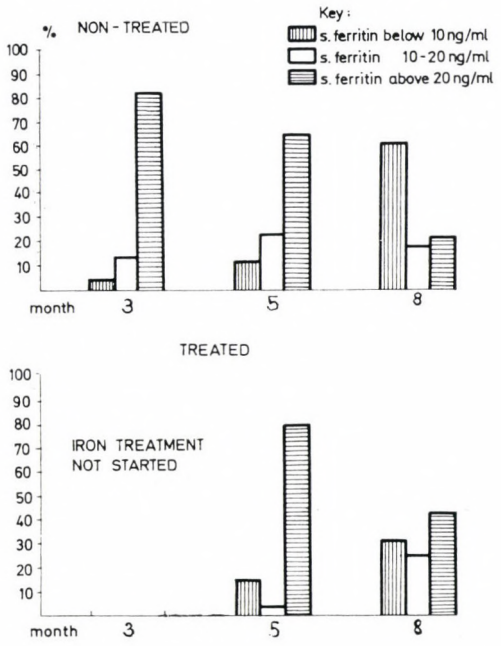

Fig. 2. Incidence of iron deficiency in iron-treated and non-treated pregnant women

in month 8,79% of the untreated and 56% of the iron-treated subjects were deficient in iron. In the third trimester, the frequency of extremely low iron stores is rather high. The serum ferritin value was below 10 ng/ml in 31% of the iron-treated group, and 61% in the non-treated group. Thus, the subjects were not given as much iron as would have been required to satisfy their iron demand.

Discussion

The serum ferritin level offers exact information about the iron stores in the organism. The test allows to trace the changes in iron metabolism in the pregnant organism, while the Hb and serum iron levels failed to furnish reliable information. Our studies showed that at a Hb value of 11.0 g/dl the iron stores may absolutely be depleted. Other authors also failed to find a correlation between the serum ferritin and Hb levels [6].

Fenton et al. [7] showed that the depletion of iron stores starts in early pregnancy, suggesting an increased erythropoiesis during the first trimester. In this light it is not surprising that iron therapy started in month 3 should at most be capable of moderating but by no means of restoring the loss of iron.

Iron demand during pregnancy is generally so high, 1000 to 1200 mg that it cannot be covered from the diet and so, unless prescribed, the stores are completely depleted by month 8. The problem has not been settled to what extent iron deficiency may be detrimental to fetal development and how the maternal organism reacts to it. In 1978, the columns of the *British Medical Journal* [8] were devoted to the question. Part of the physicians recommended the early introduction of intensive iron therapy, while others, in view of the undesirable side effects of iron preparations, considered this therapy superfluous. According to Hemminki and Starfield [9], Rios et al. [10] and van Eijk et al. [11], maternal iron deficiency does not affect fetal development and the fetal iron stores. They also found that the newborns of iron-treated or non-treated mothers are born with identical stocks. The maternal Hb level is restored by two months after delivery and the iron stores reach the lower level of normal [11, 12]. Accordingly, it was concluded that iron deficiency anaemia during pregnancy is a physiological condition that does not merit attention.

Bratlid et al. [6] found no correlation between the maternal and neonatal serum ferritin levels, between newborn Hb and serum ferritin levels or between newborn birth weight and serum ferritin levels. In contrast, Fenton et al. [7] and Kelly and Macdonald [13] could show that if the serum ferritin level of pregnant women is less than 10 ng/ml, the level in cord blood is significantly lower.

MacPhail et al. [14] found a weak correlation between cord and maternal serum ferritin concentrations, but its biological significance is questionable.

The opinion that delivery itself means a decidedly bigger trauma for a gravely iron-deficient organism seems to be justified [12]. The leading article of the November 11, 1978 issue of the *BMJ* rightly declares that ". . . no population is homogeneous, and the logical approach is to attempt to identify risk factors in individuals" [8]. Recent data [15] point to a significant functional impairment of the non-anaemic iron-deficient human subject. When evaluating our results, the main problem arising was that, provided they are in need of it, pregnant women should be given sufficient iron. The minimum oral dose is 100 mg, the optimum dose range from 100 to 200 mg a day. The increased intake of iron tablets on the assumption that 30% of the iron prescribed is never taken, is calculated to have reduced the prevalence of the decrease of iron stores by 16% in the 5th, and by 23% in the 8th, month of pregnancy. Iron requirements vary considerably among individual women and there is a wide variation in dietary intake, too.

The amount of vitamin C in the diet which is known to increase iron absorption, is also at great variance.

Apart from the present results, we cannot accept as physiological the appreciable decrease or lack of iron stores in gravidae.

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Cytological and Histological Classification of Multiple Myeloma

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In a retrospective study 202 cases of multiple myeloma (MM) were studied cyto- and histomorphologically. In 62 cases only bone marrow smears, in 60 only histological preparations and in 80 both types of material were available. According to the differentiation of myeloma cells, a plasmocytic (n = 127), a mixed cellular (n = 35) and a plasmoblastic type (n = 32) could be distinguished. Besides, 8 cases with predominance of giant cells were found (giant cell MM). The comparative cytological and histological typing into the four types of MM proved to be reproducible. The medium survival time and survival curves of the described types showed a statistically significant difference. The survival times were 39.7 months with the plasmocytic type, 16.1 months with the mixed cellular type, and 9.8 months with the plasmoblastic type. The prognostic relevance of the morphological classification was supported by the frequency of extramedullary infiltrates (20 % in the plasmocytic, 67 % in the mixed cell, 69 % in the plasmoblastic and 100 % in the giant cell type).

*

Introduction

Multiple myeloma (MM) is a proliferation of plasma cells synthesizing and secreting immunoglobulins (Ig). Thus, it may be considered a B-cell non-Hodgkin's malignant lymphoma [41, 86]. In recent years many pathomorphological (cytology, histology, enzyme chemistry) [7, 8, 18, 25, 44, 45, 65, 80, 87, 88], transmission and scanning electron microscopical [9, 11, 12, 13, 20, 26, 28, 29, 51, 59, 78, 79, 83], immunochemical and immunocytochemical [10, 21, 39, 40, 48, 56, 60, 67, 68, 72, 84, 91] studies have dealt with the different aspects of MM. The rate of Ig production by myeloma cells [58, 74, 75] and their proliferation characteristics have also been investigated [23, 64, 70, 77, 92]. One more feature described is the asynchronism of differentiation between the rather immature nucleus of the myeloma cells and their cytoplasm which shows signs of maturation [29, 52].

Owing to their doubtful clinical relevance, the classifications of MM on morphological grounds have been unsuccessful [95, 96]. Surprisingly, to this category of classifications belong those in which differentiated and undifferentiated types are distinguished [3, 4, 20, 27, 35, 58, 82] despite the fact that casuistic reports are indicative of a poorer prognosis of the undifferentiated types (myelo-

sarcoma, plasma cell sarcoma) [2, 27, 31, 38]. This seems to represent an exception to other diseases where morphological criteria are clearly related to prognostic features.

In the present study we shall describe a cytological and histological classification of MM which is reproducible and, on the basis of follow-up data, the defined types seem to be of clinical relevance.

Material and Methods

In the present study 202 cases of MM observed between 1967 and 1978 by the Medical Polyclinic and Department of Pathology, Medical Academy, Erfurt (n = 146), and by the Institute of Pathology, University Medical School, Pécs (n = 56) were analyzed.

In 142 cases the bone marrow smears stained according to Giemsa and Pappenheim were studied, and the degree of differentiation of the myeloma cells was determined. Coded preparations were examined independently by three observers. A cytological study of both bone marrow smears and sections was performed in 80 cases. In a further 61 cases several cytological examinations were done in the course of the disease. In these the problem of a change from one type to another was investigated.

To determine the average survival time, *viz.* the survival ratio, the life table method [100], and for statistical comparison of the standard errors Greenwood's formula was applied.

In 101 cases, in which necropsy was performed, the frequency of extramedullary infiltrations in relation to the morphological subtypes was examined.

Results

Pathomorphological types of MM

On the basis of cyto- and histopathological assessment of the degree of differentiation of myeloma cells the following four types were distinguished (Fig. 1).

(I) *Plasmocytic (differentiated) myeloma* (Figs 2, 3). The dominant cell type is the seemingly *mature plasma cell.* Immature forms (plasmoblasts) may occur scattered or in small foci; their percentage, however, does not exceed 15%. Hyperploid uni- or multinuclear cells are rare. Thus, this type is characterized by a high number of apparently mature plasma cells. Cases with predominance of cells corresponding to *proplasmocytes* of the physiological differentiation of plasma cells were included in this group, if the number of plasmoblasts did not exceed 15%.

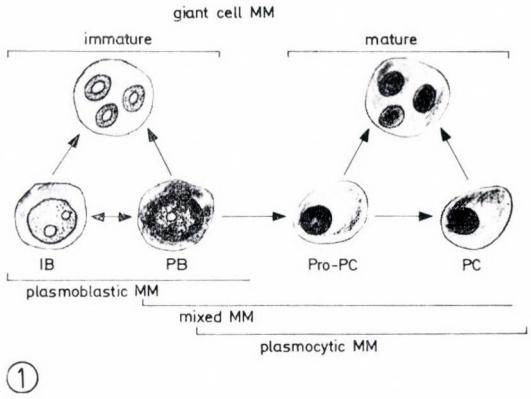
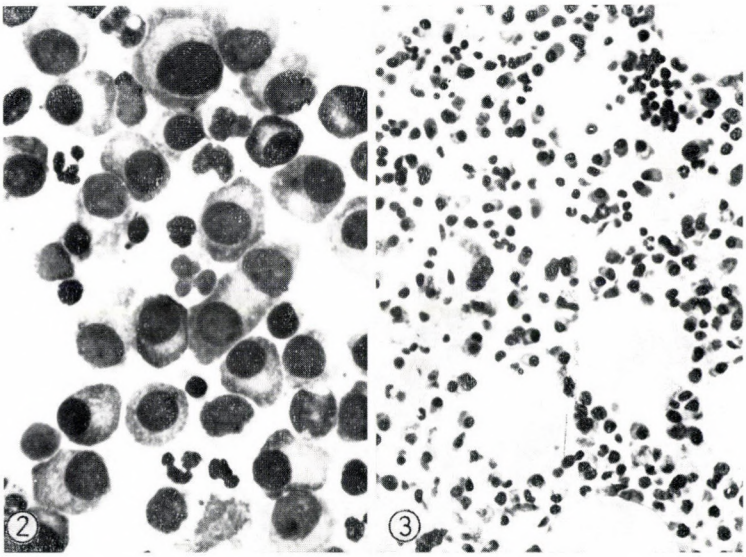


Fig. 1. Plasma cell differentiation in multiple myeloma. IB = immunoblast; PB = plasmoblast; Pro-PC = proplasma cell; PC = plasma cell

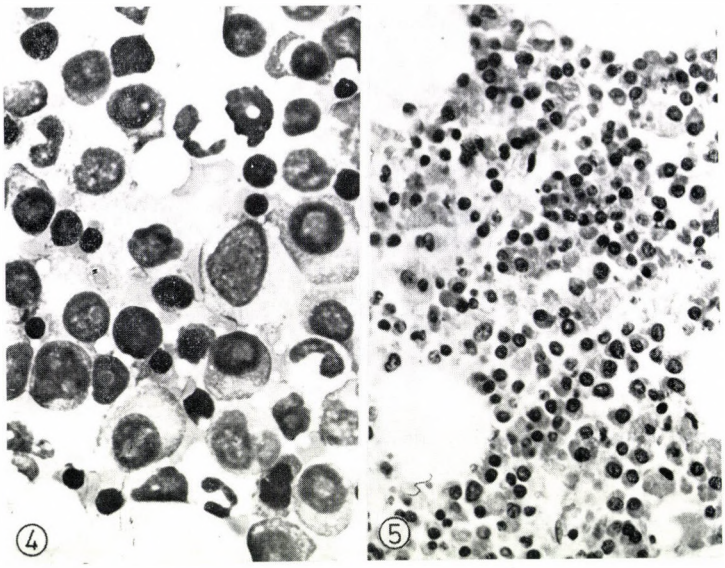


Figs 2—3. Plasmocytic MM

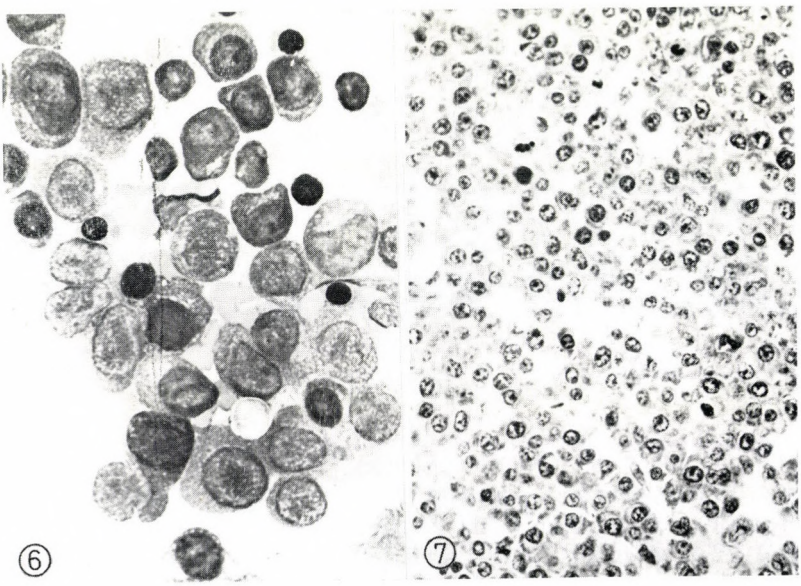
Figs 2—7. Bone marrow, smears and sections, Pappenheim and Giemsa staining, approx. × 1050 and × 270

(II) *Mixed (plasmocytic-plasmoblastic) myeloma* (Figs 4, 5). Besides mature plasma cells, the number of plasmoblasts varies between 20 and 50%. Polyploid cells are more frequent than in the previous type.

(III) *Plasmoblastic (undifferentiated) myeloma* (Figs 6, 7). The immature morphological appearance of this type can be ascribed to the predominance of plasmoblasts (over 50%). A few multinucleated giant cells may occur.



Figs 4—5. Mixed type MM (Magnification see p. 317)



Figs 6—7. Plasmoblastic MM (Magnification see p. 317)

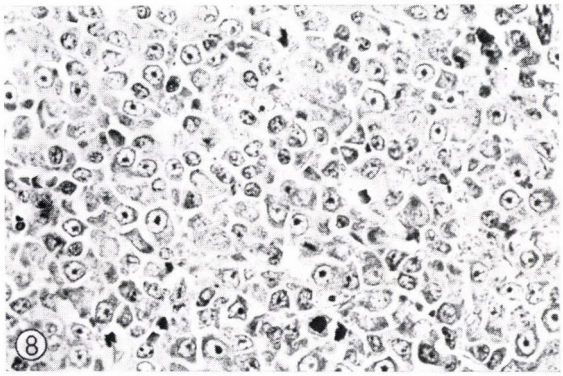
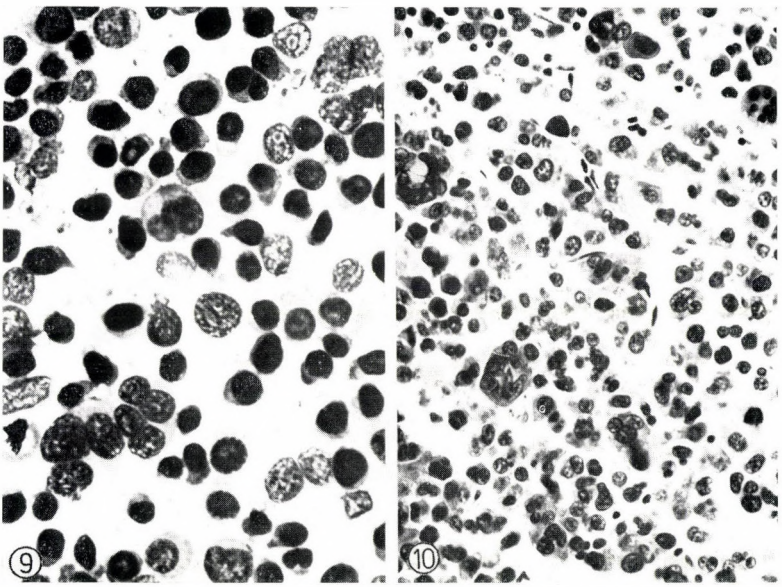


Fig. 8. Extremely immature (immunoblastic) IgG-MM, bone marrow section, Giemsa, approx. × 430



Figs 9—10. Giant cell myeloma. Pappenheim, approx. × 850 and Giemsa, × 270

In certain cases of plasmoblastic MM the cytological picture is rather immature. In smears they show one, or infrequently two, large greyish-blue nucleoli. The cytoplasm of these cells is fragile and stains smoke-grey. In bone marrow sections these cells cannot be distinguished from immunoblasts ("immunoblastic myeloma") (Fig. 8).

(IV) *Giant cell myeloma* (Figs 9, 10). The overall morphological picture of this type is that of the mixed or plasmoblastic myeloma, but the number of giant cells is usually high. According to their differentiation, giant cells may appear either as more mature, or rather immature. In this latter form the giant cell seems to correspond to the giant cells of immunoblastomas.

Table 1

Cytological classification of 142 cases

Morphologic type	No.	per cent	Age, yrs	Sex ratio (female : male)
Plasmocytic	89	63	55.8	1 : 1.6
Mixed	26	17	61.1	1 : 1.4
Plasmoblastic	21	15	53.6	1 : 2
Giant cell MM	6	5	54.0	1 : 2
Total	142	100	54.4	1 : 1.6

Table 1 shows the distribution of 142 cases according to their morphological appearance. Plasmocytic myeloma is the most frequent type (89, 63%), the second and third being the mixed (26, 17%), and the plasmoblastic type, respectively (21, 15%). The four morphological types showed no significant differences in age or sex. In Table 2 the results of histological examination and typing of the bone marrow of 121 necropsied cases are given.

Table 2

Histological classification of 121 necropsy cases

Morphological type	No.	per cent	Age, yrs	Sex ratio (female : male)
Plasmocytic	78	64	56.0	1 : 1.8
Mixed	20	17	69.2	1 : 1.6
Plasmoblastic	17	14	54.1	1 : 1.5
Giant cell MM	6	5	53.9	1 : 3
Total	121	100	55.9	1 : 1.8

Table 3

Cyto- and histological classification of 202 cases

Morphological type	No.	per cent	Age, yrs	Sex ratio (malefe : male)
Plasmocytic	127	63	54.8	1 : 1.7
Mixed	35	17	59.1	1 : 1.5
Plasmoblastic	32	16	53.1	1 : 1.6
Giant cell MM	8	4	54.0	1 : 2
Total	202	100	55.2	1 : 1.7

Table 3 summarizes the cytological and histological analyses of 202 MM cases together with the age and sex ratios. The frequency of types is almost the same as in the separate studies. The giant cell type (Type IV) represents an infrequent variant (4%) of MM.

In 14 of 19 cases, parallel bone marrow cytology and histology at the time of diagnosis resulted in identical types. The remaining 5 were classified as representing the mixed (plasmoblastic-plasmocytic) type on the basis of the cytological findings, whereas on the basis of histology 2 were described as possibly plasmocytic, and 3 as plasmoblastic (Table 4). On reexamining these cases the histological diagnoses were considered to be correct, owing to the apparent inhomogeneity of cell distribution in the marrow smears.

Table 4

Comparison of cytological and histological typing of 19 cases at the time of diagnosis

	Cytology	Histology	Final type
1. B. J.	PB – PC	PC	PC
2. K. A.	PC	PC	
3. E. P.	PC	PC	
4. E. H.	GC	GC	
5. F. K.	PB – PC	PC	PC
6. P. K.	PC	PC	
7. S. H.	PC	PC	
8. L. B.	PB – PC	PB – PC	
9. K. E.	PB	PB	
10. P. D.	PB – PC	PB	PB
11. M. E.	PB – PC	PB	PB
12. N. M.	PC	PC	
13. B. F.	PB	PB	
14. M. W.	PC	PC	
15. Z. E.	PB – PC	PB	PB
16. R. K.	PC	PC	
17. K. M.	PB – PC	PB – PC	
18. K. P.	PC	PC	
19. S. G.	PC	PC	

PC = plasmocytic
PB – PC = mixed (plasmoblastic-plasmocytic)
PB = plasmoblastic
GC = giant cell MM

Morphological classification and paraprotein types

In 81 cases, monoclonal IgG paraproteinaemia was the most frequent condition, amounting to 87% in the plasmocytic type, to 100% in the mixed, and to 61% in the plasmoblastic type. Two cases with monoclonal IgM were of the

plasmoblastic type. In 3 further cases light chain paraproteinaemia was seen (2 plasmocytic and 1 plasmoblastic), and in one the findings were indicative of a non-secreting MM (plasmocytic type with intracellular IgA).

Changes in the morphological type

In 61 cases, several morphological examinations were performed between diagnosis (sternal puncture, iliac crest biopsy) and necropsy. In 8 (13%) cases change in the morphology to a more immature type was seen. In Table 5 details of these 8 cases are given.

Table 5

Change in the morphological type in 61 cases studied several times in the course of the disease

Type at time of diagnosis	No.	Type at necropsy PC	PB-PC	PB	Total
Plasmocytic	39	32	3	4	39
Mixed	12	11	—	1	12
Plasmoblastic	10	—	—	10	10
Total	61				61

Extramedullary infiltrations

Among 101 necropsied cases in 37 (38%) were infiltrates observed in the lymph nodes, spleen, liver or kidney. In the mixed and plasmoblastic types the percentage of cases with extramedullary infiltrates was 65, *viz.* 69%, in the plasmocytic type, 20%. In all four cases of the giant cell type studied, extramedullary infiltrations were found (Table 6).

Table 6

Extramedullary organ infiltrates and morphological type

Type	Number of cases	Number of infiltrated organs		Lymph node		Liver		Spleen		Kidney	
		No.	per cent	Studied	Infiltrated	Studied	Infiltrated	Studied	Infiltrated	Studied	Infiltrated
Plasmocytic	69	14	20	25	9	55	9	52	3	61	9
Mixed	15	10	67	3	3	12	7	11	6	14	2
Plasmoblastic	13	9	69	4	3	11	2	13	7	12	1
Giant cell MM	4	4	100	3	3	4	2	3	2	3	1
Total	101	37	38	35	18	82	20	79	21	90	13

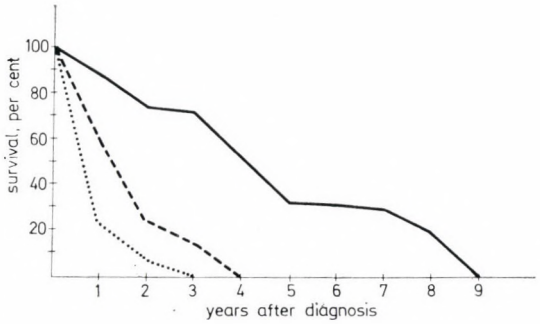


Fig. 11. Survival rates in the morphological types of MM (according to the life table method, [100)]. —— plasmocytic (n = 50), median survival 39.7 months; - - - mixed (n = 17), 16.1 months; · · · plasmoblastic (n = 16) 9.8 months

Survival rate and morphological type

In 83 cases were data available on survival. Median survival time with Type I (plasmocytic) was 39.7 months, significantly higher than with Types II (mixed cellular) and III (plasmoblastic), i.e. 16.1 and 9.8 months, respectively. The rates of survival show significant differences in the four-year period of observation ($p < 0.01$) between Type I (plasmocytic) and Types II – III (mixed cellular and plasmoblastic). Up to the 18th month after diagnosis there is also a significant ($p < 0.01$) difference in survival rate between Types II and III. None of the patients with Types II and III lived longer than 48 months, while at that time 71% of Type I were still living (Fig. 11).

Discussion

Since the introduction of intravital bone marrow examination [98] numerous studies have dealt with the morphology of the myeloma cell [7, 17, 22, 55, 73]. At first, the peculiar appearance of the cytoplasm attracted attention, resulting in designations like: Mott cells [55, 57], "morula cells" [99], "grape cells" [87], thesaurocytes [65], "flaming plasma cells" [94]. Although initially considered specific for MM, analogous cells were later described in reactive bone marrow plasmocytosis [26, 55, 73, 97, 99]. Under the electron microscope all these alterations proved to be signs of deranged immunoglobulin synthesis and/or secretion [51, 52, 53]. A few quantitative features of the cytoplasm of myeloma cells are still considered characteristic, although not pathognomonic for MM [14, 52, 81]. E.g., the perinuclear light zone (Golgi region) of reactive plasma cells is usually absent in myeloma cells, which is indicative of a highly developed endoplasmic reticulum [29, 51, 52, 53]. Similarly, the strong acid phosphatase and β-glucuronidase activity of myeloma cells is of limited diagnostic importance [18, 42, 44, 47,

88, 89]. Signs of cellular polymorphism, such as an increased nuclear-cytoplasmic ratio, loosely arranged chromatin, enlarged and multiple nucleoli, nuclear lobulation, polyploidy are also considered more or less specific for MM [7, 14, 20, 22, 25, 29, 35, 51, 81, 94, 97]. All these changes can be explained by the concept of Graham and Bernier [29], who attributed the dissociation of the maturation of the nucleus from that of the cytoplasm of the myeloma cell to a retardation of nuclear maturation.

On the basis of the above detailed cytopathological alterations of the myeloma cells, previous studies suggested that MM should be typed according to the degree of cellular differentiation [3, 4, 20, 37, 51, 54, 81]. Immature types of MM with a poor prognosis were described [2, 27, 38] and on the analogy of normal plasma cell differentiation, immature (plasmoblastic), intermediate (proplasmocytic) and mature (plasmocytic) types of MM were distinguished by both light and electron microscopic studies [3, 20, 27, 52, 81]. The prognostic relevance of these types, however, was doubtful [55, 62, 63, 94], although the various degrees of differentiation of myeloma cells were clearly established [20].

In the present study it was also assumed that on the analogy of reactive plasma cells, myeloma cells differentiated in a number of mitotic steps from a precursor cell (immunoblast or plasmoblast). The morphological classification was based on the predominance of one cell type (Fig. 1). Cytological and histological differentiation between mature and immature myeloma cells posed no problem, the histological delineation of the intermediate type (proplasmocyte) [52, 94] was, however, difficult. Nevertheless, owing to the lagging differentiation of the cell nuclei in MM, separation of this cell type does not seem to be necessary for the morphological classification of MM.

In previous studies the morphological classification of MM was hampered *(a)* by the inhomogeneity of bone marrow smears and *(b)* by the frequent occurrence of cellular atypia [55, 63, 73, 94]. On the basis of the method used in this study, i.e., analysis of the preparation by two independent observers and simultaneous cyto- and histomorphological examinations resulted in a reproducible classification. The only problem was the separation of the mixed (plasmoblastic-plasmocytic) type in a few cases.

An accumulation of the more immature types in IgA MM and in cases with light chain secretion was not seen in the present study. A few cases exhibited an immature appearance (Fig. 8). Similar types are known under various terms from the literature [2, 27, 33, 53, 61, 66]. The extremely immature precursor cells in MM are very similar to the immunoblasts of malignant lymphomas. Thus, the designation of these cases as "immunoblastic MM" seems warranted.

Cases of MM with predominance of giant cells have been described previously [16, 22, 32, 94]. Their differentiation from other types is easy. Among these cases there are two subtypes, in one of which the nuclei of giant cells are more mature and in the other rather immature (immunoblastic). The prognosis of giant cell MM is similar to that of the plasmoblastic type.

Concerning the changes of type in the course of the disease, 81 cases were

available for studying the problem. In 8 (10%) a change to a more immature type had already been described [66], in the present study the surprising constancy of the type was apparent. The reasons for the changes in the dominant cell type may be explained by a clonal selection of a more immature cell, or by loss of the ability of the cells to differentiate [23, 24].

The prognostic relevance is one of the most important requirements of a morphological classification. The morphological classifications of MM were found either of prognostic relevance, or of irrelevant prognostic value [3, 4, 5, 37, 54, 81]. In the present study, significant differences were found between the morphologic types of MM and the median survival time, *viz.* the survival rates of the patients.

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Book Review

Preleukemia. Ed. by F. Schmalzl, K.-P. Hellriegel. Springer-Verlag, Berlin—Heidelberg—New York 1979. 194 pages, 59 tables, 64 figures.

An international symposium held in Innsbruck in 1978 with the participation of 20 senior lecturers was dedicated to pathophysiologic and clinical aspects of the preleukaemic syndromes. The volume *Preleukemia* contains the contributions presented at the symposium and discussions edited by F. Schmalzl (Universitätsklinik für Innere Medizin, Innsbruck) and K.-P. Hellriegel (Medizinische Universitätsklinik, Köln). The first part of the book covers the pathophysiologic background of the preleukaemic syndromes: disturbed maturation and proliferation of the haemopoietic cells, structural anomalies and functional defects. Further papers deal with the diagnostic and prognostic value of these signs. Finally, the possible therapeutic approaches are delineated and discussed.

The symposium defined preleukaemia as an acquired disease that is characterized by haemopoietic insufficiency and terminates in acute leukaemia. Various clinical syndromes (PNH-like syndromes, refractory megaloblastic anaemias with or without increase in blast counts, chronic myelomonocytic leukaemia, myelodysplastic syndrome or pure red cell aplasia, etc.) depending on the principally affected cell types and the degree of the maturative and proliferative defects were accepted as preleukaemic states. On the other hand, several diseases and conditions (e.g. chronic myeloproliferative diseases, syndromes with increased risk of leukaemia-like Down's syndrome, Fanconi's anaemia, Louis Bar syndrome, Bloom's syndrome, the identical twin of a leukaemic patient, individuals exposed to radiation, benzene or cytostatics) were excluded. The question whether haemopoietic insufficiency was the primary event and leukaemia an error of the organism trying to correct it, or whether it represented merely a sign of preexisting leukaemia, could not be settled.

The value of diagnostic tests in preleukaemic states was thoroughly discussed at a Round Table Conference moderated by H. Heimpel. Finally the moderator pointed out that by combining several tests indicating disturbed maturation, proliferation and cytogenic anomalies, preleukaemic disorders can with great probability be diagnosed. The presented findings, however, do not give an answer to the most interesting question, whether during preleukaemia a small clone of leukaemic cells develops into the dominating cell type, or if cells are already leukaemic but attain their typical features in the course of the disease.

The participants of the symposium agreed in that the therapy of preleukaemic myelodysplastic syndromes is still an insufficiently resolved problem. Supportive measures are indicated. The kinetics of preleukaemic haematopoiesis argues against the use of cytotoxic drugs. Bone marrow transplantation has yet limited indications and the pathophysiological basis of immunotherapy is not sufficiently known.

In conclusion, the symposium on preleukaemia emphasized the importance of this syndrome and urged further studies of the factors governing the regulation of abnormal cell clones, of the immunological state and the management of preleukaemia.

The volume is up to the high standards of Springer-Verlag and is of interest to all haematologists, both clinicians and researchers.

Ilma Szász

Abstracts

Physical characteristics of human transferrin from small angle neutron scattering. P. Martel, S. M. Kim, and B. M. Powell (Atomic Energy of Canada Limited, Chalk River Nuclear Laboratories, Chalk River, Ontario, Canada). *Biophys. J. 31*, 371 (1980).

The technique of small angle neutron scattering has been used to determine the molecular shape, the volume, and the molecular weight of pooled human transferrin in an aqueous solution isotonic with blood. Analysis of the measurements assuming a spheroidal molecular shape indicates that an oblate spheroid with semi-axes of length 46.6 ± 1.4, 46.6 ± 1.4, and 15.8 ± 3.8 Å, and a molecular volume $(144 \pm 45) \times 10^3$ Å is the best simple approximation to the shape of the transferrin molecule. The radius of gyration R_g, determined from a Guinier plot is 30.25 ± 0.49 Å, in agreement with R_g calculated for the oblate spheroidal shape. The molecular weight is determined to be $(75 \pm \pm 5) \times 10^3$. The shape-independent molecular volume is found to be $(98 \pm 10) \times 10^3$ Å^3. The difference in the two volumes suggests that transferrin is not a uniform spheroid but may have a more complex shape.

A. Egyed

The kinetics of iron release from human transferrin by EDTA. Effect of salts and detergents. D. A. Baldwin (Department of Chemistry, University of Witwatersrand, Johannesburg, Republic of South Africa). *Biochim. biophys. Acta (Amst.) 623*, 183 (1980).

The kinetics of iron release from diferric human transferrin by EDTA at pH 7.4 and 37 °C has been studied. Equations have been derived which give the expected absorbance-time curves when the two sites are kinetically homogeneous or heterogeneous and these have been compared to the experimental results. In the absence of added salts or detergents, the two sites are kinetically similar but not identical with respect to iron release. However, the two sites are kinetically homogeneous in 0.02 M laurylpyridinium chloride and 2 M LiCl. In $NaClO_4$ (> 0.50 M) there are two parallel pathways for iron release; 80% of the total iron being released heterogeneously and 20% apparently homogeneously. For the simple electrolyte ions studied, the rate of iron release from diferric-transferrin increases in accordance with the lyotropic series: $SO_4^{2-} < HCO_2^- < Cl^- < < ClO_4^-$ and $Na^+ < Li^+$. Sodium dodecyl sulphate (0.02 M) and LiCl (2.0 M) increase the rate of ion release by 0.1 M EDTA by 32- and 27-fold, respectively. The results show that conformational states are attainable from which iron can be released either homogeneously or heterogeneously and from which iron release is facilitated. It is proposed that the structure of the transferrin binding site on the cell membrane and/or the presence of another molecule may promote these conformers in vivo.

A. Egyed

Identification of the binding site for transferrin in human reticulocytes. M. Steiner (Division of Hematologic Research, Memorial Hospital Pawtucket, R.I. and Brown University, Providence, R.I.). *Biochem. Biophys. Res. Commun. 94*, 861 (1980).

The receptor site for transferrin was investigated in normal human reticulocytes by

the use of photoactive 4-fluoro-3-nitrophenyl azide which was conjugated to chromatographically pure human transferrin saturated with iron. The photoprecursor-bearing protein was further treated with fluorescein isothiocyanate. The nonactivated transferrin conjugate was fully competitive with respect to binding characteristics with normal transferrin. The aryl nitrene-containing photoactivated transferrin-reticulocyte receptor complex was isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins and polypeptides were eluted from the gels and analyzed for fluorescence. A fluorescent band of 123 000 daltons was identified as possible transferrin-receptor complex. The molecular weight of the membrane receptor was estimated to be 43 000. This corresponds to the approximate weight of one of the major red cell glycopeptides.

A. Egyed

2,3-Bisphosphoglycerate in developing rabbit erythroid cells. H. Narita, K. Ikura, S. Yanagawa, R. Sasaki, H. Chiba, H. Saimyoji and N. Kumagai (Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, and Department of Internal Medicine, Faculty of Medicine, Kyoto University, Kyoto, Japan). *J. Biol. Chem. 255*, 5230 (1980).

Rabbits were made anemic to different extents by phenylhydrazine injections so as to vary the developmental stages of the erythroid cells in their peripheral blood and bone marrow. It was found that the more severe the anemia, the lower the concentration of glycerate-2,3-P_2 in the bone marrow cells and in the circulating erythroid cells. The glycerate-2,3-P_2 level was shown to rise during erythroid differentiation in a linear relationship to the hemoglobin level, corresponding to the equation $y = 0.03x + 0.29$, where y is the concentration of glycerate-2,3-P_2 (micromoles per ml packed cells) and x is the concentration of hemoglobin (milligrams per packed cells). The bone marrow cells were fractionated on a size (i.e. maturity) basis and enzyme activities in the fractionated cells were measured. The accumulation of glycerate-2,3-P_2 was found to be primarily attributable to the increase in glycerate-2,3-P_2 synthase activity. Glycerate-2,3-P_2 phosphatase and phosphofructokinase increased as the cells matured from the polychromatic stages to become reticulocytes, while pyruvate kinase and phosphoglyceromutase decreased. The changes in phosphofructokinase and pyruvate kinase appear to be advantageous for glycerate-2,3-P_2 accumulation. The two enzyme activities directly responsible for the synthesis and the breakdown of glycerate-2,3-P_2 are probably manifested by a single protein, glycerate-2,3-P_2 synthase-phosphatase.

Ilma Szász

Inhibition of red cell Ca^{2+}-ATPase by vanadate. G. H. Bond and P. M. Hudgins (Department of Physiology, Kirksville College of Osteopathic Medicine, Kirksville, Missouri). *Biochim. biophys. Acta (Amst.) 600*, 781 (1980).

The Mg^{2+}-plus Ca^{2+}-dependent ATPase (Ca^{2+}-ATPase) in human red cell membranes is susceptible to inhibition by low concentrations of vanadate. Several natural activators of Ca^{2+}-ATPase (Mg^{2+}, K^+, Na^+ and calmodulin) modify inhibition by increasing the apparent affinity of the enzyme for vanadate. Among the ligands tested, K^+, in combination with Mg^{2+}, had the most pronounced effect on inhibition by vanadate. Under conditions optimal for inhibition of Ca^{2+}-ATPase, the $K_{1/2}$ for vanadate was 1.5 μM and inhibition was nearly complete at saturating vanadate concentrations. There are similarities between the kinetics of inhibition of red cell Ca^{2+}-ATPase and ($Na^+ + K^+$)-ATPase prepared from a variety of sources; however, ($Na^+ + K^+$)-ATPase is approx. 3 times more sensitive to inhibition by vanadate.

G. Gárdos

Evidence that calcium acts as an intracellular messenger for adrenergic responses in human erythrocytes. M. J. Nelson and W. H. Huestis (Department of Chemistry, Stanford University, Stanford, Cal.). *Biochim. biophys. Acta (Amst.) 600*, 398 (1980).

Adrenergic stimulation of membrane protein phosphorylation has been studied in human erythrocytes. The adrenergic enhancement in phosphorylation of band 2 could be mimicked by the calcium-specific ionophore A23187 in the presence of 10 μm extracellular calcium. Experiments with the potassium ionophore, valinomycin, showed that potassium efflux was not the primary effector of the response. Trifluoperazine, an inhibitor of the Ca^{2+}-dependent regulatory protein, calmodulin, inhibited phosphorylation stimulation by either norepinephrine or the calcium ionophore. The norepinephrine response was observed in the absence of extracellular calcium, implicating Ca^{2+} released from cellular bound pools in mediating the response.

G. Gárdos

The transbilayer movement of phosphatidylcholine in vesicles reconstituted with intrinsic proteins from the human erythrocyte membrane. W. J. Gerritsen, P. A. J. Henricks, B. de Kruijff and L. L. M. van Deenen (Department of Biochemistry and Institute of Molecular Biology, State University of Utrecht, Utrecht, The Netherlands). *Biochim. biophys. Acta (Amst.) 600*, 607 (1980).

Vesicles have been prepared from 18 : : 1_c/18 : 1_c-phosphatidylcholine with or without purified glycophorin or partially purified band 3 (obtained by organomercurial gel chromatography). The vesicles have been characterized by freeze-fracture electron microscopy, binding studies to DEAE-cellulose, ^{31}P-NMR and K^+ trap measurements. Pools of phosphatidylcholine available for exchange have been investigated using phosphatidylcholine exchange protein from bovine liver. The protein-containing vesicles both exhibit exchangeable pools larger than the fraction of phosphatidylcholine in the outer monolayer, whereas in the protein-free vesicles the exchangeable pool is consistent with the outer monolayer. The results indicate that both glycophorin and the partially purified band 3 preparation enhance the transbilayer movement of phosphatidylcholine.

G. Gárdos

Reconstitution of human erythrocyte membrane acetylcholinesterase in phospholipid vesicles. Analysis of the molecular forms by cross-linking studies. Ch. R. Römer-Lüthi, P. Ott and U. Brodbeck (Medizinisch-chemisches Institut der Universität Bern, Bern, Switzerland). *Biochim. biophys. Acta (Amst.) 601*, 123 (1980).

Unilamellar lipid vesicles were formed upon removal of Triton X-100 with Amberlite XAD-2 from a mixture of egg phosphatidylcholine and Triton-solubilized pure human erythrocyte membrane acetylcholinesterase. A majority of large (230 nm diameter) vesicles together with a minor population of smaller (30 nm diameter) structures were observed in freeze-fracture electron micrographs. Reconstitution experiments performed with [phenyl-^{3}H(n)]-Triton X-100 showed that only one detergent molecule per 600 lipid molecules was present in the vesicles. Density gradient centrifugation showed co-sedimentation of acetylcholinesterase with the lipid vesicles. About 60% of the incorporated enzyme was directed towards the vesicle exterior and could be partially degraded by papain. Mainly dimeric acetylcholinesterase was found when the reconstituted or, alternatively, the lipid-free but Triton-solubilized enzyme were cross-linked with glutaraldehyde. Aggregates were observed when the detergent-depleted oligomeric forms of the enzyme were cross-linked. The results thus indicate that mainly the dimeric enzyme form is present in a phospholipid environment.

G. Gárdos

Association of glyceraldehyde 3-phosphate dehydrogenase with the membrane of the intact human erythrocyte. S. Keokitichai and J. M. Wrigglesworth (Department of Biochemistry, Chelsea College, University of London, London, UK). *Biochem. J. 187*, 837 (1980).

Intact human erythrocytes were exposed to low concentrations of glutaraldehyde. After washing and subsequent lysis of the cells, glyceraldehyde 3-phosphate dehydrogenase activity is found to be associated with a membrane fraction and cannot be

eluted by salt treatment. Lactate dehydrogenase activity is associated with a supernatant fraction under the same conditions. Preincubation of the intact cells under conditions designed to increase internal NADH concentrations, leads to a lower membrane-associated activity of glyceraldehyde 3-phosphate dehydrogenase after lysis.

G. Gárdos

Effects of cholesterol on lipid organization in human erythrocyte membrane. S. W. Hui, Cynthia M. Stewart, Mary P. Carpenter, and T. P. Stewart (Department of Biophysics, Roswell Park Memorial Institute, Buffalo, New York). *J. Cell Biol. 85*, 283 (1980).

The molar ratio of cholesterol to phospholipid (C/P) in human erythrocyte membrane is modified by incubating the cells with liposomes of various C/P ratios. The observed increase in cell surface area may be accounted for by the addition of cholesterol molecules. Fusion between liposomes and cells or attachment of liposomes to cells is not a significant factor in the alteration of C/P ratio. Onset temperatures for lipid phase separation in modified membranes are measured by electron diffraction. The onset temperature increases with decreasing C/P ratio from 2 °C at C/P = 0.95 to 20 °C at C/P = 0.5. Redistribution of intramembrane particles is observed in membranes freeze-quenched from temperatures below the onset temperature. The heterogeneous distribution of intramembrane particles below the onset temperature suggests phase separation of lipid, with concomitant segregation of intramembrane protein into domains, even in the presence of an intact spectrin network.

G. Gárdos

Phosphorylation and dephosphorylation of spectrin from human erythrocyte ghosts under physiological conditions: Autocatalysis rather than reaction with separate kinase and phosphatase. B. A. Imhof, H. J. Acha-Orbea, T. A. Libermann, B. F. X. Reber, J. H. Lanz, K. H. Winterhalter and W. Birchmeier (Biochemical Laboratory, Federal Institute of Technology, Zurich, Switzerland). *Proc. nat. Acad. Sci. (Wash.) 77*, 3264 (1980).

The mechanism of phosphorylation and dephosphorylation of spectrin from human erythrocyte membranes has been examined under closely physiological conditions. The results support the hypothesis that spectrin is an autophosphorylating and dephosphorylating system. Extraction from ghosts of up to 85 % of the kinase (casein kinase) suggested to catalyze the reaction only slightly reduced spectrin component 2 phosphorylation and did not affect ATP-induced changes in the ghosts' shapes. A spectrin-actin complex isolated from endocytotic inside-out vesicles under hypertonic conditions contained virtually no casein kinase activity and still exhibited a largely intact phosphorylation machinery. Photoaffinity labeling experiments indicated that spectrin component 2 fulfills the necessary prerequisite of the hypothesis — i.e., it contains its own ATP-binding site. Under various conditions, spectrin phosphorylation and dephosphorylation seem to be tightly coupled. The implications of these findings for the understanding of spectrin function and the maintenance of erythrocyte shape are discussed.

Ilma Szász

Glutathione reduces cytoplasmic vanadate. Mechanism and physiological implications. I. G. Macara, K. Kustin and L. C. Cantley (Department of Chemistry, Brandeis University, Waltham, Mass. and Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Mass.). *Biochim. biophys. Acta (Amst.) 629*, 95 (1980).

The mechanism by which cells reduce cytoplasmic vanadium(V) (vanadate) to vanadium(IV) was investigated using the human red cell as a model system. Vanadate uptake by red cells occurs with a rapid phase involving chemical equilibration across the plasma membrane and a slower phase resulting in a high concentration of bound vanadium-(IV). The slow phase was inhibited in glucose-starved cells and restored upon addition of glucose indicating an energy requirement for this process. The time course of vanadium-(IV) appearance (monitored by EPR spectroscopy of intact cells) parallelled the slow phase of uptake indicating that this phase involves vanadium reduction. The reduction of intra-

cellular vanadate to vanadium(IV) was nearly quantitative after 23 h. The intracellular reduction is not enzymatic, since a similar time course of vanadium reduction and binding to hemoglobin was observed when gluathione was added to a hemoglobin + vanadate solution in vitro. Vanadium(IV) binding to hemoglobin was reduced by addition of ATP, 2,3-diphosphoglycerate or EDTA, probably through chelation of the cation. The stability constant of the ATP-vanadium(IV) complex was determined to be 150 M^{-1} at pH 4.9. The time course of red cell vanadate uptake and reduction was followed in the concentration range in which approximately 60% inhibition of the $(Na^+ + K^+)$-ATPase is observed. It is concluded that vanadate is reduced by cytoplasmic glutathione in this concentration range and that the reduction explains the resistance of the $(Na^+ + K^+)$-ATPase to vanadium in intact cells.

Ilma Szász

Interaction of band 3-protein from human erythrocyte membranes with phospholipid monolayers at the air-water interface. D. Schubert and E. Klappauf (Max-Planck-Institut für Biophysik, Frankfurt a. M., BRD). *Hoppe-Seyler's Z. physiol. Chem.361*, 1171 (1980).

Lipid monolayers at the air-water interface were used as a model system to study the interactions of solubilized band 3-protein of the human erythrocyte membrane with phospholipids. The changes in monolayer surface pressure accompanying protein incorporation were used as a measure of the strength of the protein-lipid interactions. The following results were obtained: Both polar and apolar forces contribute to the protein-lipid interactions, the latter being predominant. The interactions strongly depend on the pH of the monolayer subphase. They are much stronger at acid than at neutral or alkaline pH. The influence of pH on the band 3-phospholipid interactions is mainly due to a pH-induced conformational change of the protein, the pK value of which is near 5.0. At all pH values studied, band 3 shows the highest affinity for phosphatidic acid and phosphatidylglycerol, the lowest for sphingomyelin and phosphatidylcholine.

Ilma Szász

Inhibition by lead of human erythrocyte $(Na^+ + K^+)$-adenosine triphosphatase associated with binding of ^{210}Pb to membrane fragments. M. L. Caspers and G. J. Siegel (Department of Chemistry and Chemical Engineering, University of Detroit, Detroit, Mich. and Department of Neurology, University of Michigan Medical Center, Ann Arbor, Mich.). *Biochim. biophys. Acta (Amst.) 600*, 27 (1980).

Fragmented human erythrocyte membranes were exposed to $PbCl_2$ for 10—40 min at 23 °C prior to $(Na^+ + K^+)$-ATPase assay. Inhibition increased with exposure time. Enzyme activity in 5 μg membrane protein was inhibited 50% after a 10-min exposure to 1.0 nmol $PbCl_2$ (25 μM final concentration) and was inhibited 100% after 40 min. When membranes at various concentrations were exposed to $PbCl_2$ for 40 min, inhibition was linear with the ratio of $PbCl_2$ to protein. Inhibition of 100% was obtained at 0.2 nmol $PbCl_2$/μg protein. A graph of activity vs. [protein] in the presence of $PbCl_2$ intercepted the abscissa to the right of the origin, indicating that lead acts as an irreversible or very slowly reversible inhibitor. Addition of 1 mM 2,3-dimercaptopropanol, 1,3-dithiothreitol, DL-penicillamine or EDTA after 40 min exposure to 100 μM $PbCl_2$ restored 45, 64, 81 and 92% of the $(Na^+ + K^+)$-ATPase, respectively. These chelators, excluding EDTA, prevented inhibition when added before $PbCl_2$. Two washings of the membrane fragments with water or 10 mM imidazole-HCl (pH 7.4) did not restore activity. ^{210}Pb bound tightly to membrane fragments and beginning of saturation was observed at 0.19 nmol Pb^{2+} bound/μg protein. This corresponded to 200 μM final concentration of $PbCl_2$ in the ATPase assay. At 0.2 nmol $PbCl_2$/μg protein (100% inhibition of ATPase), from 0.10—0.17 nmol of lead was bound per μg protein. Under the same conditions, 1 mM DL-penicillamine removed 80% of the bound lead which correlated with its restoration of ATPase activity. Pb^{2+} does not appear to denature the enzyme. The irreversible kinetics may be related to sequestration of Pb^{2+} within vesicles that interfere with the accessibility of chelators to Pb^{2+} binding sites.

Ilma Szász

Biophysical studies of erythrocyte membranes from patients with Duchenne muscular dystrophy. R. Dellantonio, F. Angeleri, M. Capriotti, G. Lenaz, G. Curatola, L. Mazzanti, E. Bertoli (Clinica Neurologica, Università di Ancona e Istituto "S. Stefano" di Porto Potenza Picena (Macerata) and Istituto di Chimica Biologica, Università di Ancona, Italy). *Italian J. Biochemistry 29*, 121 (1980).

Lipid soluble spin labels were used to probe the fluidity of membrane lipids in erythrocyte membranes from patients with Duchenne muscular dystrophy. A greater mobility of spin label motion was noticed at the surface of the Duchenne erythrocyte membranes in comparison with the similarly labeled normal human erythrocytes. Changes in the protein conformation and/or protein-lipid organization, as a consequence of an altered membrane lipid fluidity, have been demonstrated by the use of a sulfhydryl group specific protein spin label. Strongly immobilized sulfhydryl groups appear to be located both at the membrane surface and deep within the lipid bilayer. The ratio of the spectral amplitude of the spin label attached to weakly immobilized sulfhydryl groups to that of strongly immobilized SH-groups is significantly greater in Duchenne membranes, compared to those of controls. These studies suggest alterations in lipid-protein organization at the surface of Duchenne erythrocytes, indicating that Duchenne muscular dystrophy may be a disease resulting from a membrane abnormality at the molecular level.

Ilma Szász

Shape and volume changes in erythrocyte ghosts and spectrin-actin networks. R. M. Johnson, G. Taylor and D. B. Meyer (Departments of Biochemistry and Anatomy, Wayne State University School of Medicine, Detroit, Michigan). *J. Cell Biol. 86*, 371 (1980).

In response to changes in electrolyte concentration and pH, erythrocyte ghosts can exhibit some of the characteristic shapes seen in the intact erythrocyte. These shape changes are accompanied by volume changes; both are reversible, not energy dependent, and not inhibited by sulfhydryl reagents. The volume reduction can also be seen in isolated Triton-free spectrin-actin lattices, showing that this network is capable of reversible contraction. The results suggest that reversible changes in size of the underlying cytoskeleton of the erythrocyte membrane can control cell shape.

Ilma Szász

MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

B. P. L. Moore

Antibody detection and identification by AutoAnalyzer haemagglutination systems. Haematologia 14, 265—276 (1981)

The fundamental aims of antibody detection, the limitations imposed by present manual methodology, and what can be expected through the use of single-channel AutoAnalyzers are described. A combination of three different channels gives adequate power of detection and breadth of coverage for routine needs. Special techniques are also available that are useful in the study of antigen : antibody reactions and autoimmune diseases.

B. Zachara, M. Kopff

Activity of inosine triphosphate pyrophosphohydrolase in fresh and stored human erythrocytes. Haematologia 14, 277—283 (1981)

The accumulation of inosine triphosphate (ITP) in humanerythrocytes incubated with inosine depends on the activity of inosine triphosphate pyrophosphohydrolase (ITPH). High activity of this enzyme is accompanied by a low concentration of ITP and conversely. We showed that ITPH activity decreases with the prolongation of blood preservation time. As a consequence there is a lower accumulation of ITP in fresh erythrocytes incubated in a medium containing high concentrations of inosine, pyruvate and phosphate (IPP) than in red blood cells preserved at 4 °C. Synthesis of ITP in erythrocytes incubated in IPP medium being so intensive, it seems possible that during incubation an intermediate accumulates which decreases ITPH activity.

B. Zachara, I. Zakrzewska, Z. Maziarz, W. Gaszyński, N. Wachowicz

Concentration of 2,3-diphosphoglycerate and adenosine triphosphate in erythrocytes following acute blood loss in the beagle. Haematologia *14*, 285—291 (1981).

The effect of experimental anaemia induced by phlebotomy and the effect of infusion of various fluids on the 2,3-DPG, ATP and total organic phosphate (P_{org}) concentration were examined in the red cells of beagles made anaemic by withdrawal of 30% of the circulating blood. Particular groups of dogs 30 min after phlebotomy were infused with own blood, blood with inosine, pyruvate and phosphate (IPP), Fluigel and Fluigel with IPP. The changes were most distinct in dogs which were not infused with any fluid. After 24 hours the content of 2,3-DPG, ATP and P_{org} increased by 12, 27 and 20%, respectively, in comparison with the results obtained before blood loss. Twenty-four hours following the reinfusion of own blood, a slight increase occurred in the 2,3-DPG and P_{org} content. Infusion of Fluigel caused a decrease of the phosphate compounds concentration after 3 and 6 hours and then the concentration almost returned to normal. Infusion of own blood as well as Fluigel enriched with inosine, pyruvate and phosphate did not lead to any change in 2,3-DPG concentration. The red cells of dogs do not utilize inosine for 2,3-DPG or nucleotide synthesis. The increased cardiac output seems to be the main mechanism for oxygen transport to tissues after an acute haemorrhage.

G. Lelkes, Gy. Lelkes, K. Natonek, S. R. Hollán

Intracellular "labyrinths" in acute leukaemia. Do they indicate the maturational ability of leukaemic cells? Haematologia *14*, 293—306 (1981).

The occurrence and ultrastructure of the so-called "labyrinths" were investigated in the bone marrow cells from 13 patients with preleukaemia and acute leukaemia. Except for one patient, labyrinths were found in the myeloid cells from patients with preleukaemia and acute myelogenous leukaemia. Labyrinths could not be detected in lymphoblasts from patients suffering from acute lymphoblastic leukaemia or in plasma cells from a patient with plasma cell leukaemia. In patients with preleukaemia and smoldering leukaemia, labyrinths could be detected in all differentiation forms, from myeloblasts to mature polymorphonuclear granulocytes. Since it is improbable that labyrinths develop in consequence of exogenous effects, their presence in the mature granulocytes points to the *in vivo* maturational ability of leukaemic precursors in certain cases of acute myelogenous leukaemia and preleukaemia.

K. Német, K. Andrássy, V. Mann, E. Füzes, L. Kocsár, I. Simonovits

Iron stores in healthy pregnant women as determined by immunoradiometry of their serum ferritin levels. Haematologia *14*, 307–313 (1981)

Immunoradiometric determination of the serum ferritin levels allows to obtain the true value of the volume of iron stores in pregnant women. To complete our earlier examinations, in 752 primigravidae aged 20 to 29 years, the serum ferritin level has been determined in the 3rd, 5th and 8th month of pregnancy. One hundred and fifty-three of the subjects were given iron therapy, while 599 received no iron during pregnancy. The haemoglobin value begins to decrease in early pregnancy, reaches its minimum in month 5 and stays at this level until month 8. In the primigravidae, the mean haemoglobin level decreaed to 12 g/dl by the 5th month of pregnancy and persisted at that level until delivery. Serum ferritin and iron values decreased continuously from the beginning until the end of pregnancy. The decrease in serum iron level was essentially less than that in the serum ferritin level. In spite of the 12.1 g/dl haemoglobin value considered normal and of the about normal mean serum iron value, the serum ferritin level was extremely low in 10% of the pregnant women, a proof of depleted iron stores. In about another 10%, haemoglobin values higher than 11 g/dl were found, with serum ferritin levels of 5 ng/ml or even lower. In this latter group, the serum iron level and the saturation index already pointed to iron deficiency in months 5 to 8 of the pregnancy. Our assumption that iron dosage was insufficient in an appreciable part of subjects given iron therapy, was well founded. It is concluded that the overwhelming part of pregnant women is in need of iron therapy from the 3rd month of pregnancy.

K. Wutke, M. Várbíró, K.-D. Rüdiger, G. Kelényi

Cytological and histological classification of multiple myeloma. Haematologia *14*, 315–329 (1981)

In a retrospective study 202 cases of multiple myeloma (MM) were studied cyto- and histomorphologically. In 62 cases only bone marrow smears, in 60 only histological preparations and in 80 both types of material were available. According to the differentiation of myeloma cells, a plasmocytic (n = 127), a mixed cellular (n = 35) and a plasmoblastic type (n = 32) could be distinguished. Besides, 8 cases with predominance of giant cells were found (giant cell MM). The comparative cytological and histological typing into the four types of MM proved to be reproducible. The medium survival time and survival curves of the described types showed a statistically significant difference. The survival times were 39.7 months with the plasmocytic type, 16.1 months with the mixed cellular type, and 9.8 months with the plasmoblastic type. The prognostic relevance of the morphological classification was supported by the frequency of extramedullary infiltrates (20% in the plasmocytic, 67% in the mixed cell, 69% in the plasmoblastic and 100% in the giant cell type).

MAGYAR
TUDOMÁNYOS AKADÉMIA
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Haematologia 14 (4), pp. 339–359 (1981)

Thrombin and Haemostasis: Regulation of the Biological Functions of Thrombin

(Facts and perspectives)

R. MACHOVICH, I. HORVÁTH

Second Institute of Biochemistry, Semmelweis University Medical School, Budapest, Hungary

(Received November 19, 1980)

Thrombin plays an essential role in the maintenance of haemostatic balance through several biochemical reactions. Its functions as well as the regulation of its activities seem to be decisive in controlling thrombus formation. The present paper endeavours to give a brief summary of the control of thrombin activity and the regulation of biological actions of thrombin. As to the control of the amount of the active enzyme, three possible ways are discussed, i.e. prothrombin synthesis, activation of prothrombin, and inactivation of thrombin by plasma proteinase inhibitors (antithrombin III, α-2-macroglobulin, α-1-proteinase inhibitor). Regarding the regulation of the various actions of thrombin, the most important biological targets of this enzyme are summarized, namely interaction with fibrinogen, activation of Factor XIII, Factor IX, Factor VIII, Factor V and prothrombin, and the binding to platelets, fibroblasts and endothelial cells. The limits of our present knowledge about the determination of the possible reaction routes catalyzed by thrombin are also considered. Finally, a tentative hypothesis is put forward to explain how the endogenous modulators like heparin determine the biological functions of thrombin.

*

Maintenance of the haemostatic balance is the result of several well-regulated reactions in the circulating blood. The essential "aim" of these reactions is to control the formation of an active enzyme: the thrombin. Under normal circumstances there is no measurable amount of thrombin present in the plasma. Increase in the production or activity of thrombin, however, may result in formation of a clot. Thrombus formation inside the vessels may, in turn, lead to thromboembolic disease which, besides cancer, is one of the most common serious medical problems of our time.

Thrombin contributes to maintenance of the haemostatic balance through several biological reactions [1–4 and references therein]. Its primary function is to act as a catalyst in the conversion of fibrinogen to fibrin. The fibrin clot formed, however, is not the final form of the thrombus. In nearly all cases, aggregates of platelets can also be found in it. Moreover, the size and stability of the clot is further modified by another enzyme, Factor XIIIa. Thrombin has an essential role in some additional reactions, i.e. its binding to platelets initiates a series of events within the cells and its reaction with Factor XIII results in activation of the above proenzyme. In addition, thrombin binds to endothelial cells, inducing the

release of prostacyclin and several other biochemical reactions. Thrombin influences its own formation through reactions with prothrombin, Factor V and Factor VIII. Besides, more than ten other biological functions of thrombin are known.

In spite of the facts that (i) thrombin has various biological functions; (ii) similarly to several other serine proteinases, thrombin cleaves arginyl-glycine bonds; (iii) there is a sequence homology between thrombin and the other proteinases of blood coagulation, thrombin has a high specificity for its proteolytic activity (see later). Since thrombin plays a key role in maintaining haemostasis (if there are not sufficient active thrombin molecules present, haemorrhagic diseases may develop or if their activity is too high, it can lead to thrombosis), the control of its activity and the regulation of its biological functions are important.

The aim of this review is to give a brief summary of our present knowledge and some new viewpoints regarding (A) the control of thrombin activity, and (B) the regulation of biological functions of thrombin.

A. Control of thrombin activity

Thrombin activity is regulated in at least three ways (Fig. 1), *viz.* by controlling prothrombin synthesis, prothrombin activation leading to thrombin formation, and inactivation of the unrequired amounts of thrombin.

1. Prothrombin synthesis

Prothrombin is a plasma protein of about 70 000 molecular weight, synthesized in the liver. It is composed of a single polypeptide chain containing approximately 8% carbohydrate [2, 5]. Removal of the carbohydrate portion has no effect on activation but it increases the rate of hepatic uptake of the molecule *in vivo* [6]. Prothrombin is one of the four clotting proteins that require vitamin K for biosynthesis. Vitamin K is necessary for the formation of specific calcium binding sites, γ-carboxylglutamic acid residues in the prothrombin molecule. Abnormal prothrombin, synthesized in the absence of vitamin K, binds less than one calcium ion per mole of protein at 1 mM calcium concentration [7]. As to the calcium binding sites, they are located at the amino-terminal region of the prothrombin in fragment 1, which is a glycopeptide released during prothrombin activation. Approximately 20 Ca^{2+} bridges link to the phospholipid the 20 carboxyl groups of the 10 γ-carboxylglutamyl residues of prothrombin fragment 1 [8, 9]. Thus, abnormal prothrombin lacks biological activity because this calcium-mediated binding site to phospholipid is absent.

Plasma prothrombin levels, approximately 100 μg/ml, are normally held within a narrow range. The mode of regulation of the plasma prothrombin level is not known. Liver microsomes, isolated from hypoprothrombinaemic animals, show an increased rate of prothrombin synthesis after vitamin K administration

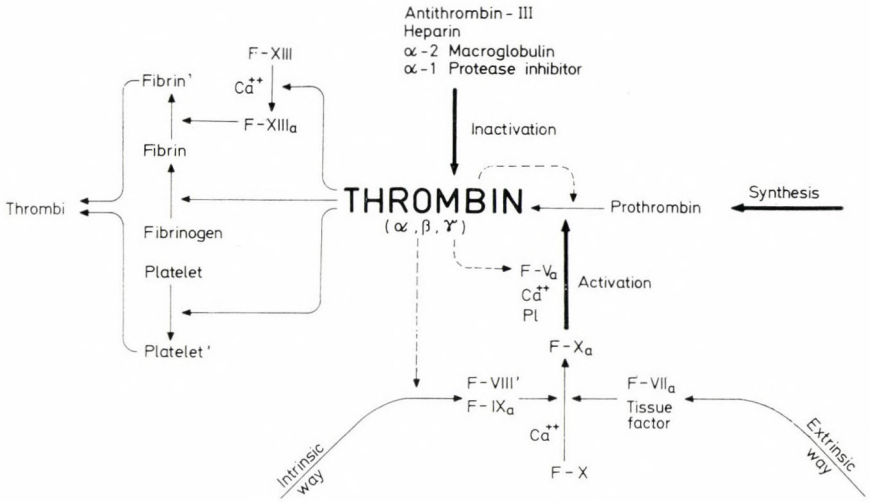

Fig. 1. Regulation of thrombin activity. F = Factor; a = activated; Pl = phospholipid

[10]. This elevation in rate is maintained until the plasma levels of prothrombin are restored to normal. There are no data available that would give any indication on how these levels might be regulated by the plasma prothrombin concentration, and this particular area remains an interesting field for further investigations.

2. *Regulation of prothrombin activation*

(a) *Thrombin and prothrombin*

The activation of prothrombin *in vitro* [3] takes place in several steps (Fig. 2). The first one appears to be the cleavage of a large glycopolypeptide, fragment 1·2 (M_r* 33 500) from the amino-terminal end of prothrombin by Factor Xa. This reaction gives rise to a single chain protein called prethrombin 2 (M_r 38 000). In the second step of activation, prethrombin 2 is cleaved by Factor Xa to form thrombin.

Thrombin[1], a serine proteinase, consists of a light chain (M_r 5700) and a heavy chain (M_r 32 000), held together by a disulfide bond. The heavy chain contains three intrachain disulfide bridges, carbohydrate and the active serine residue

* M_r stands for molecular weight.

[1] The complete primary structure of human α-thrombin has been reported [92]. It is initially produced as a molecule having two chains linked by disulfide bridges and being composed of a 49 residue A-chain and a 259 residue B-chain. The active site serine, residue

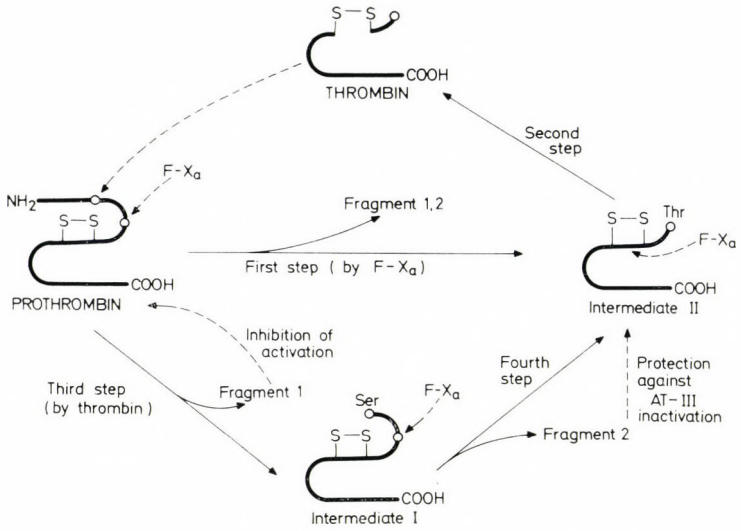

Fig. 2. Mechanism and control of activation of prothrombin. Abbreviations as in Fig. 1. Intermediate I = prethrombin 1, Intermediate II = prethrombin 2. (From ref. [3])

with the histidine and aspartic acid residues participating in the charge relay system.

The first traces of thrombin formed react with prothrombin (third step) and cleave a peptide shorter than cleaved by Factor Xa, called fragment 1 (M_r 23 000), containing the γ-carboxylglutamic acid residues which are primarily responsible for binding prothrombin to phospholipid *via* Ca^{2+} ions [11]. In addition, prethrombin 1 (M_r 50 000) is formed, which is readily converted into prethrombin 2 by Factor Xa (fourth step) resulting in an additional peptide, the so-called fragment 2 (M_r 12 700). Fragment 2 also binds calcium, and has been shown to be the Factor V binding portion of the prothrombin molecule [12, 13]. As the first traces of thrombin give rise to the formation of prethrombin 1, very small amounts of fragments 1 · 2 are formed. Accordingly, thrombin can control its own formation from prothrombin. Moreover, the fragments released during activation are not inert [3, 5, 14], as fragment 1 inhibits prothrombin activation, especially when approximately 50% of prothrombin has been converted into thrombin and

254, is identified on the B-chain. Partial proteolysis at arginine 122 of α-thrombin leads to the formation of β-thrombin, resulting in a three-chain protein. A second cleavage at lysine 203 yields γ-thrombin producing a four-chain protein. The chains are held together by disulfide bridges. All three active site residues (ser 254, his 92, asp 146) must remain in an active configuration in both β- and γ-thrombins, because they remain catalytically active toward low molecular weight substrates, although both β- and γ-thrombins lose most of the fibrinogen clotting activity [93]. The biological significance of β- and γ-thrombins is not known.

enhances its esterolytic activity, without affecting its clotting effect. The biological function, if any, of this activation of thrombin is questionable, but the protective effect of fragment 2 binding to thrombin against antithrombin III inactivation seems to be an important factor in controlling the localization of clot formation [15].

These reactions can further be modified by contributing accessory components to Factor Xa in the process of prothrombin activation. Phospholipid binds Factor Xa as well as prothrombin in the presence of calcium and accelerates thrombin formation [16]. Further activation can be obtained in the presence of an additional protein, Factor Va. To illustrate this point we mention that addition of phospholipid to Factor Xa and Ca^{2+} increases the activation rate of prothrombin 50-fold, while in the presence of Factor Va, without phospholipid, the rate rises 350-fold. When the complex is complete, i.e. Factor Xa, Factor Va, phospholipid, calcium and prothrombin are together, the relative activation rate changes between 1600 and 19 000, depending on the concentration of the reactants [12, 17]. This means that, at least, three orders of magnitude rate-enhancement occurs in such a complex, suggesting a very rapid reaction *in vivo*. Moreover, the rate-enhancement depends on the relative concentration of both Factor Xa and Factor Va, i.e. a maximum reaction rate is obtained when the two factors interact stoichiometrically [18]. Thus, activation of the other precursor, i.e. Factor XII, Factor XI, Factor IX and Factor VIII, controlling Factor Xa formation from Factor X, also determines the rate of prothrombin activation (for additional details see Refs [3, 4]). These steps of the coagulation-cascade, however, are also influenced by thrombin.

(b) *Thrombin and Factor V*

Factor V activity increases substantially upon brief exposure to thrombin, suggesting that Factor V, like many other proteins of blood coagulation, is subject to activation by a proteolytic mechanism. The increase in Factor V activity may be as high as 80-fold [13]. Recent results show that thrombin catalyzes the proteolysis of the single chain Factor V by cleavage of the parent molecule (M_r 330 000) to intermediate (M_r 205 000 and M_r 150 000) followed by cleavage of the latter, leading to end products possessing apparent molecular weights of 94 000 and 74 000. The generation of Factor Va activity coincides with the second cleavage [18].

(c) *Thrombin and Factor VIII*

In the "prothrombinase-complex", activated Factor X is necessary for prothrombin activation. In normal plasma, however, only the precursor form of this serine proteinase is found. To become active, a complex of Factor IXa, phospholipid, calcium and Factor VIII is required. It has been demonstrated that Factor

VIII activity can further be increased, about 10 to 30-fold, by thrombin [19, 20]. It has been suggested that during activation a limited proteolysis takes place on Factor VIII molecule, too [20].

(d) *Thrombin and Factor IX*

As mentioned above, for the activation of Factor X, besides Factor VIII, another serine proteinase, Factor IXa, is required. Under normal circumstances, this enzyme exists in an inactive form, called Factor IX. During its activation by Factor XIa, in the presence of calcium, the single chain precursor protein is converted to a two-chain active enzyme. Thrombin can also generate Factor IXa activity from purified Factor IX. Thus, besides other functions, thrombin may potentiate the effect of Factor XIa on the activation of Factor IX in plasma [21].

(e) *Prothrombin activation and the plasma inhibitor system*

The "overall-regulation" of prothrombin activation by the end-product, thrombin, is summarized schematically in Fig. 3 (see also Fig. 2). These reactions, however, are further controlled by the natural inhibitor system of the plasma, mainly antithrombin III. Antithrombin III inactivates thrombin, hence regulates its activity as well as its control of its own activation. Moreover, this inhibitor is able to inactivate directly the other serine proteinases of blood coagulation [22], namely Factor Xa, Factor IXa, Factor XIa, Factor XIIa and plasmin (Fig. 3). These reactions are further accelerated by a natural polysaccharide, heparin. (The nature of the inhibition as well as the role of heparin will be discussed later.)

At present, the rate-limiting step in the activation of prothrombin, the significance of the relative ratio of the various clotting factors including thrombin

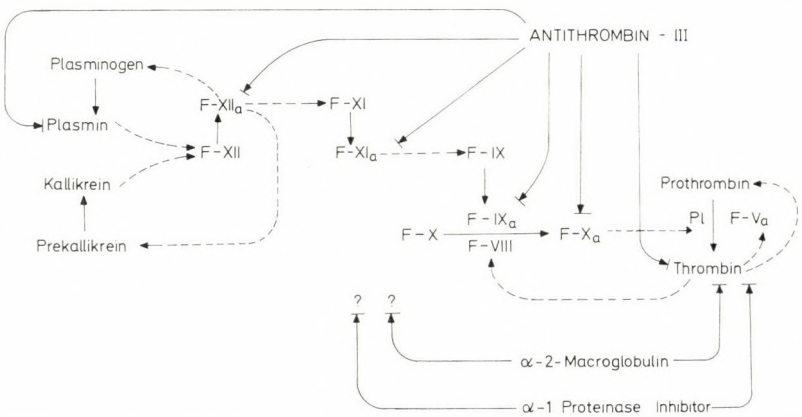

Fig. 3. Controls of thrombin formation. Abbreviations as in Fig. 1

and the sensitivity of the clotting enzymes to the inhibitor, antithrombin III, is not known. The role in these reactions of the other plasma inhibitors, α-2-macroglobulin and α-1-proteinase inhibitor, is also obscure.

3. Inactivation of thrombin

A third way to control thrombin activity is the inactivation of the excess enzyme molecules. In plasma, a number of proteinase inhibitors exist [23]. Among them, three inhibitors seem to be responsible for the majority of thrombin inactivation, and are found in high molar concentrations. These are: α-2-macroglobulin, α-1-proteinase inhibitor and antithrombin III. As to their relative effectiveness, antithrombin III is responsible for about 70% of the total thrombin inactivating capacity of the human plasma [24, 25]. *In vitro* measurements of the inactivation rate of thrombin by purified inhibitors and calculations from the *in vivo* concentration of the three plasma inhibitors suggest that α-1-proteinase inhibitor is 3 times, whereas antithrombin III is 15 times more potent than α-2-macroglobulin [26]. It is not, however, possible at present to reach a final standpoint regarding the importance *in vivo* of the plasma inhibitors.

(a) *α-2-macroglobulin*

The level of α-2-macroglobulin in plasma is about 3 μM [26]. Its inhibitory spectrum is very broad, in that it appears to interact with, and inhibit a variety of, proteinases including thrombin [26, 27, 28, and references therein]. Its plasma concentration is influenced by various pathological conditions, hormones, etc.

Looking at the structure of α-2-macroglobulin, it consists of subunits (M_r 185 000) which form a protein complex of about 700 000 molecular weight [29]. During interaction with trypsin or thrombin, a fragment (M_r 85 000) appears [29], suggesting a limited proteolysis of the inhibitor molecule. The mechanism of inhibition of thrombin by α-2-macroglobulin is not known exactly. There are conflicting reports about the complex formed, namely whether one molecule of inhibitor binds one or two enzyme molecules. The inhibition of thrombin activity develops only towards fibrinogen substrate, whereas in the presence of small molecular weight synthetic substrates, the enzyme activity is preserved, although its kinetics are affected [30, 31]. It has been supposed that the mechanism of inhibition of thrombin activity is sterical, i.e. the active centre of the enzyme does not react with α-2-macroglobulin.

(b) *α-1-proteinase inhibitor (α-1-antitrypsin)*

Of the proteinase inhibitors present in plasma, α-1-proteinase inhibitor is found in the highest molar concentration, i.e. 42 μM [26]. Attention has been focussed primarily on the inhibition of trypsin and chymotrypsin, with few reports as to the effect on thrombin. A series of contradictory reports were published earlier as to the question whether or not thrombin is inactivated by the α-1-pro-

teinase inhibitor. At present, it is acceptable that α-1-proteinase inhibitor inactivates thrombin [28, 32], forming a 1 : 1 stoichiometric complex, in which the enzyme loses its activity [26]. Under conditions when excess thrombin is present, both the enzyme/inhibitor complex and the free α-1-proteinase inhibitor are subjected to proteolysis [26]. The inhibitor is a glycoprotein with approximately 50 000 molecular weight [33]. Its carbohydrate component is supposed to play a role in the inhibitor's metabolism since the half-life of α-1-proteinase inhibitor in plasma decreases from 16 hours to 30 min when it is absent [34]. As to the mechanism of its action, it takes place in two steps. First, hydrolysis of the inhibitor occurs at a lys-thr bond, releasing a peptide (M_r 8000). Thereafter, a stable complex is formed between the "activated inhibitor" and the enzyme [35]. For this function, the inhibitor has to contain lys residues, since their modifications result in inactivation of the inhibitor [36]. It is supposed that a covalent bond forms between the hydroxyl group of the active serine of the enzyme and a carboxyl group of the inhibitor [26, 35]. The reaction is slow as compared to the thrombin/antithrombin III reaction [26, 28, 32]. Because of the high concentration of α-1-proteinase inhibitor in plasma, especially in conditions of antithrombin III deficiency, it may play an important role in the regulation of thrombin activity.

(c) *Antithrombin III (Heparin cofactor)*

Antithrombin III, the main natural inhibitor of thrombin, exists in plasma at a concentration of 4.5 μM [26], which is approximately two times higher than the prothrombin level. Its half-life in the blood circulation is 2.8 days [37].

Antithrombin III is a single chain glycoprotein containing three disulfide bridges and having a molecular weight of 65 000 [38–40]. It shows an ellipsoid form with semi-axes of 1.9, 3.7 and 5.2 nm [41].

The biological function of antithrombin III is rather wide; it inactivates a series of serine proteinases including all clotting enzymes of blood coagulation [22]. As to the mechanism of action, only thrombin inactivation is well documented. It has been demonstrated that the inhibitor forms a 1 : 1 stoichiometric complex with the enzyme [42, 43] and in this complex, thrombin loses its clotting as well as its esterolytic activity [38]. Antithrombin III is also termed "progressive antithrombin", since the inhibition of thrombin activity is not instantaneous.

(d) *Regulation of thrombin inactivation by heparin*

The kinetics of thrombin inactivation by antithrombin III are changed by the natural polysaccharide, heparin. The exact structure of heparin, especially the sequences of the saccharide units, has not been determined. The most reliable information is that the polysaccharide, with a molecular weight between 6000 and 20 000, consists of repeated units of N-sulfated as well as O-sulfated or acetylated glucosamine, L-iduronic acid and unsulfated D-glucuronic acid residues, which are joined by α-1-4-glycosidic linkages. As to the role of heparin in thrombin

inactivation, it is known that it facilitates the formation of a complex of thrombin and antithrombin III. Although the effect of heparin on thrombus formation has been known for more than half a century, the exact mechanism of its action could not be elucidated. The understanding of the exact role of heparin, however, is important from two points of view. First, heparin is synthesized by mammalian cells and is widely distributed in the different organs, hence its regulatory function seems to be very probable. Secondly, heparin is an essential drug in the prevention and treatment of thromboembolic diseases.

Regarding the mechanisms of action of heparin on the reaction between thrombin and antithrombin III, two hypotheses are generally mentioned in the literature (Fig. 4). According to the "inhibitor-fitting" hypothesis, heparin binds to antithrombin III and activates it [42]. The other, the "enzyme-fitting" hypothesis, gives the primary importance to a thrombin/heparin interaction which, in turn, induces a conformational change in the enzyme, rendering it more susceptible to the inhibitor [44]. Both hypotheses are supported by several findings. Namely, (i) the binding of heparin to these proteins is tight: Kd for thrombin is 1.5×10^{-9} M [45], for antithrombin III is 4.4×10^{-7} M [46]; (ii) the modification of lys or arg residues of antithrombin III [42, 47] as well as of thrombin [47–51] inhibits the rate-enhancing effect of heparin on the thrombin/antithrombin III reaction. Although kinetic analyses support the primary importance of the thrombin/heparin interaction [52–55, 99], the mentioned data suggest an apparent catalytic role of heparin in the reaction between thrombin and antithrombin III [47, 56]. According to our theoretical consideration [100], heparin fulfils qualitatively several criteria of a cat-

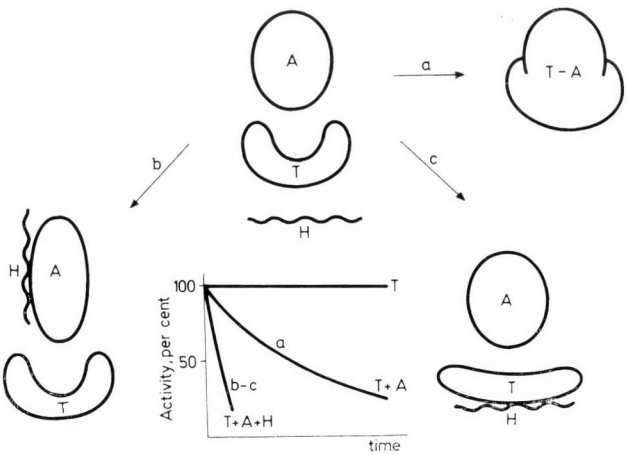

Fig. 4. Hypotheses for heparin action. Reaction *a* symbolizes the slow interaction between thrombin (T) and antithrombin III (A) resulting in an irreversible complex (T-A) in which thrombin loses its activity. Reaction *b* and *c* symbolize the "inhibitor-fitting" hypothesis and the "enzyme-fitting" hypothesis, respectively. The reaction rate-enhancement (b−c) by heparin (H) is a consequence of a conformational change either in the inhibitor or in the enzyme

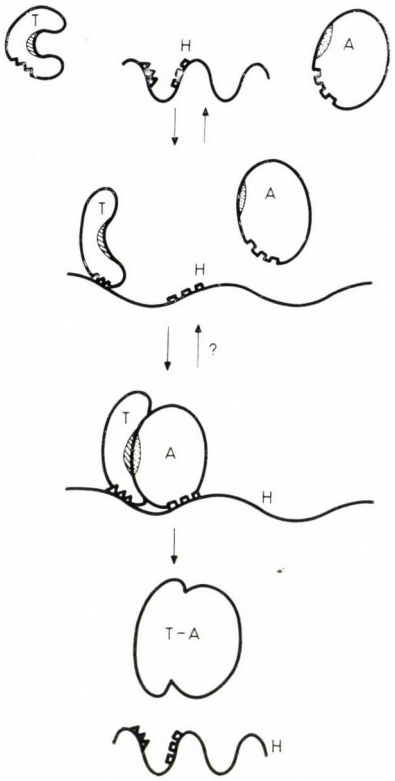

Fig. 5. Mechanism of heparin action in the reaction between thrombin and antithrombin III. Thrombin (T) binds to the non-specific binding site of heparin (△△△) thereby resulting in a conformational change of the protein as well as of heparin (H). This reaction renders the active site of thrombin more susceptible to its inhibitor. Antithrombin III (A) bound to the specific binding site of heparin (□□□) reacts with thrombin, forming a ternary complex. Since the binding to heparin of the thrombin/antithrombin III complex (T-A) is less strong than that of the thrombin or antithrombin III alone, the complex is released as long as free thrombin molecules exist. In the complex, the enzyme becomes inactive. The free heparin released can react again with a new thrombin molecule, acting thereby as a catalyst

alyst: binding nature, reaction rate-enhancement (increasing activation entropy), recyclization of the heparin molecule in the reaction (effectiveness of non-stoichiometric amount of polysaccharide), Michaelis–Menten saturation kinetics, and specificity. The catalysts in biological systems, however, are enzymes, i.e. proteins, and such a function would be unusual for a polysaccharide. It is proposed to call this type of reaction "cataloid-acceleration" and the modulator "catalor". On the basis of these data, a model is suggested for the mechanism of action of heparin (Fig. 5). Such a function of heparin may be interesting from another point of view, as well (see later in paragraph "Heparin as an endogenous modulator").

As to the generalization of the proposed model presumably the apparent catalytic role of heparin is not restricted to the thrombin/antithrombin III reaction. It seems to be valid for two other enzymes of blood coagulation, i.e. Factor Xa and plasmin [52, 57–59, 100]. This is most probable, since heparin has various repeating sequences, which may contain structures for the recognition of different proteins. If so, some saccharide groups build up the units of information.

B. Regulation of biological actions of thrombin

As mentioned earlier, thrombin can react rather specifically with several proteins, proenzymes and various cells. The main targets of its action are summarized in Fig. 6.

1. Thrombin-fibrinogen reaction

Fibrinogen, a glycoprotein of about 340 000 molecular weight, is synthesized in the liver, secreted into blood circulation, where its concentration is approximately 3 mg/ml, and has a half-life of three days. The dimension of fibrinogen appears to be about 90×450 Å. It is composed of three pairs of nonidentical polypeptide chains called $A\alpha$, $B\gamma$ and γ. The chains are held together by disulfide bridges [60, 61, and references therein].

When thrombin is formed, it converts fibrinogen, a soluble plasma protein, to an insoluble gel called fibrin. Fibrin formation is due to the release by limited

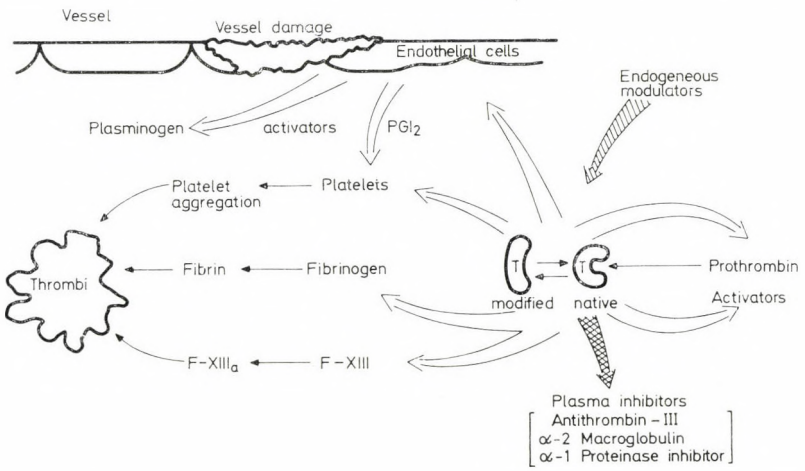

Fig. 6. Possible reactions of thrombin. Native thrombin means thrombin formed during prothrombin activation in the "prothrombinase complex". A change in its conformation, induced by an other molecule, is designated as modified thrombin. Endogenous modulators are supposed to control the conversion between native and modified state of thrombin. Abbreviations as in Fig. 1. PGI_2 denotes prostacyclin

proteolysis of four fibrinopeptides from the amino-terminal ends of the fibrinogen molecule. The reaction is highly specific, namely while trypsin splits approximately 300 bonds in fibrinogen, thrombin catalyzes selectively the hydrolysis of four bonds in the same molecule [62]. The action is stimulated by the presence of calcium ions. The sequence of events in fibrin formation may be subdivided into three phases: proteolysis, polymerization and stabilization. In the proteolytic phase, thrombin attacks four arginyl-glycine bonds in the Aα and Bβ chains, liberating a pair of peptides called fibrinopeptide A and B [63]. Fibrinopeptide A is released at a faster rate than fibrinopeptide B, and this may be one of the critical steps leading to fibrin formation. The γ-chain is not hydrolyzed during proteolysis. The remaining parent molecules (fibrinogen minus fibrinopeptides) called fibrin monomers, enter the second phase, that of polymerization. This reaction is characterized as being a non-enzymatic, self-assembly process, resulting in end-to-end and lateral aggregations. Polymers formed in the absence of calcium ions are soluble in dilute acid or base and 6 M urea solutions. This polymer is also very susceptible to degradative enzymes such as plasmin. It is stabilized, however, in the presence of another plasma enzyme, Factor XIIIa (Laki–Lorand factor). This is the third phase, the phase of stabilization (cf. below).

2. Factor XIII activation by thrombin

Factor XIII is a plasma glycoprotein composed of two pairs of nonidentical subunits, called a and b [64 and references therein]. Their concentration in plasma is about 10 μg/ml. The a-subunits (M_r 75 000) and the b-subunits (M_r 88 000) form a biologically inactive tetramer having a molecular weight of 320 000 [65]. Factor XIII has also been isolated and characterized from platelets. This latter protein has no b-subunits, although its a-subunits are identical in size and amino acid composition with the a-subunits of plasma Factor XIII [65]. These data suggest a regulatory function of b-subunits.

In the presence of thrombin and calcium ions, Factor XIII is converted to an enzyme possessing transglutaminase activity. The activation is as follows: In the first step, thrombin cleaves an arg-gly bond in each of the a-subunits, liberating two activation peptides [65, 66]. In the next step, the tetrameric intermediate dissociates into a catalytic dimer, designated a'2-dimer and an inactive b'2-dimer. This reaction requires the presence of calcium ions. Activation of Factor XIII from platelets by thrombin occurs at a significantly faster rate than that of the plasma enzyme, suggesting that the b-subunits control the rate of enzyme formation by thrombin [65].

When Factor XIIIa is formed, it establishes cross-linkages in fibrin, resulting in a tough, insoluble clot which is resistant to lyses. The cross-linkage is due to the formation of about six ε(γ-glutamyl) lysine bridges between a glutamine side chain in one fibrin monomer and a lysine side chain in another fibrin monomer [67, 68].

As to the function of the plasma- and platelet-enzyme, one may conclude that such a control mechanism yields a fast enzyme function when there is a need for it. On the other hand, it protects fibrinogen under normal haemostatic conditions.

3. *Binding of thrombin to endothelial cells*

Normal haemostasis results from a complex series of biological events which occur in immediate response to blood vessel injury. Since thrombin plays a key role in haemostasis and thrombosis, its interaction and influence on endothelial cells is critical. Thrombin has been documented to bind tightly to endothelial cells, with a Kd of 10^{-10} M. It has been suggested that approximately 3×10^3 specific binding sites exist on a cell. For binding, the active centre of the enzyme is not needed [69]. As to the function of this interaction, information available in the literature is scarce. Recent reports have, however, shown that thrombin may release prostacyclin from endothelial cells [70, 71]. Another important finding is that thrombin bound to endothelial cells can be released by heparin (unpublished data).

4. *Platelet receptors for thrombin*

The essential functions of platelets involve their response to various stimuli. One of the important stimuli is thrombin [73, 74, 98 and references therein]. The binding of thrombin and the generation of a signal are separate processes [75]. There are, at least, two thrombin binding sites on the platelets: a high affinity one with about 800 sites/cell with a Kd of 1.5×10^{-9} M, and a low affinity one, with about 3×10^4 sites/cell with a Kd of 3×10^{-7} M [76]. Experiments with chemically modified, as well as with α-, β- and γ-thrombins indicate that the high affinity binding site interacts with the fibrinogen binding region of the thrombin molecule [76, 77]. It is important to know that heparin inhibits the high affinity binding [76]. A direct correlation can be found between the ability of the different forms of thrombin to clot fibrinogen and to influence platelet function. In general, thrombin of high fibrinogen clotting activity is a potent inducer of platelet aggregation and release reaction, while species of a low clotting ability are poor inducers of aggregation and release reaction [77].

The platelet release reaction is a specific process by which certain platelet constituents (ADP, ATP, Ca^{2+}, 5-hydroxytryptamine, platelet factor 4, etc.) are secreted to the extracellular phase. It is an essential step in the sequence of events leading to the formation of the dense, adhesive aggregates of platelets observed in thrombosis. This secretory process can be induced by a variety of agents such as ADP, collagen, epinephrine and thrombin. The actual mechanism of release, or the mechanism by which the initial stimulus triggers the release, is not known. It is, however, accepted that in most secretory cells calcium mediates the stimulus-secretion process [74]. It is remarkable that 1 NIH unit of thrombin is able to induce the release of about 50 pM Ca^{2+} from 7×10^8 platelets/ml [78].

5. Thrombin binding and mitogenic stimulation

Thrombin stimulates DNA synthesis and cell division in non-proliferating fibroblasts [79, 80 and references therein]. The binding of thrombin involves regions of α-thrombin, distinct from the catalytic apparatus that are related, if not identical, to regions required for fibrinogen recognition, although the mitogenic activity of thrombin on these cells requires the intact catalytic centre [80]. It presumably cleaves a specific protein component. There are about 2×10^5 thrombin receptors/cell with a single affinity constant of about 10^9 M^{-1} on the cell surface [80]. Furthermore, in human fibroblasts thrombin stimulates the production and release of a major surface-associated glycoprotein, fibronectin [81].

Since thrombin is generated after tissue damage, these functions of thrombin may be important in wound healing and tissue repair [81, 82].

What determines the choice among the possible reaction routes catalyzed by thrombin?

Under normal conditions, there is no measurable amount of thrombin in blood circulation. The essential question is, therefore, what happens to the first thrombin molecules formed, i.e. which natural substrate is its first target? This question (especially when the intrinsic pathway is triggered) is important from the point of view of understanding the biological functions of thrombin. The question, however, is difficult to answer. Although all of the concentrations of substrates for thrombin in plasma are known, their Kd or Km have not been determined[2]. Even if we had these data, we could not answer the basic question, since the known plasma concentrations of the various components are not applicable to the actual situation as they are not distributed uniformly in plasma. They form complexes with phospholipid, calcium and several proteins and in these "compartments" there are extreme changes in concentrations of the components resulting in alterations of reaction rates of several orders of magnitude. To illustrate the problems, we present some relevant findings from the literature. If we want to predict the possible reaction ways of thrombin on the basis of its "substrate"-concentrations in plasma, the data are: α-1-proteinase inhibitor ($\sim 4.0 \times 10^{-5}$ M) > fibrinogen ($\sim 10^{-5}$ M) > antithrombin III ($\sim 4.5 \times 10^{-6}$ M) > α-2-macroglobulin ($\sim 3 \times 10^{-6}$ M) > prothrombin ($\sim 1.2 \times 10^{-6}$ M) > Factor IX ($< 10^{-7}$ M) > Factor XIII ($\sim 3 \times 10^{-8}$ M) ≈ Factor V ($\sim 3 \times 10^{-8}$ M) ≥ Factor VIII ($\sim 2 \times 10^{-8}$ M) > thrombin binding site on endothelial cells[3] in capillary ($> 10^{-8}$ M) > thrombin

[2] The known binding affinities of thrombin are as follows: Kd for endothelial cells ≈ 10^{-10} M [69], for antithrombin III 10^{-10} M [83], for platelets 1.5×10^{-9} M [76], and the Km for fibrinogen Aα-chain ≈ 5×10^{-6} M [94].

[3] Calculation of the "concentration" of thrombin binding sites of cells in plasma is for platelets: 2.5×10^8 cells/ml with about 800 binding sites/cell [76] results in about 2×10^{11} sites/ml. Since 6×10^{23} molecules/l gives an about 1 M solution, the concentration of thrombin binding site on platelets could be about 3×10^{-10} M in plasma. For endothelial cells: assuming that two endothelial cells with 6×10^3 binding sites [69] belong to a 2×10^{-11} ml (accepting that the capillary and the red cell are nearly equivalent in diameter) the concentration of thrombin binding capacity in a capillary would be about 10^{-8} M.

binding site on platelets[3] ($\sim 3 \times 10^{-10}$ M). According to this order, the first thrombin molecule formed would react with plasma inhibitors, fibrinogen or prothrombin. Although α-1-proteinase inhibitor is present in the highest concentration in plasma, the main inhibitor of thrombin is antithrombin III (see paragraph "Inactivation of thrombin"). On the basis of these data, antithrombin III seems to be an essential "substrate" of thrombin. Since antithrombin III concentration in plasma is about 4.5 μM and the Kd for thrombin is about 10^{-10} M [83], it seems that inhibition would be so close to completion at equilibrium that the remaining thrombin levels would always be insignificant. This, however, may not necessarily be so. The level of prothrombin in plasma is about 2 μM, which means that if, for example, 10% of it were activated[4], giving a total thrombin generation of 20 units/ml, then we can estimate a minimum equilibrium level after inhibition of 0.5 milliunit/ml. This concentration of thrombin may be within the range of an effect on the proenzymes of blood coagulation [83]. There are other situations as well, where the equilibrium nature of inhibition could be of much greater importance, i.e. in prothrombin complexes where antithrombin III concentration is relatively low as compared to the high capacity for thrombin generation.

Although it is not possible to predict whether thrombin interacts primarily with fibrinogen, antithrombin III or prothrombin (it depends, very probably, on the conditions *in vivo*), the problem discussed above seems to be interesting since it has stimulated several hypotheses (cf. below).

Endogenous modulators of thrombin

The biological functions of thrombin are determined not only by the concentrations of the different factors and the binding affinity for the various components. There is a possibility that a change in the conformation of thrombin induced by endogenous factors, favours a certain way of reaction, whereas it decreases the probability of others. If so, it seems reasonable to search for cell and/or plasma components which may modify thrombin conformation, thereby determining a special reaction way. Although there are some data in the literature regarding the modification of thrombin [28, 77, 84, 85, 97], the discussion of their biological role is nowadays reduced to conjectures. Nevertheless, a possible candidate of natural origin may be proposed for such a modulator function.

Heparin, as an endogenous modulator

Heparin, as detailed earlier, is synthesized by mammalian cells and has a high affinity to thrombin (Kd $\approx 1.5 \times 10^{-9}$ M). Its plasma concentration has to be lower than 10^{-8} M. During therapeutical administration, however, the level may be as high as 10^{-5} M. Thus, heparin may function in a wide range of concen-

[4] Experiments suggest that the maximum amount of thrombin generated during coagulation in human plasma, is about 10% of the prothrombin [96].

ɛration. The binding of heparin to thrombin results in a conformational change of the enzyme [45, 48–51, 53, 86–88). Thrombin, bound to heparin, retains its activity toward fibrinogen substrate [86]. Thus, heparin may be really considered as a modulator molecule for thrombin, especially as the inactivation of heparin-bound thrombin by antithrombin III is accelerated 100- to 200-fold, whereas its stability against thermal denaturation or inactivation by α-1-proteinase inhibitor and α-2-macroglobulin is increased [56, 89]. The binding of the thrombin/heparin complex to platelets is also less than that of the free enzyme [76].

The possible endogenous modulator role of heparin may be interesting from another point of view. Heparin, and some other heparin-like molecules, i.e. heparan sulfate, dermatan sulfate, having also heparin-like anticoagulant activity [51, 90, 91], could serve as a surface on the cell membrane [95] for a catalyst-like function (see also the paragraph on "Regulation of thrombin inactivation by heparin"), Thus, on the cell membrane, various proteinases could be bound tightly. After reacting with their plasma inhibitor, antithrombin III, the newly formed complexes are more readily released since their binding to the heparin-like surface is less tight than that of their components (see also Fig. 5). Such a function of a modulator molecule, like heparin, may result in protection of cells against proteolytic damage.

Accordingly, thrombin may function differently depending on its conformation (Fig. 6), i.e. a natural modulator, for example heparin, may influence the reaction ways.

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Cytomorphology and Lysosomal Enzyme Activity of the Haemopoietic and Reticuloendothelial System of the SJL/J Mouse

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The cytomorphology and lysosomal enzyme activity of the haemopoietic and reticuloendothelial system (RES) of SJL/J mice, which develop a spontaneous reticulum cell neoplasm type B (RCN-B), were studied. The cytological findings during development of the disease were a predominance of medium-sized lymphocytes and an increase in the number of hyperbasophilic cells and plasma cells in the RES. These pathological findings were compared to the changes due to ageing in control groups of healthy C3H/eB mice. Acid phosphatase activity increased in the RES, whereas that of β-D-glucuronidase remained unchanged during development of the disease. These findings indicate that the RCN-B of the SJL/J mice bear a certain resemblance to Hodgkin disease in man.

Introduction

SJL/J, an inbred mouse strain developed at the Jackson Laboratory, Maine, USA, was found by Murphy in 1963 [22] to have a 90% incidence of spontaneous reticulum cell neoplasm type B (RCN-B) at a mean age of 13 months. Histologically, this neoplasm resembled Hodgkin disease in man, according to Dunn's classification [11]. The disease of SJL/J mice has been subjected to extensive histopathological [21, 22] and immunological [3, 10, 14, 27] investigation. Viral aetiology [32] and the effect of different carcinogens [13, 15, 21] were also examined. But to the best of our knowledge, no cytomorphological and cytochemical investigations of the development of RCN-B in the SJL/J have been performed to date. In this study we analyzed the cytomorphology and the cytochemistry of the lysosomal enzymes of the haemopoietic and reticuloendothelial system of SJL/J mice and in control groups of C3H/eB mice. Our aim was to find out whether the disease of the SJL/J was a proper cytological model for research of Hodgkin disease, and whether the changes in enzymatic activity and cytomorphology, if they existed, were primary or age related.

Materials and Methods

SJL/J and C3H/eB mice 3–4 months and 17 months of age, supplied kindly by Prof. N. Haran-Ghera of the Weizmann Institute, were used. Blood and organs

for examination were taken at the same hour every morning in order to eliminate diurnal variations [5].

Mice were bled from the retro-orbital venous plexus, and bone marrow was taken from the femur and prepared for examination with the cytocentrifuge [28]. After the death of the mouse, a careful dissection was done, and smears and touch preparations were taken from the spleen, thymus, cervical and inguinal lymph nodes, tumour mass and ascites.

Blood counts were done with haemocytometer and differential counts after May–Grünwald–Giemsa staining. The RES and bone marrow preparations were fixed in methanol and stained with Giemsa.

For cytological evaluation of the RES, a semi-quantitative scoring method, with values ranging from 0 to 3 was used. This resulted in relative values, not suitable for statistical evaluation.

For lysosomal enzyme activity, two representative enzymes were studied: 1) Acid phosphatase activity was demonstrated by the method of Barka and Anderson, and specific inhibition with L(+) tartrate [2]. Fresh air-dried smears were fixed for 30 seconds in a solution of 60% acetone at 4 °C and incubated at 37 °C for 30 minutes. 2) β-D-glucuronidase was studied by the method of Lorbacher et al. with a modification of the counterstaining: Harris haematoxylin was used instead of methyl green [20]. Fresh air-dried smears were fixed in a solution of methanol-formalin (3 : 7) at 4 °C for 60 seconds and incubated at 37 °C for 60 minutes. Cytochemical activity was evaluated by a semi-quantitative scoring method, which took into account the intensity of staining in the positive cells, and the relative amount of these cells in each group of cells. Scores ranged from 0 to 4 (0 = absence of activity; 4 = very strong activity).

For scoring, the slides were coded to avoid bias. Student's t test was used to determine the statistical significance of the blood and bone marrow counts.

Results

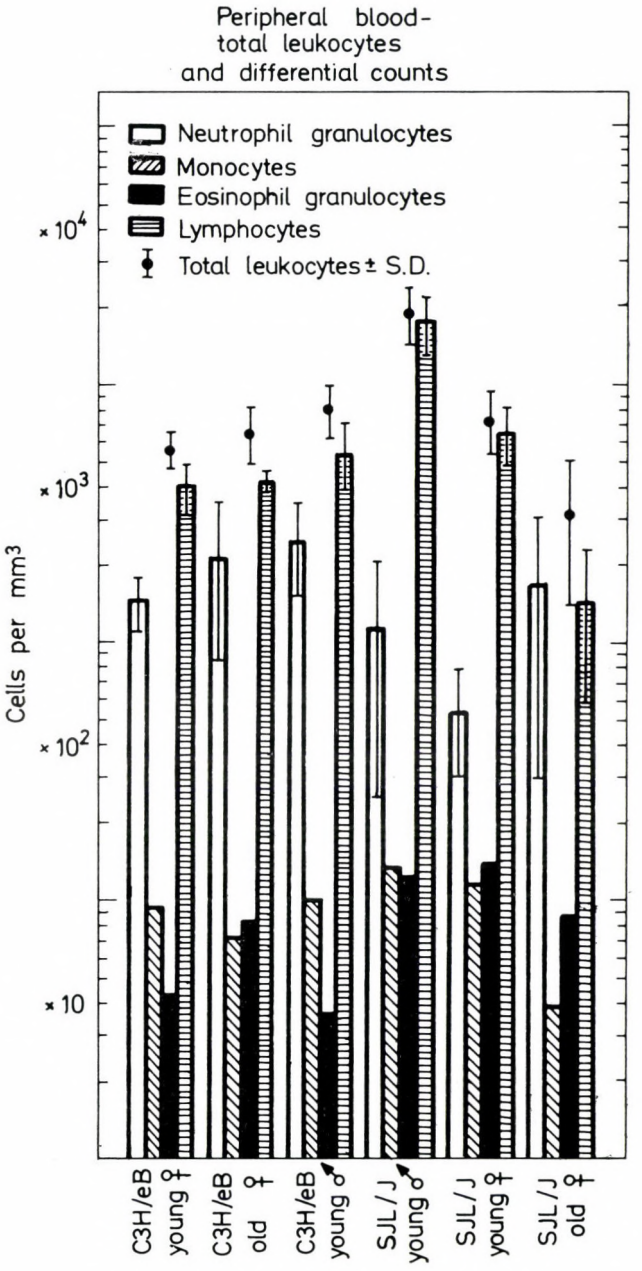
Clinical Signs

The sick SJL/J mice appeared weaker and less alert compared to old C3H/eB mice. Some of them suffered from severe dyspnoea. Physical examination revealed hepato-splenomegaly and sometimes cervical lymphadenopathy. At autopsy there was generalized lymphadenopathy and enlargement of the whole RES.

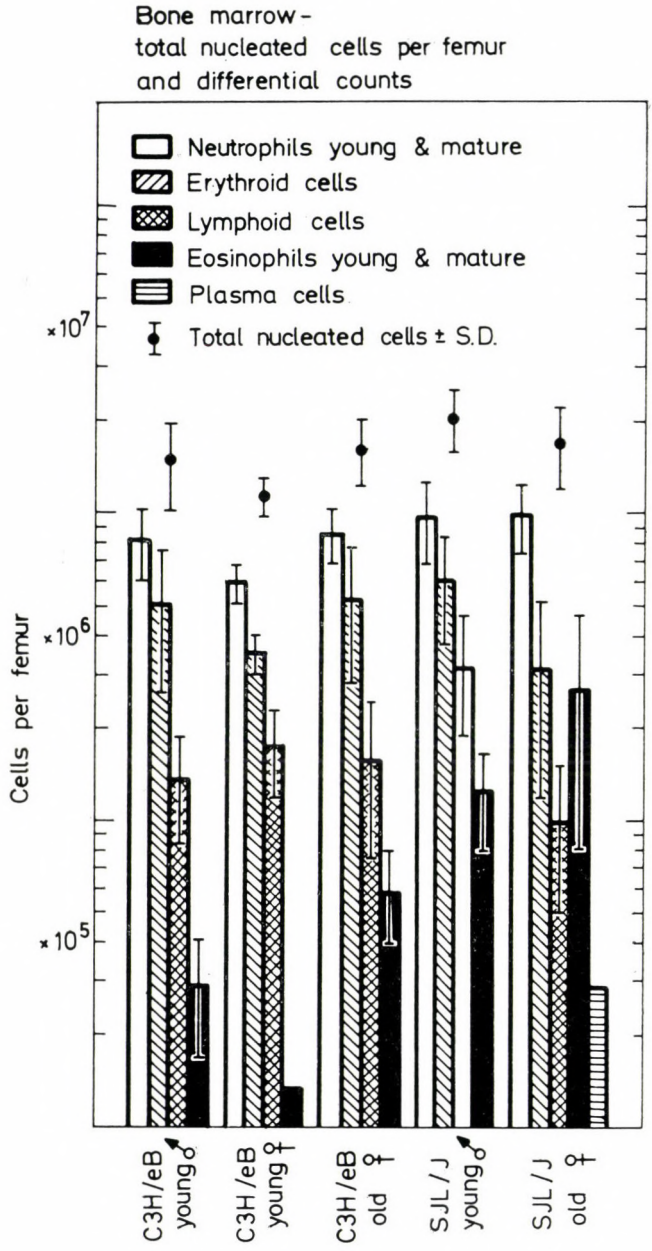
Cytomorphology

A. *Haemopoietic system*

1. *Peripheral blood* (see Histogram 1). The important findings were leukocytosis in young male SJL/J mice in comparison with the other groups ($p < 0.001$), and leukopenia in the sick old SJL/J mice ($p < 0.01$). The leukopenia was the


Peripheral blood-
total leukocytes
and differential counts
Neutrophil granulocytes
Monocytes
Eosinophil granulocytes
Lymphocytes
Total leukocytes ± S.D.
× 10⁴
× 10³
× 10²
× 10
Cells per mm³
C3H/eB young ♀
C3H/eB old ♀
C3H/eB young ♂
SJL/J young ♂
SJL/J young ♀
SJL/J old ♀

Histogram 1


Bone marrow -
total nucleated cells per femur
and differential counts
Neutrophils young & mature
Erythroid cells
Lymphoid cells
Eosinophils young & mature
Plasma cells
Total nucleated cells ± S.D.
×10^7
×10^6
×10^5
Cells per femur
C3H/eB young ♂
C3H/eB young ♀
C3H/eB old ♀
SJL/J young ♂
SJL/J old ♀

Histogram 2

result of marked lymphopenia ($p < 0.01$), 44.2% lymphocytes in the sick mice compared to 90% in the young. On the other hand, there was a neutrophilia of 52% in the sick mice, which was significant in comparison with the young female SJL/J ($p < 0.05$). There was no difference in the number of eosinophils between the young and the sick mice. The high eosinophil count in the young SJL/J compared to the young C3H/eB was equalized with age increase in both groups.

2. *Bone marrow* (see Histogram 2). There was a significant difference between the young SJL/J and the sick SJL/J in the erythroid ($p < 0.05$) and the lymphoid series ($p < 0.001$). The difference in the lymphoid series was marked, especially in the percentage of these cells in bone marrow: 5.8% in the sick compared to 16.2% in the young mice. There was eosinophilia in both strains, increasing with age but the number of eosinophils in the young SJL/J was significantly higher, even when compared to the old C3H/eB mice ($p < 0.01$). The eosinophil count in the sick SJL/J mice was so high that it almost equalled the number of normoblasts. A remarkable phenomenon was the large number (1.7%) of plasma cells in the sick SJL/J. In the other groups there were only a few plasma cells in the bone marrow.

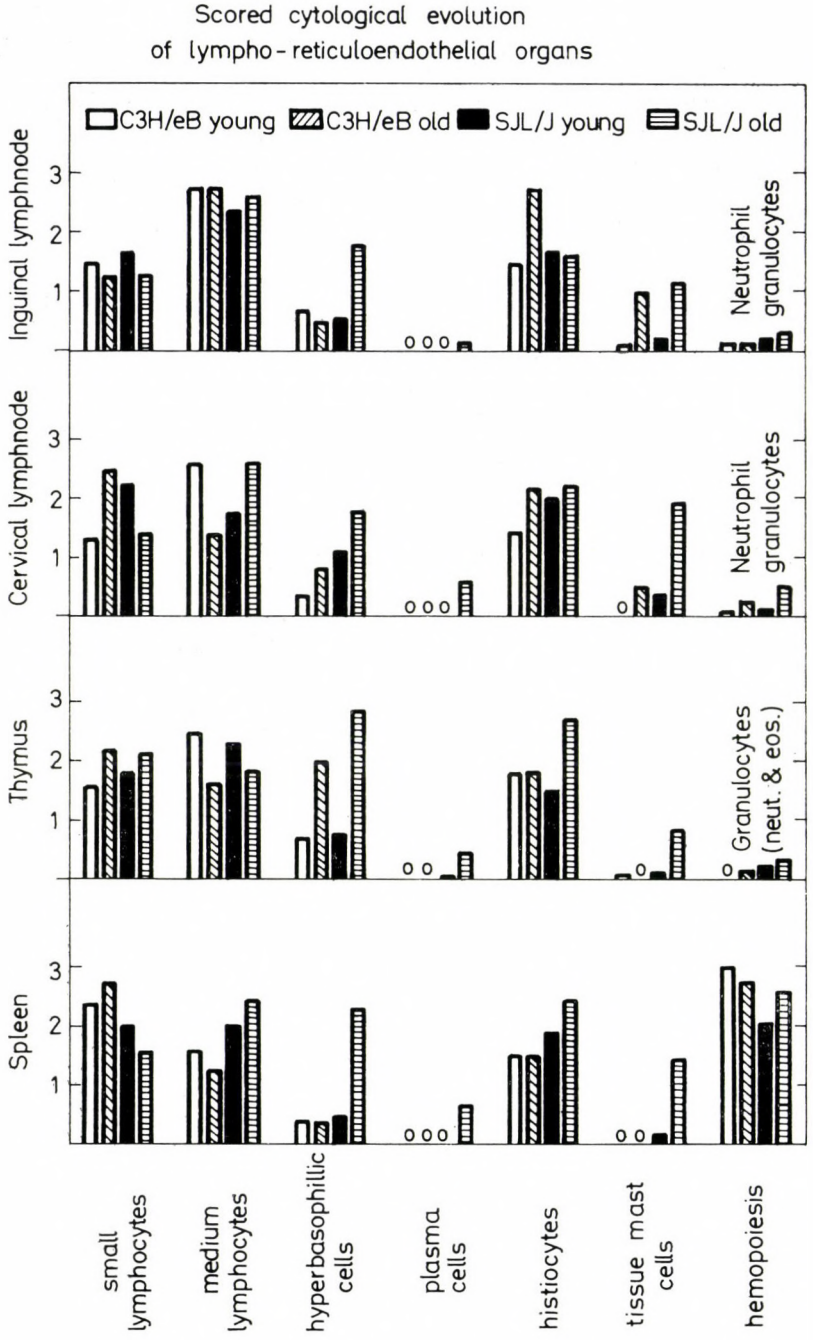
B. *Reticuloendothelial system (see Histogram 3)*

Several cell types were observed in the RES: small lymphocytes, medium lymphocytes, hyperbasophilic cells (immunoblasts), plasma cells, histiocytes, tissue mast cells, haemopoietic cells and/or granulocytes.

1. *Spleen.* The lymphocytic compartment in the SJL/J mice was composed mainly of medium lymphocytes, especially in the sick SJL/J mice, whereas in C3H/eB mice it was composed mainly of small ones. Another striking difference between the sick SJL/J and the other groups was in the high number of hyperbasophilic cells, plasma cells and tissue mast cells. In the C3H/eB mice these cells were negligible as compared to other cells. The number of histiocytes in the spleen of SJL/J mice was higher than in that of the C3H/eB mice, with a tendency to rise in the sick SJL/J mice. There was almost no difference in haemopoiesis among the groups examined.

2. *Thymus.* There was a considerable similarity in the lymphocytic and hyperbasophilic components between the young mice of both strains and the old ones, but the number of hyperbasophilic cells in the sick SJL/J mice was greater than in the old C3H/eB mice. The lymphocytic component of the sick SJL/J mice in the thymus was different from that of the spleen, the small lymphocytes being the majority. Another remarkable finding was the increase in histiocytes in the sick SJL/J mice, some of them resembling Reed–Sternberg cells (Fig. 1). The number of plasma cells and tissue mast cells in the sick SLJ/J mice was not high, but it was striking compared to the other groups of mice.

3. *Cervical lymph nodes.* The main lymphocytic component in the young SJL/J mice consisted of small lymphocytes, whereas in the sick SJL/J mice medium lymphocytes were the majority. There was a tendency to a greater number of hyperbasophilic cells in the SJL/J strain, while in the sick mice the number was


Scored cytological evolution
of lympho-reticuloendothelial organs
C3H/eB young
C3H/eB old
SJL/J young
SJL/J old
Inguinal lymphnode
Cervical lymphnode
Thymus
Spleen
Neutrophil granulocytes
Granulocytes (neut. & eos.)
small lymphocytes
medium lymphocytes
hyperbasophillic cells
plasma cells
histiocytes
tissue mast cells
hemopoiesis

Histogram 3

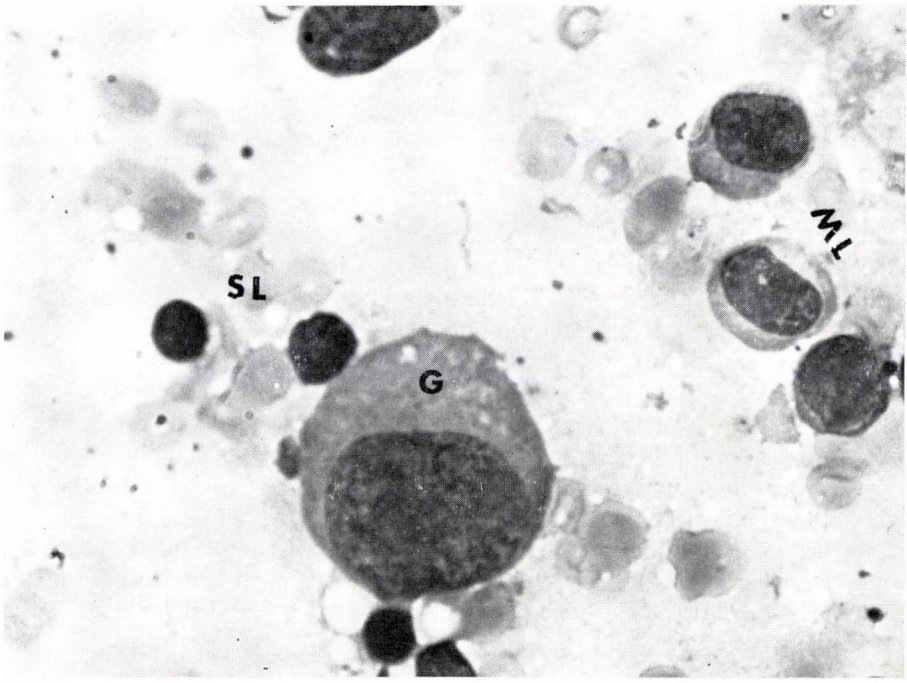


Fig. 1. Sick SJL/J thymus. SL — Small lymphocyte. ML — Medium lymphocyte. G — Giant cell resembling Reed–Sternberg cell. ×800 (Stain, methanol Giemsa)

even greater. The sick SJL/J mice differed from the other groups in the number of plasma cells and tissue mast cells, whereas the number of histiocytes was similar in all groups.

4. *Inguinal lymph nodes.* The lymphocytic composition was similar in all the groups, the majority being medium lymphocytes. The number of hyperbasophilic cells was highest in the sick SJL/J mice, whereas the number of plasma cells was negligible in all groups. A striking finding was the large number of histiocytes in the old C3H/eB mice.

5. *Tumour mass.* Half of the sick SJL/J mice each had a giant tumour mass, either mesenteric or mediastinal. These masses had equal numbers of small and medium lymphocytes. There was a large number of hyperbasophilic cells and histiocytes, and a small number of plasma cells. Some of the histiocytes were multinucleated, and some of them were giant cells. Some giant bare nuclei with prominent nucleoli were also present. Randomly, cells resembling Reed–Sternberg cells were found. Tissue mast cells, neutrophil granulocytes and eosinophils were seen occasionally.

C. *Cytochemistry (see Histogram 4)*

The cytochemical activity of β-D-glucuronidase and acid phosphatase in the positive cells was seen in one of 3 forms: coarse or delicate red granules (one or a few) scattered in the cytoplasm (characteristic of lymphocytes), diffuse staining in the whole cytoplasm, and diffuse-granular staining in the whole cytoplasm (the last two forms are characteristic of histiocytes). We have to emphasize that by means of cytochemical staining we were unable to differentiate between the different cells in the RES. Therefore, the marker for differentiation was the cell size; small and medium cells were identified as lymphocytes, and the large ones as histiocytes.

1. *Acid phosphatase.* All peripheral blood and bone marrow cells were negative in the SJL/J and CeH/eB strains. In the young SJL/J mice no activity was seen in the lymphocytes of the RES, except for the spleen in which the activity was very weak. In the sick SJL/J mice the spleen lymphocytes exhibited weak activity. On the other hand, the histiocytes showed a strong activity (except for the thymus); this activity rose in the sick SJL/J mice in the whole RES, including the thymus and the tumour mass. Even in ascites there was some acid phosphatase activity in histiocytes. The activity of the enzyme was almost completely inhibited by L(+)-tartrate. In the old mice of both strains strong intercellular activity was seen in the RES (Fig. 2A).

2. *β-D-glucuronidase.* Activity was weak in the RES of the young C3H/eB mice, in both lymphocytes and histiocytes. This activity vanished in the old C3H/eB mice. In the SJL/J mice, activity was more or less similar in young and old mice; activity was weak in lymphocytes, and medium to strong in histiocytes (Fig. 2B). Peripheral blood and bone marrow cells were negative in both strains.

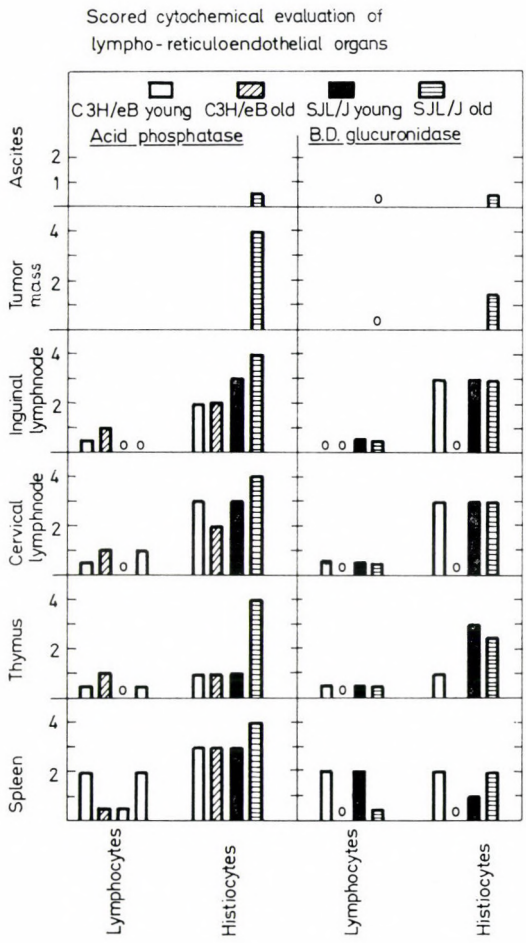
Discussion

The purpose of this study was to evaluate the reliability of RCN-B of the SJL/J mice as a cytological and cytochemical model of Hodgkin disease in man, and to find out whether the changes found were inherent or secondary to the development of the disease.

Cytologically, the peripheral blood of the sick mice showed lymphopenia, neutrophilia, and in some cases eosinophilia. In the bone marrow we found lymphopenia, a decline in erythropoiesis, and an elevation of the proportion between the myeloid and erythroid series (from 1.6 in the young mice to 3.14 in the sick mice). These findings are largely similar to those observed in the haemopoietic system in Hodgkin disease in man [29]. In the bone marrow of the sick mice, eosinophilia and an increased number of plasma cells were also present.

The lymphopenia in the haemopoietic system could be attributed to a decreased lymphopoiesis in the bone marrow and to a possible exhaustive mobilization of lymphocytes to the RES which showed an increase of the lymphocyte

population during development of the disease. Cleason et al. [6] showed migration of SJL/J B-lymphocytes from the bone marrow to the thymus – supporting our assumption. Contrary to the lymphopenia in sick mice, there was lymphocytosis



Histogram 4

in the young SJL/J mice which was probably inherent. This lymphocytosis increased the probability for the mice to develop an acquired lymphoproliferative disorder. Yet, the fact that the disease appeared at a certain age in a very high percentage of mice [22] does not exclude the possibility that it is congenital in this strain.

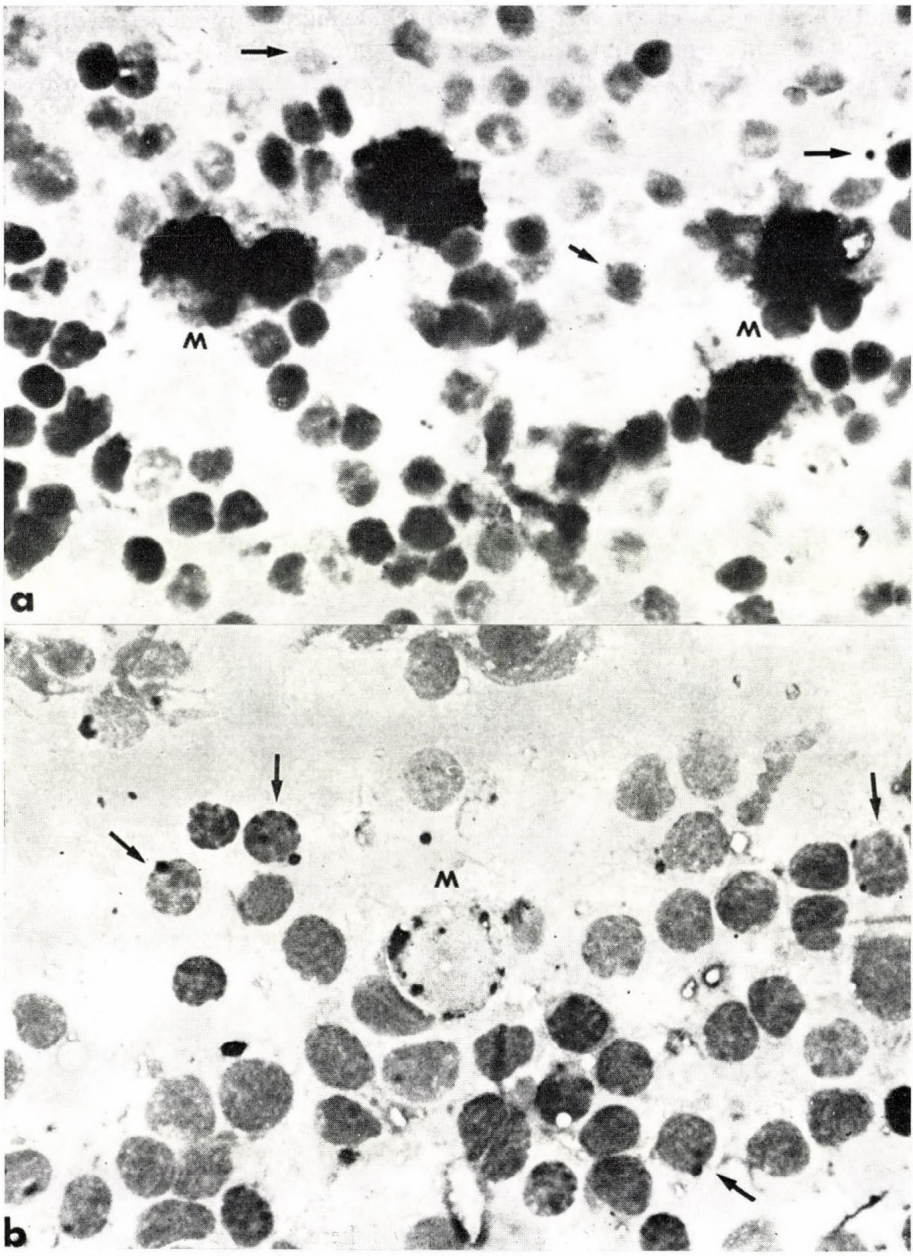


Fig. 2A. Acid phosphatase in a cervical lymph node smear of a sick SJL/J mouse. M — Macrophages with strong diffuse staining. (→) Small and medium lymphocytes with granular staining. ×800

Fig. 2B. β-D-glucuronidase in a thymus smear of a sick SJL/J mouse. M — Macrophage with granular-diffuse activity. (→) — Small and medium lymphocytes with granular staining. ×800

Meshorer [21] claims that the development of RCN-B in the SJL/J mice is directly related to the eosinophilia in the bone marrow. We think that the eosinophilia in the bone marrow of the sick SJL/J mice is not unique to this strain. It appears to be age-related, as in the C3H/eB mice, though the basic level is higher in the young SJL/J mice. The different eosinophil counts in the bone marrow of young mice of the two strains are probably inherited, whereas the eosinophilia in the aged mice is probably due to non-specific antigenic stimuli. The differential count of eosinophils in the peripheral blood is in accordance with the findings of Crispin [7] and Dunn [11]. A special phenomenon is the high plasma cell count in the bone marrow of sick SJL/J mice. The connection between this phenomenon and the development of the disease is not clear.

The representative RES organs, from the cytomorphological point of view, are the spleen and lymph nodes. There is a change towards the predominance of medium-sized lymphocytes and an increase in the number of hyperbasophilic cells, plasma cells and tissue mast cells. Moreover, there appear cells resembling the Reed–Sternberg cells. Thus, the cellular composition of the RES in the sick SJL/J mice resembles that in humans with Hodgkin disease [12, 24, 29]. It is important to note that such a cellular composition in man or in mice is not a proof of malignancy but could be a reaction to specific or non-specific inflammation. The cytological distinction between Hodgkin disease and reactive lymph nodes is based on the presence of Reed–Sternberg cells [24, 29]. Nevertheless, this is not pathognomonic of Hodgkin disease, because Reed–Sternberg-like cells are found in infectious mononucleosis too [12]. Therefore, in the RCN-B there is the same dilemma as in Hodgkin disease regarding the exact role of lymphocytes and reticulum cells and identification of the malignant cell. Della-Porta et al. [8] suggested that the term "composite lymphoma" be used instead of RCN-B, to indicate not only the polymorphic association of neoplastic and inflammatory cells but also the possibility of an association of different neoplastic elements.

We found an increase in the number of histiocytes in the spleen and thymus of the sick SJL/J mice, and the same was observed in the healthy old C3H/eB mice. It seems, therefore, that the increasing number of histiocytes in mice is either age-related or an expression of non-specific stimuli but not a consequence of the disease. It should be noted that there is no increment in histiocytes in lymph-nodes of patients with Hodgkin disease [12].

The thymus lymphocyte population was somewhat different from that of other RES organs. Ben-Yaakov and Haran-Ghera [3] showed that the involution of the SJL/J thymus stops at the age of 7–9 months, and that thereafter the thymus increases in weight due to re-population with B-lymphocytes. Cleason et al. [6] found that these B-lymphocytes originated in the bone marrow. This observation agrees with our observations of the considerable increase in hyperbasophilic cells in this organ. Dumont [10] claimed that the alterations in the lymphocyte constitution of the SJL/J thymus were related primarily to ageing and not to the development of the RCN-B.

The fact that there were no differences in extra-medullary haemopoiesis in

any of the groups of mice provides further support for the conclusion that RCN-B is not a haemopoietic disease but an RES disease, like Hodgkin disease in man [24, 25, 29]. Della-Porta et al. [8] too, did not find obvious involvement of peripheral blood or bone marrow even in animals with advanced disease. They suggested that the paraproteinaemia sometimes occurring in the disease of SJL/J mice [27] together with the cytological and ultrastructural findings justifies calling the tumour immunoblastic sarcoma with plasmoblastic differentiation.

In most of the studies on lymphocytes in Hodgkin disease the activity of the lysosomal enzymes acid phosphatase and β-glucuronidase was normal in peripheral blood [18, 23, 30, 31, 33], spleen and lymph nodes [16, 23, 33], irrespective of the histological stage of the disease. Some investigators, however, found a low acid phosphatase activity in the lymphocytes of lymph nodes [1] and peripheral blood [19]. The histioreticular cells in Hodgkin disease display a strong acid phosphatase activity compared to the activity in lymphocytes [24]. In our study only acid phosphatase activity was significant in the development of the disease in the SJL/J mice, namely there was an increase in the activity of this enzyme in lymphocytes and histiocytes. This finding agrees with the observations of other authors [4].

The findings regarding β-D-glucuronidase activity were different in that β-D-glucuronidase had disappeared from the RES of old C3H/eB mice. The reason for this is not quite clear, but the finding may partly support studies showing the absence of β-D-glucuronidase activity in mice [9, 26], and those studies may have been done on adult mice.

In conclusion, from the cytomorphological and cytochemical points of view, these spontaneous tumours bear a certain resemblance to Hodgkin disease in man. This was expressed in both the haemopoietic system and the RES. From the clinical point of view one may compare the findings in SJL/J mice to stage III–IV in Hodgkin disease. Yet, it is worthwhile emphasizing that we are dealing with inbred strains of mice, in which development of a disease might be solely genetic [17]. On the other hand, in man there are no data showing solely a genetic aetiology. Therefore, there is a possibility that immunological disturbances, which were not found to contribute to the development of the SJL/J disease, may play an additional role in the development of the disease in man [10, 14].

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Predictive Value of Lymphocytotoxic Test and Platelet Aggregometry for the Effect of Transfused Platelet Concentrates

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Sera of patients with thrombocytopenic diathesis before platelet transfusion were evaluated using donor's platelets in the aggregometry test and donor's lymphocytes in the lymphocytotoxicity test. In most cases the results of both tests were in agreement, although sometimes, despite strongly positive results of LCTT, the Agr was negative.

Both tests showed a similar value for the prediction of the effectiveness of platelet concentrates: Agr in 80% and LCTT in 71% of cases.

Introduction

The effect of transfusion of platelet concentrates in thrombocytopenic patients has unquestionably been demonstrated. In often transfused recipients a loss of effectiveness due to alloimmunization is, however, frequent. Resistant patients should be transfused with HLA-matched platelets [5, 7, 9, 11, 17, 18]. Sometimes it is not possible to find HLA-compatible donors, or the patients fail to respond to HLA-matched platelets. This requires techniques for cross-matching [3, 19, 20]. A number of tests are currently being studied by several authors. They are: aggregometry [19, 20] and lymphocytotoxicity [3] as well as indirect binding assays of alloantibody uptake by platelets with fluorescein- [1, 2], iodine- [12] or alkaline phosphatase- [6] labelled anti-immunoglobulin reagents. There are also tests with radioactive reagents [4, 8] and the recently described method with carboxyfluorescein diacetate [15]. The purpose of the present study was to assess the usefulness of the lymphocytotoxic test (LCTT) and platelet aggregometry (Agr) for predicting the effectiveness of transfusion of platelet concentrates.

Material and Methods

Thirty-eight patients with thrombocytopenia ($<20\,000/\mu l$) and haemorrhagic diathesis in the course of acute myeloplastic leukaemia (34 patients) or exacerbations of chronic myeloid leukaemia in blast crisis (4 patients) received 68 platelet concentrates from random donors matched for ABO and Rh antigens. In all pa-

tients the thrombocytopenia was due to chemotherapy. Platelet concentrates were prepared by the double centrifugation method or with Hemonetics 30 cell separator. The number of platelets in the concentrates was 1 to 4.5×10^{11}.

LCTT was performed by the international standard technique [10]. Screening of recipient sera was done in LCTT against a panel of lymphocytes. Before transfusion, the patient sera were cross-matched with donor lymphocytes in LCTT. The intensity of LCTT was evaluated in arbitrary units as follows: 0, less than 10% stained cells; 1, 10 to 30%; 2, 30 to 60%; 3, 60 to 90%; 4, more than 90% stained cells. Agr was performed during 8 min according to Wu [20] with a standard platelet aggregometer in which 0.2 ml of the recipient heat-inactivated serum (56 °C, 30 min) was added to 0.5 ml of donor platelet-rich plasma. The following scale of arbitrary units based on the extent of light transmission was used: 0, below 5%; 1, 5 to 15%; 2, 15 to 25%; 3, 25 to 50%, and 4, above 50%. The platelet concentrates selected for transfusions showed negative or weak positive reactions in the above-mentioned test. Only in life-threatening diathesis were such platelets transfused which gave a positive LCTT in spite of negative or weak positive Agr.

The effect of the transfused concentrates was evaluated by the 1 h and 20 h post-transfusion platelet increment and regression of decreasing intensity of haemorrhagic diathesis. Platelet counts were done by phase-contrast microscopy. In order to compare the response to transfusion of different recipients, the following formula was used to adjust the increment (Inc) to one unit given to a recipient with a square meter body surface area (BSA):

$$\text{corrected Inc} = \frac{\text{post-transfusion platelet count} - \text{pre-transfusion platelet count}}{\text{No. of platelets transfused} \times 10^{11}} \times \text{BSA}.$$

The effect was regarded to be good if after one hour the corrected Inc exceeded $10\,000/\mu l \times m^2/10^{11}$ platelets, and after 20 hours it exceeded $5000/\mu l \times m^2/10^{11}$ platelets [4, 19].

The usefulness of cross-matching was assessed on the basis of an agreement of the results of the tests with the corrected increment of platelets after transfusion. The results were considered false positive if a good recovery of the transfused platelets was observed despite a positive result of the cross-matches, and false negative if no good recovery was observed despite a negative result of the cross-matches.

Results

The results of LCTT and Agr were compared in 137 cross-matches (Fig. 1). In most cases the results were in agreement, especially the negative ones, but sometimes the Agr was negative in spite of a strongly positive result of the LCTT.

Aggregometry (№ of experiments)		Lymphocytotoxic test (№ of experiments) dead cells, per cent 0-15 negative	15-50 weak positive	50-100 positive	Total
light transmission, per cent 0-5 negative		88	10	9	107
5-25 weak positive		8	4	9	21
25-50 positive		0	0	9	9
Total		96	14	27	137

Fig. 1. Comparison of lymphocytotoxic test and platelet aggregometry. ///// Result of LCTT in agreement with Agr

In Table 1, the corrected results of the post-transfusion platelet increment, clinical improvement and post-transfusion reactions were summarized in the following groups of patients. Group I, 32 transfusions in patients who had no lymphocytotoxic alloantibodies, the LCTT cross-match was negative, and the Agr cross-match was not done; Group II, 18 transfusions in patients without detectable lymphocytotoxic alloantibodies and with negative cross-matches done by both LCTT and Agr; and Group III, 6 transfusions in patients with detectable lymphocytotoxic antibodies against panel lymphocytes, in whom the LCTT and Agr cross-matches were negative. No significant differences in the effectiveness of transfusions were observed in these groups.

Table 1
Post-transfusion platelet increments and clinical response in comparison to the results of lymphocytotoxic test and platelet aggregometry

Group of patients	No. of transfusions	Lymphocytotoxic alloantibodies	Cross-match		1 h corrected increment >10 000/μl	20 h corrected increment >5 000/μl	Clinical assessment			Mean post-transfusion corrected increment platelet count/μl ×m²/10¹¹	
			LCTT	Agr			improvement	without improvement	post transfusion reaction	1 h	20 h
					% of transfusions		% of transfusions			$\bar{X}\pm SE$	$\bar{X}\pm SE$
I	32	−	−	n.d.	70	49	3	82	0	14530 ±1450	7900 ±2040
II	18	−	−	−	100	61	6	66	1	18030 ±1920	10730 ±1980
III	6	+	−	−	83	50	17	50	0	16480 ±2850	7390 ±4370

Table 2
Post-transfusion platelet increment and clinical response in patients with positive cross-match

No.	Patient	Lymphocytotoxic alloantibodies	Cross-match: lymphocytotoxicity in arbitrary units (No. of donors: 1 2 3 4 5 6 7)	Cross-match: aggregometry in arbitrary units (No. of donors: 1 2 3 4 5 6 7)	Corrected increments pl. count/μl $\times m^2/10^{11}$ 1 h	Corrected increments pl. count/μl $\times m^2/10^{11}$ 20 h	Clinical improvement	Post-transfusion reaction
1	K. Z.	weak	2	0	19 800	13 200	+	+
2	K. Z.	weak	2	0	16 300	4 700	+	+
3	K. Z.	weak	1	0	26 000	14 900	+	+
4	R. G.	strong	3 0 0 0	0 0 0 0	5 900	3 000	+	−
5	C. A.	−	0 0 0 0	2 2 1 0	25 100	n.d.	?	−
6	M. J.	−	0 0 0 0	2 1 0 0	5 600	5 600	+	−
7	B. B.	weak	1 0 2 1	1 0 0 0	5 800	1 800	+	+
8	B. Z.	weak	2 1 0 0 0 0	1 0 0 0 0 0	16 900	14 300	±	−
9	B. W.	weak	2 1 1 0 0 0 0	1 0 0 0 0 0 0	12 800	12 100	?	−
10	M. A.	strong	[4 4] 4 4 4 4	[3 3] 1 0 0 0	11 800	8 700	−	−
11	L. M.	strong	4 4 3 1 0 0	2 2 1 1 0 0	12 200	n.d.	++	−
12	A. M.	strong	4 4	2 2	18 000	n.d.	+	+

In brackets: not transfused platelet concentrates.

In Table 2 the results are shown of 12 transfusions in patients who had received platelet concentrates despite positive results of the cross-matches with one or both methods.

In 9 patients the cross-match was weak positive in LCTT (Nos 1–4) or Agr (Nos 5 and 6) or in both these tests (Nos 7–9). In 6 patients the clinical effect of the transfusion was good, although in 3 cases the increase in platelet count was unsatisfactory. Four transfusions were followed by post-transfusion reactions with chills and fever; these patients had weak lymphocytotoxic antibodies.

In the 3 remaining cases (Nos 10–12) strong lymphocytotoxic antibodies were found against the panel lymphocytes and in the cross-match, but the Agr was not always positive. Because of life-threatening purpura, platelet concentrates were transfused, with the exception of those who showed the strongest aggregation with the recipient serum. In all these patients a good recovery of the platelets was observed, which was accompanied in 2 patients by a good clinical result.

Figure 2 shows the results of platelet concentrate transfusions in cases with negative and positive Agr and LCTT cross-matches done. The crosses on the diagrams correspond to cases in which the tests predicted incorrectly the result of the transfusions, which means that they were false negative or false positive. In Agr in 29 out of 36 cases (80%) the results of the transfusion was predicted correctly. In 3 cases out of the remaining 7, the results of Agr were false negative,

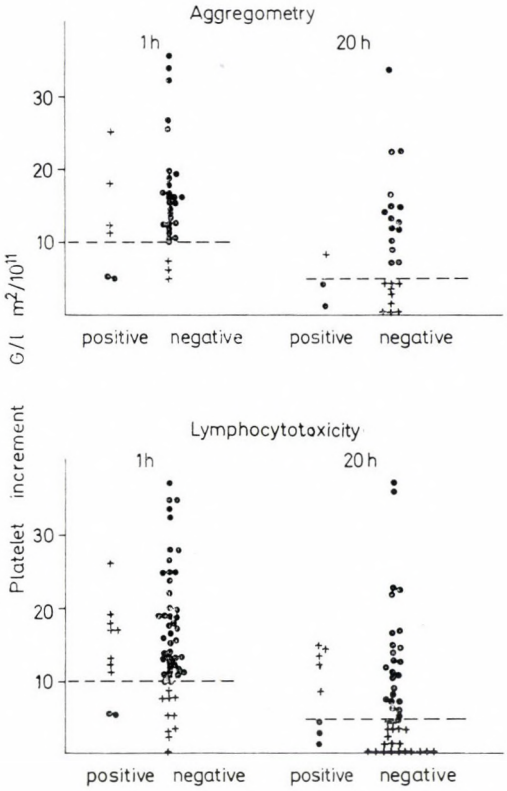

Fig. 2. Platelet increment 1 and 20 h after transfusion of platelet concentrates with negative or positive (incompatible in at least 25% of donors) cross-match. *Points:* Cases in which the tests predicted correctly the result of the transfusion; *Crosses:* Cases in which the tests predicted incorrectly the result of the transfusion; *Dashed line:* Limit of corrected platelet increment

and in 4 they were false positive. The LCTT predicted correctly the result of the transfusion in 47 out of 66 cases (71%); in 8 cases it was false positive, while in 10 cases it was false negative.

Discussion

The above-presented evaluation of LCTT and Agr applied for predicting the effectiveness of platelet concentrate transfusion showed a good usefulness of both tests but, sometimes, even a joint evaluation of both cross-matches could not guarantee the correct prediction of the result. Both tests were found to be useful in a similar proportion of cases, since with the Agr it was possible to predict cor-

rectly the outcome of the transfusion in 80%, while with LCTT this prediction was possible in 71% of the cases. The usefulness of LCTT was 73% according to Wu et al. [19] and 76% according to Filip et al. [3]. Also, the number of false negative and false positive results of LCTT in the experience of both these authors was similar as in our material. On the other hand, the usefulness of Agr according to Wu et al. [19] was slightly higher, 90%, and according to Filip et al. [3], slightly lower, 66%, than in our material. The percentage of false negative and false positive results in our material was similar to that of Wu et al. [19], while Filip et al. [3] obtained a greater proportion of false negative results but failed to observe any false positive ones.

A favourable effect of platelet transfusion was obtained in some cases with strong lymphocytotoxic antibodies, but with negative or weak positive Agr. This effect may have been due to the demonstration of antibodies in the LCTT against antigen absent or less strongly expressed on the transfused platelets than on the lymphocytes, for instance DR antigens or some A locus or B locus antigens of the HLA system [13, 16].

Interpretation of a positive Agr with negative LCTT is more difficult. It seems doubtful that Agr would demonstrate in such cases only antibodies against platelets, since the studies of Slichter [14] in dogs have demonstrated that antibodies against non-transplantation antigens develop after platelet transfusion much later than do the antibodies against HLA antigens.

An interesting fact is the more frequent occurrence of post-transfusion reactions in the group of patients with weak positive LCTT and negative Agr. On the other hand, no reactions were observed when the LCTT was strongly positive. This observation suggests that the post-transfusion reaction was caused by antibodies against non-HLA antigens, perhaps by antibodies against antigens specific for granulocytes.

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Platelet Function Abnormalities in Acute Leukaemia

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Studies on platelet function *in vitro* were done in fourteen patients with different kinds of acute leukaemia. Quantitative clot retraction was abnormal in all patients. Aggregation was different with the various agents tested. Availability of platelet factor 3 was abnormal in six patients. All patients were studied prior to chemotherapy. Two patients were studied in complete remission, as well; they showed an almost normal platelet function. Another patient without remission after induction treatment displayed a platelet function worse than in the initial study.

The results suggest that the defect in leukaemic platelets is due to the leukaemic process itself.

*

The haemorrhagic syndrome is a common complication in patients with acute leukaemia (AL) and it often contributes to the rapid fatal outcome of this disease. The severe thrombocytopenia is the primary abnormality responsible for the bleeding manifestations [13]. Qualitative platelet disorders, too, have been described [5, 8, 9, 16, 17] but only few studies have been devoted to this problem, perhaps as a consequence of the thrombocytopenia encountered in the disease.

A study of some qualitative platelet parameters has been done in 14 patients with various forms of AL.

Material and Methods

Fourteen patients with AL, 9 males and 5 females ranging between 3 and 89 years of age, were studied. Diagnosis and classification of leukaemias were based on the cell type and according to the standards of the French-American-British (FAB) Co-operative Group [1]. These included 4 patients with acute lymphoblastic leukaemia (ALL-L1), 5 with acute myeloblastic leukaemia without maturation (AML-M1), 2 with acute myeloblastic leukaemia with maturation (AML-M2), and 3 with myelomonocytic leukaemia (AML-M4). All patients were studied prior to chemotherapy. A patient with ALL-L1 and a patient with AML-L1 were studied also in complete remission and another with AML-M4 was studied after induction treatment but without remission. ALL induction chemotherapy regimen consisted of intravenous vincristine (1.5 mg/m^2/dose), and

daunorubicin (1 mg/kg/dose) weekly during four weeks, with prednisone (40 mg/m^2/day) orally for a month. AML induction chemotherapy regimen consisted of a series of six-day courses of vincristine (1.5 mg/m^2/dose) on the first and fourth days, and daunorubicin (1 mg/kg/day) and cytosine arabinoside (2 mg/kg/day) given intravenously for six days.

The results were compared with those from 12 normal controls.

Blood samples

Samples ranging from 40 to 100 ml of blood, depending on the initial platelet count, were drawn with 3.8% sodium citrate as anticoagulant (ratio 9 : 1). Immediately after venipuncture the samples were centrifuged at 1000 r.p.m. for 5 min. The separated plasma was centrifuged again at 3000 r.p.m. for 10 min. Platelet-rich plasma (PRP) was prepared, resuspending the platelets in the necessary volume of plasma in order to give a concentration of 200×10^9/l. Platelet-poor plasma (PPP) was obtained by centrifuging blood at 4000 r.p.m. for 30 min. The PRP and PPP of patients and controls were processed in the same way. Platelets from 2 patients and one control were washed three times with 0.9% saline solution in order to eliminate the plasma components [10]. Patient platelets were resuspended in normal plasma, and normal platelets were resuspended in a patient's plasma, following a brief incubation at room temperature for 10 min, and aggregation was measured again.

In all determinations, plastic or siliconized material was used.

Platelet function

Bleeding time was measured as described by Ivy [15]. Platelet counts in peripheral venous blood were done by phase microscopy [3]. Quantitative clot retraction was estimated by Breddin's method [4] using PRP at concentrations of 200×10^9/l. Availability of platelet factor 3 (APF-3) was estimated by Hardisty's test [14]. Platelet aggregation was studied using a single channel aggregometer at 37 °C attached to a recorder according to the method of Born [2] and O'Brien [18]. As aggregating agents we used adenosine diphosphate (ADP) (Sigma Chemicals) in final concentration of 8 μg/ml plasma; epinephrine in final concentration of 1.85 μg/ml plasma; ristocetin (Lundbeck) in final concentration of 2 mg/ml plasma, and collagen. Collagen suspension was prepared from 100 mg of desiccated bovine tendon (Sigma Chemicals) with 5 ml of 0.9% saline solution, and diluted to 1 : 50 final concentration. A volume of 0.45 ml of PRP was placed in the cuvette and 0.05 ml of the different aggregating agents was added in each case. The percentage of aggregation was calculated by the formula,

$$\frac{\text{initial OD} - \text{final OD}}{\text{initial OD}} \times 100 \quad (\text{OD} = \text{optical density}).$$

Table 1
Qualitative platelet tests in fourteen patients with AL

Patient	Diagnosis	Age-Sex (years)	Platelet* count (10^9/L)	B. T. (min)	C. R.* (per cent)	APF-3	Aggregation (per cent)				B. D.
							ADP***	Epi-nephrine**	Col-lagen*	Ristocetin	
1	ALL-L1	19-M	60	3	56	A	71	25	20	75	Yes
2	ALL-L1	9-M	140	1.30	40	A	75	75	75	65	Yes
3	ALL-L1	3-M	30	5	60	A	48	25	0	80	No
4	ALL-L1	19-M	30	3	80	N	80	50	0	88	No
5	AML-M1	72-M	50	2.30	36	A	28	21	45	80	No
6	AML-M1	44-F	14	8	36	N	52	31	28	39	Yes
7	AML-M1	45-M	46	3	60	A	57	68	13	77	No
8	AML-M1	44-F	60	15	20	N	56	46	41	75	Yes
9	AML-M1	46-M	20	3	35	N	80	31	28	80	No
10	AML-M2	46-M	40	8	60	N	62	45	25	71	Yes
11	AML-M2	51-F	26	10	36	N	76	26	26	78	Yes
12	AML-M4	39-M	60	5	80	N	84	81	34	89	No
13	AML-M4	89-F	18	3	40	N	82	0	28	80	Yes
14	AML-M4	44-F	40	5	50	A	40	34	0	47	Yes
	Controls		210±50	3±2	90±5	N	76±9	65±15	71±14	86±16	

* $p < 0.001$
** $p < 0.01$
*** $p < 0.05$
B. T. = Bleeding time
B. D. = Bleeding diathesis
C. R. = Clot retraction

Other tests

Prothrombin time [20] and activated partial thromboplastic time [19] were measured in all patients.

Statistical analysis

Conventional methods were used for the calculation of the arithmetic mean and standard deviation. The significance of differences between mean values was tested by Student's *t* test.

Results

Thrombocytopenia was associated with a considerably abnormal platelet function. All patients showed deficient clot retraction ($p < 0.001$). APF-3 was abnormal in 6 cases and bleeding time was prolonged in 4 patients but without statistical differences from the controls (Table 1).

Aggregation was different with the various agents tested (Table 1). Thirteen patients demonstrated abnormal collagen-induced aggregation ($p < 0.001$) while 10 had impaired aggregation with epinephrine ($p < 0.01$). Thus, with 4 exceptions, an abnormal aggregation response to collagen was always associated with abnormal epinephrine-induced aggregation. Abnormal aggregation with collagen was shown by prolonged lag phase in 4 patients, while in all cases the percentage of aggregation was impaired, and both the first and second waves were diminished or missing (Fig. 1). Aggregation with ADP was impaired in 7 of the 14 patients (Table 2) ($p < 0.05$), and 3 cases showed a slightly but not significantly decreased ristocetin-induced aggregation. The 2 patients (1 and 9) in complete remission showed improvement in their platelet function. The case studied after induction treatment without complete remission (patient 12) showed more alterations than in the initial study (Table 3, Figs 2, 3).

The results of aggregation of washed platelets from a control and patients 4 and 6, were similar to the initial study (Table 4).

We found a good correlation between bleeding diathesis and abnormalities in bleeding time, clot retraction and aggregation induced by collagen and epinephrine, but not with the other tests performed.

Prothrombin time and activated partial thromboplastic time were normal in all patients.

Discussion

The present results show that in patients with AL the platelet function has one or more defects.

Four patients had prolonged bleeding time with different abnormal qualitative platelet tests, but 10 with normal bleeding time also had some platelet dis-

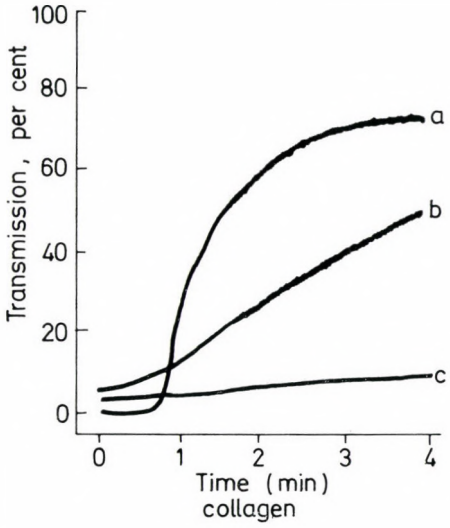

Fig. 1. Collagen-induced aggregation showed diminished or missing aggregation in two patients. a = control; b = patient 5; c = patient 4

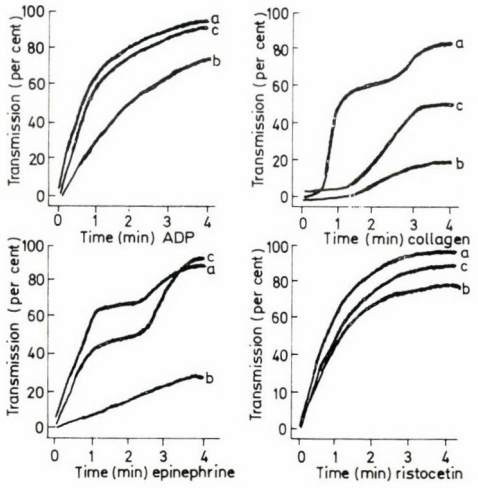

Fig. 2. Patient 1: Comparative study before and after treatment in complete remission. a = control; b = before treatment; c = after treatment

function. Clot retraction was deficient in all patients, therefore, under our conditions it was the best method to characterize platelet disfunction. Platelet aggregation by different aggregating agents was impaired in all patients. Both collagen and epinephrine act on the platelet surface "receptors" releasing ADP which initiates

Table 2

ADP, epinephrine, collagen and ristocetin induced aggregation

Patient	Diagnosis	ADP***	Epinephrine**	Collagen*	Ristocetin
		(per cent)			
1	ALL-L1	71	25	20	75
2	ALL-L1	75	75	75	65
3	ALL-L1	48	25	0	80
4	ALL-L1	80	50	0	88
5	AML-M1	28	21	45	80
6	AML-M1	52	31	28	39
7	AML-M1	57	68	13	77
8	AML-M1	56	46	41	75
9	AML-M1	80	31	28	80
10	AML-M2	62	45	25	71
11	AML-M2	76	26	26	78
12	AML-M4	84	81	34	89
13	AML-M4	82	0	28	80
14	AML-M4	40	34	0	47
	Controls	76±9	65±15	71±14	86±16

* $p < 0.001$
** $p < 0.01$
*** $p < 0.05$

Table 3

Comparative study before and after induction treatment

Patient	Diagnosis	Aggregation				B. T. (min)	C. R. (per cent)	APF-3
		ADP	Epinephrine	Collagen	Ristocetin			
		(per cent)						
1	ALL-L1							
	Initial study	71	25	20	75	3	56	A
	Complete remission	88	87	48	83	5	80	A
9	AML-M1							
	Initial study	80	31	28	80	3	35	N
	Complete remission	80	76	52	80	3	80	N
12	AML-M4							
	Initial study	84	81	34	89	5	80	N
	No remission	67	44	0	71	2.30	95	N

B. T. = bleeding time.
C. R. = clot retraction.
APF-3 = availability of platelet factor 3.

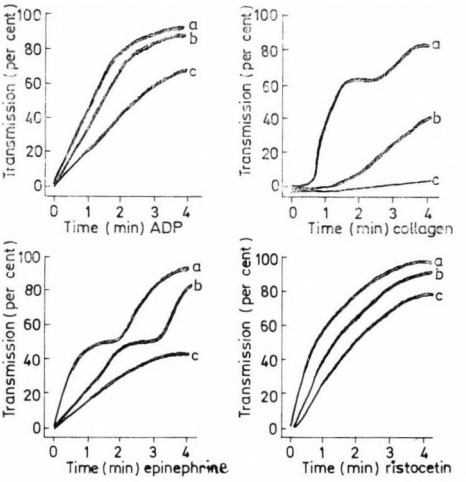

Fig. 3. Patient 12: Comparative study before and after induction treatment without remission. a = control; b = before treatment; c = after treatment

aggregation [6, 11, 12]. The defective aggregation of leukaemic platelets with collagen and epinephrine may be considered an indication of receptor abnormality in the release mechanism or may be due to a lack of storage pool nucleotides. All these types of defects have been reported in leukaemic platelets [5, 8, 9, 11] and any of them could explain the results obtained in our patients. Aggregation

Table 4

Aggregation platelet study with washed platelets

Patient		Aggregation			
		ADP	Epinephrine	Collagen	Ristocetin
		(per cent)			
4 (ALL-L1)	Control	88	85	75	80
	Patient (initial study)	80	50	0	88
	Patient's platelets + Control's plasma	80	52	6	88
	Control's platelets + Patient's plasma	93	85	72	88
7 (AML-M1)	Control	88	85	75	80
	Patient (initial study)	52	31	28	80
	Patient's platelets + Control's plasma	60	32	32	35
	Control's platelets + Patient's plasma	82	85	73	78

with ADP was impaired in 7 cases. This abnormality has also been reported [8, 9]. Three patients had slightly impaired ristocetin-induced aggregation, but it was not possible to study the von Willebrand factor and other elements of platelet aggregation with that agent [7, 16, 21].

Aggregation of washed platelets from one control and two patients suggests that the anomaly observed in our patients is due to an intrinsic defect of leukaemic platelets and not to secondary plasmatic phenomena.

The 2 patients studied in complete remission had almost normal platelet function, and one patient without remission after induction treatment had a worse platelet function than in the initial study. These results also suggest that the defect in leukaemic platelets may be due to the leukaemic process itself and that it improves when complete remission is obtained with cytostatics, but further data are needed to confirm these results.

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The Haemostatic System Components of Neointima Forming in Polyester Aortic Grafts in the Early Postoperative Period

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The aim of the studies was to determine the content of certain components of the haemostatic system in the neointima of a polyester graft implanted in the dog abdominal aorta over a 7-day period of observation.

The neointima of an aortic polyester graft in the early postoperative period possesses a high thromboplastic activity, greater than that of the artery or vein, and containing prothrombin, active thrombin, platelet aggregating substances and heparin neutralizing substances. The plasminogen activator activity in the neointima of the polyester graft is also considerably higher than in the intima of arteries and veins.

Introduction

Studies of the biochemical changes taking place in the luminal surface of prostheses and the chemical substances and enzymes contained in the material accumulated there, are essential in order to elucidate the mechanisms of the sealing of graft interstices, the formation of neointima, healing of the prosthesis and maintenance of its patency.

Whether or not the graft is successful depends to a great extent on the course of these processes during the first few hours and even minutes after implantation [2, 24]. During this immediate post-implantation period, the components of the haemostatic system play a dominant role. Studies of the subject are, however, scarce and fragmentary [18, 20].

The aim of the present study was to determine the content of certain components of the blood clotting system, blood platelet aggregating substances and the plasminogen activator in the neointima of polyester grafts in the early postoperative period.

Materials and Methods

The experiments were carried out on 18 male dogs, weighing 22–26 kg, aged 2–4 years. A section of the abdominal aorta of each dog was excised between the renal arteries and the aortal bifurcation. The excised section was

then replaced by end-to-end implantation of a polyester prosthesis made of Dallon (knitted artificial vessels of ethylene polyteraphthalate fibre produced by the Research and Development Centre of the Knitting Industry, Łódź, Poland). The inside diameter of the prosthesis was 8 mm and its length 8 cm. No drugs affecting the haemostatic blood system were administered before, during, or after the operation. The dogs were divided into 3 experimental groups of 6 animals each. The polyester grafts and sections of the normal aorta above the graft and abdominal sections of the inferior vena cava were excised from each dog, in group I one hour, in group II, 24 hours, and in group III, one week after implantation. These vascular sections were cut open longitudinally, washed out with icy 0.9% solution of NaCl and thoroughly dried with adsorbent gauze. The intima was then dissected from the aorta and vein and the intimal layer closely adhering to the prosthesis was scraped off with a metal curette. To the material thus obtained 0.9% NaCl solution was added in a ratio of 1 : 9 (w/v) after which the material was homogenized in a Potter homogenizer. The homogenate was centrifuged at 3000 *g* for 30 minutes. The composition of the supernatant was established by determining the total protein content by the method of Lowry et al. [13]. The supernatant was used for most of the tests. Only for determination of thromboplastic activity was used the supernatant from which prothrombin had been removed by adsorption on $BaSO_4$ [10]. The substrate plasmas used for determination of the various clotting system and fibrinolysis components in the homogenates were platelet-poor citrated canine plasma, prothrombin-free (by adsorption on $BaSO_4$) canine plasma and platelet-poor citrated human plasma. The thromboplastin source was a suspension of rabbit brain produced by the Warsaw Serum and Vaccine Laboratory.

The thromboplastic activity of the homogenates was determined by measuring the clotting time in a test system composed of 0.2 ml citrated canine plasma and a 0.2 ml mixture consisting of equal volumes of prothrombin-free homogenate and 0.05 M $CaCl_2$. The prothrombin content of the homogenates was determined by measuring the clotting time in a test system of 0.2 ml of prothrombin-free canine plasma after the addition of 0.1 ml homogenate and 0.2 ml of a mixture containing equal volumes of thromboplastin from rabbit brain and 0.05 M $CaCl_2$. Thrombin activity in the homogenates was determined by measurement of clotting time of 0.2 ml prothrombin-free canine plasma under the influence of 0.2 ml of the homogenate. Blood platelet aggregation was tested by the method of Niewiarowski and Thomas [15] in a test system consisting of 2 volumes of platelet-rich plasma containing 250 000 mm^3 platelets and 1 volume of intima homogenate. Antiheparin activity was determined by the Popławski and Niewiarowski method [16].

Plasminogen activator activity in the homogenates was determined by the euglobulin test in which the fibrinogen and plasminogen source was citrated human plasma [7]. To 0.5 ml of the homogenate, 10 ml of icy distilled water and 0.5 ml citrated human plasma were added. After acidification with 1% acetic acid to pH 5.3, the mixture was left for 12 hours at a temperature of 4 °C. The

precipitate separated by centrifugation was dissolved in 0.5 ml Palitsch borate buffer solution pH 7.6 clotted by adding 0.5 ml of 0.05 M $CaCl_2$ containing 1 u/ml thrombin. The fibrinolysis time of the clot formed was determined in a water bath of 37 °C. All control measurements were carried out in the same way, with the only difference that instead of the homogenate, an equal volume of 0.9% NaCl solution was added in each test.

For statistical calculations, analysis of variance and Duncan's test were applied.

Results

The neointima of aortal polyester grafts one hour and one day after the implantation displayed a high thromboplastic activity being 6 times greater than that of the arteries and 4 times greater than the thromboplastic activity of the veins, and on the seventh day, the activity was 9 and 6 times greater, respectively (Table 1). The graft neointima contained prothrombin and on the first and seventh days of the experiment also active thrombin, thus components not present in the arterial and venous intima. On the first and seventh days of the experiment the graft neointima homogenate aggregated blood platelets. Antiheparin activity was higher in the polyester graft than in the artery and considerably higher than in the vein. The plasminogen activator occurred in the graft neointima as early as 1 hour after the implantation. The activity of this activator rose rapidly, reaching one day after the beginning of the experiment, an activity 4 times greater than that of the artery and 2 times greater than that of the vein. One week after implantation the plasminogen activator content fell. The total protein content of the graft neointima was twice as high as that of the arterial and venous intima.

Discussion

The components of the haemostatic system comprise the greater portion of the material covering the luminal surface of the prosthesis directly after its implantation and the release of blood flow. They also play an important role in the formation of the neointima. Our results show that thromboplastins, prothrombin, thrombin, heparin neutralizing substances and plasminogen activators are present in the material.

A transmission and scanning electron microscopic study showed that the formed neointimal layer contained fibrin, blood platelets, erythrocytes and small numbers of leukocytes [25].

Studies *in vitro* showed that the synthetic fibres form an active surface on which blood plasma clotting factors become activated [2, 5] and adhesion of blood platelets occurs [2, 6], accompanied by the release of platelet clotting factors 3 [23] and 4 [6]. The surface of the activated platelets provides the site

Table 1

Haemostatic components contained in intima of polyester graft, artery, and vein

Test	Polyester graft			Artery	Vein	Control
	1 hour	1 day	1 week			
Thromboplastin activity (s)	30.3* (27–33)	34.0* (32–36)	19.8* (18–21)	176.7 (170–190)	125.0 (120–135)	210 (200–220)
Prothrombin content (s)	95.7* (54–170)	31.8* (23–45)	26.8* (21–35)	>600	>600	>600
Thrombin activity (s)	>600	55* (51–62)	55* (47–70)	>600	>600	>600
Platelet aggregation time (s)	>600	36.8* (34–40)	37.0* (30–50)	>600	>600	>600
Antiheparin activity (s)	24.7* (23–27)	17.2* (16–18)	20.3* (19–22)	32.0 (27–35)	57.0 (52–62)	60 (48–72)
Plasminogen activator activity (min)	178.3* (130–240)	72.5* (60–90)	108.3* (90–130)	287.5 (280–295)	142.0 (125–170)	230 (220–240)
Protein (mg/ml)	2.52* (2.00–3.00)	2.15* (1.75–2.90)	2.43* (2.20–2.60)	1.23 (0.95–1.50)	1.02 (0.70–1.30)	0

* Statistically significant differences as compared with artery and vein ($P < 0.001$).

for adsorption of prothrombin and other plasma clotting factors. As a consequence, active thrombin appears and fibrin is formed on the luminal surface of the prosthesis after its implantation and the release of blood flow. Studies *in vitro* seem to indicate that the thrombin formed becomes adsorbed to the surface of the blood platelets [21], to the fibrin clot [12] and to the surface of the polyester graft [2]. The simultaneous appearance of thrombin and the induction of platelet aggregation by the homogenate of the graft neointima indicates that thrombin is responsible for the process of aggregation. The binding of plasminogen activators to the fibrin clot [22] demonstrated *in vitro* indicates that the activators in the graft may originate from the blood plasma.

Another source of active substances in the haemostatic process which takes place in the layer coating the polyester graft during the early post-operative period is provided by leukocytes and erythrocytes which adhere to the fibres of the prosthesis [2] and become enclosed in the fibrin clot. These cells contain thromboplastins, plasminogen activators and heparin neutralizing substances [4, 11, 14, 17].

The blood clotting factors activated on the luminal surface of the prosthesis may play a role in the intravascular activation of the coagulation process, which may provoke thrombus formation and even induce disseminated intravascular coagulation [9]. Despite the presence of active clotting factors in the neointima and the existence of a hyper-coagulative state of the plasma clotting factors [8], activation of the coagulation process is not propagated and the patency of the graft is preserved if the natural control mechanisms of the blood coagulation system are functioning normally. Such control mechanisms include inhibitors of blood coagulation [1] and the reticuloendothelial system [19] which enables the organism to eliminate the activated products and factors. An important role in the maintenance of graft patency is played by local conditions, above all the blood flow rate [3], and the plasminogen activators contained in the graft neointima [7, 18, 20].

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Lycurim (R-74) in the Therapy of Chronic Lymphocytic Leukaemia

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Lycurim was used in the therapy of 41 CLL patients, in 34 a positive clinical effect was found. The effect was marked in the cases of CLL associated with autoimmune haemolytic anaemia.

The immunodepressive property of Lycurim was confirmed by immunological investigation. It has been shown that the levels of IgG, IgA and IgM fall after Lycurim treatment of CLL patients. At the same time the T-lymphocyte count decreases and a tendency for EAC rosette-forming B-lymphocyte decrease is marked.

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The results of modern therapy of chronic lymphocytic leukaemia (CLL) cannot be regarded as satisfactory, in spite of effective cytostatics for treating such cases (chlorambucil, cyclophosphamide, mannomustine, etc.). Thus the clinical study of new drugs creates definite possibilities in the perfection of CLL therapy.

One such drug is Lycurim (R-74), a 1,4-di(2-methyl-sulphonyloxy-ethyl-amino)-1,4-didesoxyerythrite-dimethyl-sulphonate, synthesized in 1962 in Hungary by T. Horváth and L. Vargha.

The clinical effect of Lycurim in the cases of malignant tumours was studied by Petrányi [3], Sellei [5], Polubiec et al. [6]. Borisov et al. [1] published the results of a cooperative study of Lycurim and found a high degree of its effectiveness on patients suffering from CLL, malignant lymphomas as well as in certain localizations of cancer.

Petrányi and Szobor [4] described a marked immunodepressive action of Lycurim which resulted in its successful application in such diseases as myasthenia gravis, dermatomyositis, etc. An experimental study of the immunodepressive property of Lycurim showed an influence of the drug on cellular immunity, whereas humoral immunity was less depressed [2].

The present paper describes a clinical and haematologic study of the effect of Lycurim in cases of CLL and of its influence on cellular and humoral immunity.

Patients and Methods

Lycurim was administrated to 41 patients with CLL (26 males, 15 females) from 39 to 72 years of age; 19 of the patients had autoimmune haemolytic anaemia (AIHA).

The patients were subjected to the usual clinical and haematologic examinations including peripheral blood counts and a morphological study of the bone marrow. The following studies were performed to determine the effect of Lycurim on the immune status of the CLL patients:

– the level of immunoglobulin fractions by electrophoresis in agar gel;

– the concentration of the serum immunoglobulins G, A, M and D by radial immunodiffusion [7] using Sevac sets (CSSR);

– the quantity of EAC-rosette-forming lymphocytes, characterizing the level of B-lymphocytes in peripheral blood;

– the number of E-rosette-forming T-lymphocytes.

Lymphocytes for E- and EAC-rosette formations were isolated on Ficoll-Verografin gradient at 400 *g*. Lymphocytes were washed three times in Hank's balanced salt solution. The E-rosette-forming reaction was performed by incubation for 5 min at 37 °C with sheep red cells (SRC); at the centrifugation the incubation was extended for one hour at 20 °C.

To obtain EAC-rosettes, the lymphocytes were incubated for one hour with SRC, sensitized with rabbit IgM antisheep antibodies and a mouse complement at 37 °C, with centrifugation 5 min after the beginning of the incubation period. Then, in both cases the pellet was resuspended, fixed with 0.6% glutaraldehyde and smears were prepared for calculating the percentage of rosette-forming cells [8].

The control group included 30 healthy persons of middle age.

Clinical and haematological characteristics of CLL patients

The patients were divided into the following groups, according to the duration of the disease:

duration up to one year	10 patients
1–3 years	16 patients
3–6 years	12 patients
6–9 years	3 patients

In 10 patients, Lycurim was the first cytostatic drug. The other 31 patients had received chlorambucil, cyclophosphamide, mannomustine, X-ray therapy, or other cytostatics before Lycurim.

All patients had moderate symptoms: feebleness, subfebrility, perspiration, weight loss, anorexia. The majority exhibited enlargement of the lymph nodes, spleen or liver. Red blood cell count in 10 patients was normal; 31 were anaemic, of which 19 had autoimmune haemolytic anaemia (AIHA), 12 had reduced haemoglobin concentration and erythrocyte count.

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The haemoglobin level varied from 21 g/l to 100 g/l, erythrocyte count from 0.6×10^{12}/l to 3.0×10^{12}/l.

Most patients evidenced marked leukocytosis and lymphocytosis.

The leukocyte count in 12 patients was 246.0×10^9/l – 743.0×10^9/l; in 13 patients, 103.0×10^9/l – 200.0×10^9/l; and in 14 patients, 26.0×10^9/l – 92.0×10^9/l.

Two patients with a low leukocyte count (5.0×10^9/l – 6.0×10^9/l) were also treated with Lycurim.

The thrombocyte count before taking Lycurim was normal in 13 patients; 10 patients had an initial thrombocytopenia (from 55.0×10^9/l to 94.0×10^9/l); and in 18 patients the thrombocyte count varied from single ones to 50.0×10^9/l.

Lycurim dosage

Lycurim was given intravenously in doses of 30–60 mg daily in courses of 120 to 1470 mg, depending on individual sensitivity.

In the majority of cases with anaemia, thrombocytopenia or fever, the patients received corticosteroids simultaneously. It was impossible to stop corticosteroid treatment since the patients had received hormonal therapy before Lycurim. During Lycurim therapy the dose of corticosteroids was decreased.

Results

Immediate effect. Twenty-five patients showed marked improvement directly after treatment, displaying a normal temperature, decrease of perspiration, improvement of appetite and increase of weight. Enlargement of the lymph nodes decreased or disappeared altogether, the liver and spleen became smaller and in some patients impalpable.

The blood indices improved. The haemoglobin level became normal, from 80.7 ± 5.4 g/l to 112.7 ± 3.5 g/l ($p < 0.001$), the erythrocyte content changed from $2.59 \pm 0.21 \times 10^{12}$/l to $3.5 \pm 0.15 \times 10^{12}$/l ($p < 0.05$). The leukocyte count decreased and the leukocyte formula improved, the granulocyte rate increased to 20–40%. The thrombocyte number also increased, from $53.5 \pm 9.4 \times 10^9$/l to $116.7 \pm 15.6 \times 10^9$/l ($p < 0.001$).

The above-mentioned effect was attained in 14 patients treated with 420–1380 mg of Lycurim. The rest showed a marked improvement after smaller doses of the drug.

The most striking effect was seen in leukocyte number. The leukocyte count of 14 patients decreased from $282.1 \pm 58.2 \times 10^9$/l to $60.5 \pm 18.7 \times 10^9$/l ($p < 0.05$) after Lycurim treatment. Those who received smaller doses showed a decrease of the leukocyte count from $75.0 \pm 16.8 \times 10^9$/l to $23.2 \pm 8.7 \times 10^9$/l ($p < 0.05$).

The effect was less pronounced in 9 CLL patients. All of them, however, showed general improvement, normal temperature and a 25 to 50% decrease of

the enlarged peripheral lymph nodes, liver and spleen. The peripheral blood indices improved somewhat, the haemoglobin level rose from 88.2 ± 7.6 g/l to $103.7 \pm \pm 5.3$ g/l ($p > 0.05$); the erythrocyte count from $2.74 \pm 0.36 \times 10^{12}$/l to $3.34 \pm \pm 0.38 \times 10^{12}$/l ($p > 0.05$). The leukocyte count decreased to $66.1 \pm 13.9 \times 10^9$ from $149.4 \pm 33.1 \times 10^9$/l ($p > 0.05$).

Six of the patients showed no change. It is necessary to mention that in two patients treatment was begun at a low initial leukocyte count, and a small dose (210–240 mg) of the cytostatic resulted in marked leukopenia (1.8×10^9/l). Therefore, treatment with Lycurim of these patients was immediately stopped. The size of the lymph nodes, liver and spleen was unchanged.

The remaining 4 patients received a sufficient dosage of Lycurim (630–1470 mg) but showed no clinical effect. Leukocyte number rose from $151.1 \pm 39.8 \times \times 10^9$/l to $188.3 \pm 68.1 \times 10^9$/l ($p > 0.05$), and the disease progressed markedly. One of these patients died, the cause was a progressive haemorrhagic syndrome.

An initial resistance to Lycurim was noted in one patient who had not previously received any cytostatic therapy. The rest of this group might have had a crossed resistance because of earlier treatment with different chemotherapeutics.

Special attention should be paid to the fact that an initial thrombocytopenia was not considered to counterindicate Lycurim treatment. 15 out of 23 patients with marked thrombocytopenia showed after Lycurim treatment a significant increase of the thrombocyte count (from $42.1 \pm 5.5 \times 10^9$/l to $126.67 \pm 16.99 \times \times 10^9$/l; $p < 0.001$). Four patients showed no change in the thrombocyte count, while the remaining 4 showed a continuous decrease in the count during treatment (from 22.0×10^9/l – 94.0×10^9/l to 6.0×10^9/l – 66.0×10^9/l).

Period of somatic compensation after treatment. This constituted a period of 7–14 months for 16 patients, 4–6 months for 7 patients, 1–3 months for 5 patients. Three CLL patients after Lycurim treatment underwent splenectomy which prevented the evaluation of the after-effects. Four patients treated with Lycurim did not report for haematologic control.

Lycurim in the therapy of CLL with immune complications. Taking into consideration the immuno-depressive action of Lycurim it seemed advisable to use it in patients suffering from CLL with immune complications. Thirteen out of 19 patients with symptoms of AIHA showed a marked clinical effect after Lycurim treatment, with disappearance of the haemolysis and improvement of the red blood cell indices, i.e. a normalization of the bilirubin and reticulocyte levels, increase of the haemoglobin level (from 65.8 ± 6.6 g/l to 115.4 ± 3.6 g/l; $p < 0.001$) and erythrocyte count (from $2.02 \pm 0.27 \times 10^{12}$/l to $3.67 \pm 0.13 \times \times 10^{12}$/l; $p < 0.001$).

A less pronounced effect was marked in 3 CLL patients with AIHA. In these, Lycurim caused a slight improvement of the haemolytic symptoms and of red blood cell indices, i.e. the haemoglobin level rose from 40 – 80 g/l to 65 – 93 g/l; the erythrocyte count from 1.3×10^{12}/l – 2.2×10^{12}/l to 2.1×10^{12}/l – 2.9×10^{12}/l. Two patients in this group displayed neither clinical nor haematologic improvement after Lycurim.

Lycurim action on the immune status in CLL. Thirty-one CLL patients were studied for immune status before and directly after Lycurim treatment. The results are shown in Table 1. As can be seen, CLL patients showed marked changes in the immunological indices. The results of agar gel electrophoresis showed a definite decrease of the β_2 and γ-globulin fractions in both cases (to 2.3 ± 0.2 g/l and 12.5 ± 1.3 g/l respectively).

Table 1

Effect of Lycurim on immunological status of CLL patients

		Immunoglobulins (g/l)			
		IgG	IgA	IgM	IgD
Healthy persons		13.90±0.64	3.82±0.19	1.18±0.06	0.05±0.02
Pre-treatment		11.75±1.51	2.35±0.39	0.90±0.17	0.05±0.02
	p_1	>0.05	<0.05	>0.05	>0.05
Post-treatment		7.12±1.30	1.26±0.36	0.41±0.12	0.02±0.01
	p_1	<0.05	<0.05	<0.05	>0.05
	p_2	<0.05	<0.05	<0.05	>0.05

		Immunoglobulin fractions (g/l)		EAC rosette forming lymphocytes (%)	E rosette forming lymphocytes (%)
		β_2	γ		
Healthy persons		2.8±0.1	15.3±0.6	26.6±2.7	53.0±2.1
Pre-treatment		2.3±0.2	12.5±1.3	48.5±2.8	14.3±2.1
	p_1	<0.05	<0.05	<0.05	<0.05
Post-treatment		2.2±0.5	8.8±1.3	37.8±5.2	7.7±2.4
	p_1	>0.05	<0.05	>0.05	<0.05
	p_2	>0.05	<0.05	>0.05	<0.05

p_1: statistical comparison with normal data of healthy persons.
p_2: statistical comparison with data before treatment.

Less marked are the changes in the various classes of immunoglobulins. Thus, the average IgG level does not change. Detailed studies of the character of the distribution of IgG concentration in CLL makes it, however, possible to divide the patients into two groups, those with a lowered or normal level of IgG and those with a higher level. The IgA content of serum definitely decreased to 2.35 ± ± 0.39 g/l ($p < 0.05$), whereas the decrease in IgM was insignificant ($p > 0.05$).

CLL patients show a definite increase of the B-lymphocyte content which have surface markers for Ig and C3 (EAC-cells), to 48.5 ± 2.7% ($p < 0.05$). This increase occurs as a result of an accumulation in peripheral blood of the leukaemic

B-lymphocyte population of monoclonal origin. Simultaneously, the content of E-rosette-forming T-lymphocytes decreased to 14.3 ± 2.1% ($p < 0.05$), although the absolute T-lymphocyte count was unchanged.

After Lycurim treatment, a significant alteration of the immunological indices occurs in the blood of CLL patients.

The β_2-globulin fraction content did not change while the γ-globulin level fell below the normal range (8.8 ± 1.3 g/l; $p < 0.05$). Further, the IgG level showed a definite fall after Lycurim therapy to 7.12 ± 1.30 g/l, a value considerably lower than the normal one. In the same way changed the other serum immunoglobulin levels, IgA and IgM fell to 1.26 ± 0.36 g/l and 0.41 ± 0.12 g/l, respectively and the concentration of each immunoglobulin differed from the normal ($p < 0.05$).

After Lycurim therapy a certain insignificant fall occurred in the count of B-lymphocytes which forms EAC-rosettes ($p < 0.05$).

The T-lymphocyte content in peripheral blood also decreased considerably under the influence of Lycurim (to 7.7 ± 2.4%; $p < 0.05$).

Discussion

In spite of the existence of a number of cytostatics effective in CLL, the study of new ones is extremely important, especially from the point of view of secondary resistance, inevitably arising as a result of lengthy treatment with conventional cytostatics.

One of the new antileukaemic drugs with a pronounced lymphotopic action is Lycurim. The investigation presented above proved the high efficacy of Lycurim in CLL cases, and especially in cases of anaemia due to a compromise of bone marrow function and immunological complications. Lycurim therapy brought about considerable improvement in the general condition of the patients, a decrease of the size of peripheral lymph nodes, liver and spleen and a fall of the leukocyte count. The failure of the drug in the case of six patients may be explained as follows. In two patients with a low initial leukocyte count the development of leukopenia did not permit a completion of the therapy. Three patients had received an active cytostatic therapy before Lycurim treatment and the failure of the drug may be explained by the development of cross resistance due to the previous alkylating therapy. Only one patient showed an initial resistance to Lycurim.

It may be concluded that Lycurim is characterized by a high effectiveness in CLL therapy.

The majority of the patiens needed a total dose of 900 to 1000 mg. Some cases were, however, more sensitive, showing a significant decrease of the leukocyte count after smaller doses (180 to 300 mg).

Taking into consideration such an individual sensitivity to the drug, treatment should be conducted under thorough control of the peripheral blood. Specific attention should be paid to patients with a normal or subleukaemic initial leukocyte level. In addition, the fact that the leukocyte count continues to fall for two weeks after cessation of therapy should be borne in mind.

The immunosuppressive activity of the drug stipulates the treatment with Lycurim of immune complications of CLL. CLL with AIHA treatment caused a definite increase in the haemoglobin level and the erythrocyte and thrombocyte counts. A number of cases showed a complete normalization of these values after Lycurim therapy.

The effect of Lycurim on the immune status of CLL patients has been supported by immunological studies. A definite decrease of serum immunoglobulins and T-lymphocytes has been shown to occur after Lycurim therapy of CLL patients.

The change in the number of B-lymphocytes which form EAC-rosettes is evidently connected with the influence of the drug on the monoclonal leukaemic B-lymphocytes.

Our study has shown that treatment of CLL with Lycurim results in a definite antitumoural and immunodepressive effect. Its depressive activity involves both humoral and cellular immunity.

The above-mentioned properties of Lycurim permit us to recommend it for wide clinical use in CLL patients. Lycurim is a drug that can be used in tumourous cases of CLL as well as in cases of AIHA complications.

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Changes in Marrow Myelopoietic and Lymphoid Cell Counts after Repeated Cyclophosphamide Administration in the Rat

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Rat marrow myeloid and lymphoid counts were estimated after repeated doses of cyclophosphamide injected intraperitoneally. It was found that in order to evaluate the bone marrow response, a quantitative approach is a necessity. Myelotoxic and lymphotoxic effects in the bone marrow following repeated doses of cyclophosphamide were markedly similar to suppression which followed the restriction of food consumption. Relationships between rat white blood and marrow counts were analyzed. Convenience of determination of marrow cellularity for measurement of myelotoxic effects is discussed with respect to studies with repeated administration of cytostatic agents.

Introduction

Administration of cytostatics leads to inhibition of the function and proliferation of the myelopoietic system accompanied by leukopenia and increased susceptibility to bacterial infections. Knowledge of the intensity of myelopoietic system impairment and its regeneration are of a great practical value [6, 18]. Szmigielski and Jeljaszewicz [18] evaluated various haematological methods for the investigation of myelosuppression in rabbits. They determined rabbit marrow myelopoietic mass by mobilization of the bone marrow reserve pool.

Rats are the most common laboratory animals used in drug toxicology which include haematological examinations. A quantitative evaluation of rat bone marrow morphology determined by femoral cellularity [10, 15] provided accurate results in the investigation of myelotoxic effects after a single dose of cyclophosphamide [2], other cytostatic agents [5, 7] or irradiation [9]. Rats are more suitable than rabbits for such investigations because a quantitative preparation of their bone marrow is technically easier in a sufficient number of animals.

A method of evaluation of the rat marrow myelopoietic system after repeated doses of a drug is, however, missing. Studies with repeated administration of a drug to animals always precede its use in human therapy [21]. This report concerns changes in rat marrow myelopoietic morphology after repeated intraperitoneal doses of cyclophosphamide [2, 10, 12, 18]. As the number of nucleated marrow cells depends on an influence of lymphoid cells [11], variations in marrow lymphoid cell counts were also studied.

Materials and Methods

Experiments were performed on adult male Wistar rats of 210–240 g body weight bred at our Institute. They were housed under constant temperature (22 ± 2 °C) and light conditions (a light/dark cycle of 12L/12D, lights on at 07.00 and off at 19.00 h); tap water and food pellets (Velaz DOS 2b) were available *ad libitum* except for the group with restriction of food consumption. Rats were allowed to adapt for 2–3 weeks before being used for this study at 8.0 ± 0.5 weeks of age.

Blood was drawn from the tail with about 1 mm long tips cut off at 08.00 h (±20 minutes). Leukocytes were counted electronically (Celloscope 101 Coulter). White blood cell differential counts were performed on Pappenheim stained smears, counting at least 100 leukocytes. Each rat was sacrificed by cervical traction at 09.00 h (±60 minutes) and bone marrow cells from the right femur were expressed into an aqueous solution of 154 mM NaCl, 4.1 mM K_2EDTA, and 20 mM formaldehyde. Marrow nucleated cell counts were performed using a haemocytometer and Türk's solution. Differential marrow cell counts were determined for each animal by scoring at least 500 nucleated cells on the panoptically stained imprint from the left femur.

Cyclophosphamide (Cyclophosphamid, Jenapharm Ankerwerk, Rudolstadt, GDR) was dissolved in saline (154 mM NaCl) and 1 ml/200 g b.w. was injected intraperitoneally within 30 minutes. Five experimental groups of rats were used. In group 1, 5 mg/kg/day of cyclophosphamide was administered for 28 consecutive days. This dose is twofold higher than the therapeutic one recommended as a minimal dose in toxicological studies with repeated drug administration [21]. It had no lethal effect. In group 2, a dose of 15 mg/kg/day was injected for 21 consecutive days. This dose had highly toxic effects; 25% of the animals died within 21 days, and all the animals which were not sacrificed died within 28 days after drug withdrawal. In group 3, intact rats with restricted food consumption were given 10 g of pellets/animal/day (= mean food consumption between 14th and 21st day of drug administration in the dose of 15 mg/kg/day) was included in this experiment. Group 4 contained intact rats without restricted food consumption. They consumed 22 g/animal/day. Group 5 contained rats treated with saline. They consumed 18 g/animal/day, as in the cyclophosphamide group of 5 mg/kg/day.

Values are presented as mean ± standard error of mean for 6–10 animals each except absolute counts of marrow myelopoietic cells in the control (intact) animals which were calculated for 34 rats. 0 day of drug withdrawal means 24 hours after the last injection. Comparison of data was done using the two-tailed Mann-Whitney U test with Milton's [13] tables ($2\alpha = 0.05$).

Results

Relative myelopoietic cell counts increased after cyclophosphamide administration in both dose groups (Figs 1 and 2). The increase was more marked

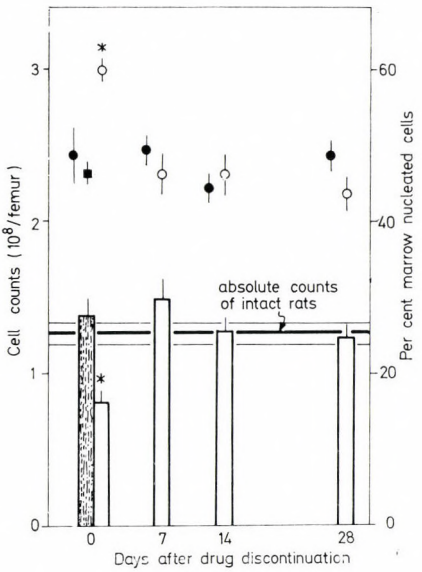

Fig. 1. Femoral myelopoietic cell counts after discontinuation of 5 mg/kg/day of cyclophosphamide. Relative counts (points): ● intact (control) rats, ■ saline, ○ cyclophosphamide; absolute counts (histograms): blank columns — cyclophosphamide, marble columns — saline; ✳ statistically significant as compared with intact (= control) rats

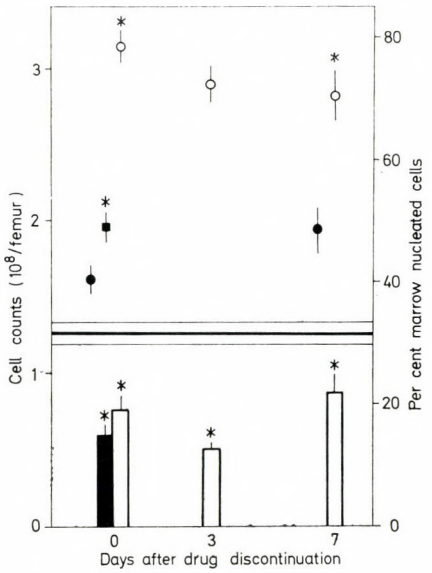

Fig. 2. Femoral myelopoietic cell counts after discontinuation of 15 mg/kg/day of cyclophosphamide. □ and black columns — rats with restricted food consumption; other symbols as in Fig. 1

after the higher dose. Relative counts of marrow myelopoietic cells in the rats treated with 5 mg/kg/day decreased to the normal limit within 7 days of drug withdrawal but they persisted above the normal limit in rats treated with 15 mg/kg/day (Figs 1 and 2).

Absolute total myelopoietic cell counts decreased in both dose groups. The decrease was near to the lower limit of the normal range. (Absolute counts of all intact animals were pooled as no significant age-dependent variations were observed from 11 to 16 weeks of age.) After the higher dose, the decrease of the absolute marrow myelopoietic cell counts intensified for 3 days after drug withdrawal, and after the 5 mg/kg/day dose, the absolute marrow cell counts were in the normal range 7 days after drug withdrawal (Figs 1 and 2).

Repeated injections of saline did not affect either the relative or the absolute marrow myelopoietic cell counts (Fig. 1). There was a significant increase in the relative and a decrease in the absolute myeloid cell counts after restriction of food consumption; the decrease of absolute counts was similar as after applications of cyclophosphamide in the 15 mg/kg/day dose (Fig. 2). Variations of the total (Figs 1 and 2) and individual myeloid cell types (Figs 3–8) were similar.

Absolute counts of circulating segmented neutrophils slightly increased after 5 mg/kg/day cyclophosphamide dose and they returned to normal in 4 weeks after

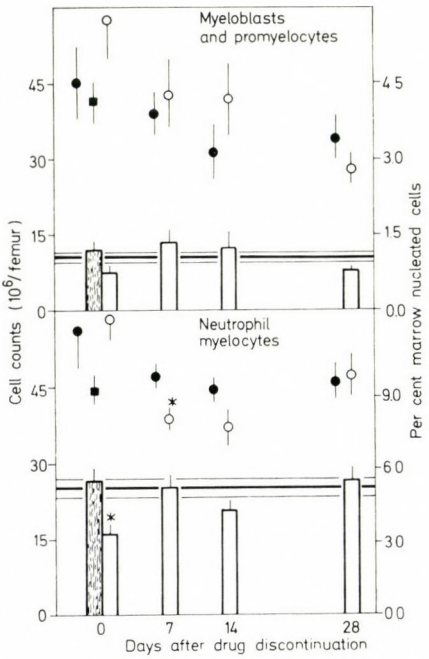

Fig. 3. Femoral dividing myeloid cell counts after discontinuation of 5 mg/kg/day of cyclophosphamide. Symbols as in Fig. 1

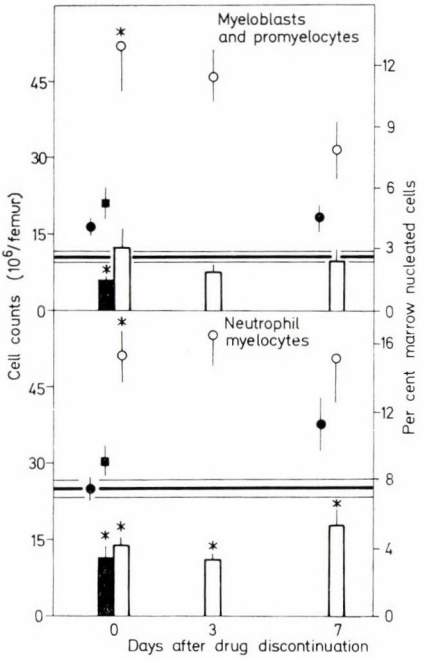

Fig. 4. Femoral dividing myeloid cell counts after discontinuation of 15 mg/kg/day of cyclophosphamide. Symbols as in Fig. 1

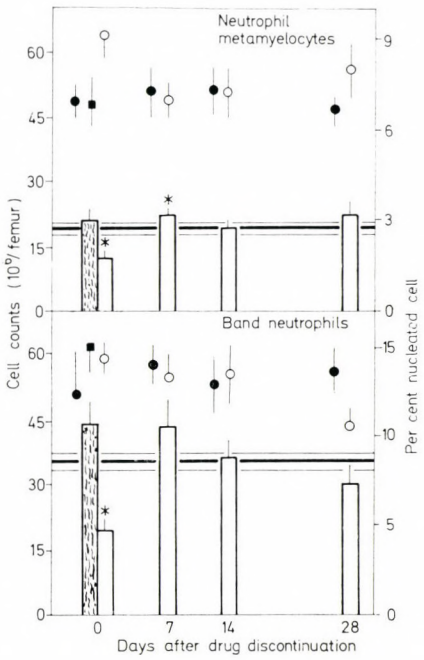

Fig. 5. Immature non-dividing myeloid cell counts in femoral marrow after discontinuation of 5 mg/kg/day of cyclophosphamide. Symbols as in Fig. 1

drug withdrawal (Fig. 7). Absolute counts of circulating segmented neutrophils increased markedly after cyclophosphamide withdrawal in the 15 mg/kg/day group (Fig. 8). The changes of absolute counts of circulating and marrow segmented neutrophils were not correlated in any dose group. Circulating hypersegmented neutrophils were observed in all rats treated with the higher dose and they persisted until death in this group. Sporadic circulating neutrophils with nuclear fragments or more expressed granulation were also found in this group. Hypersegmented neutrophils were observed after 28 days of 5 mg/kg/day cyclophosphamide but they disappeared after cyclophosphamide withdrawal.

Absolute and relative marrow lymphoid cell counts decreased markedly in the 5 mg/kg/day group (Table 1) and these cells were missing in the femoral marrow in the 15 mg/kg/day group (Table 2). Full recovery of marrow lymphoid cell counts took 7 days after discontinuation of cyclophosphamide in the 5 mg/kg/day group while in the 15 mg/kg/day group the counts improved but full recovery could not be observed as all animals died. The decrease of absolute marrow lymphoid cell counts was observed also in the rats with restriction of food consumption (Table 2). Absolute blood lymphocyte counts seemed to reflect the suppression

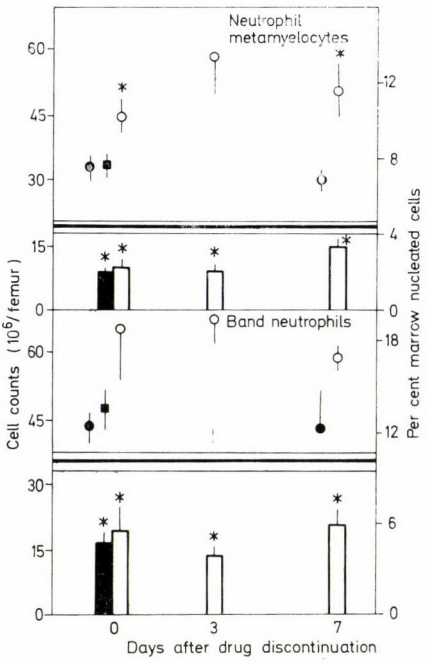

Fig. 6. Immature non-dividing myeloid cell counts in femoral marrow after discontinuation of 15 mg/kg/day of cyclophosphamide. Symbols as in Fig. 2

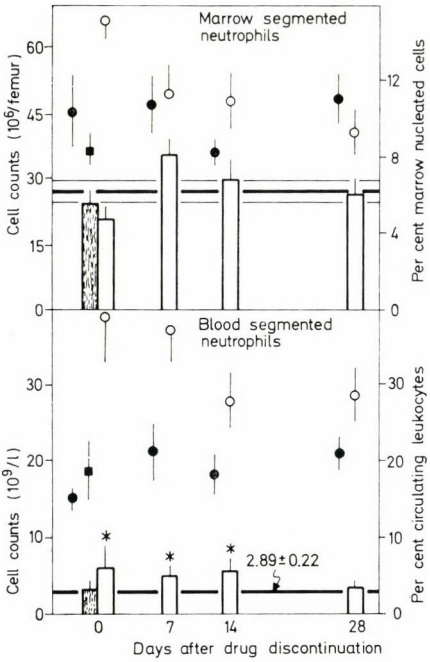

Fig. 7. Marrow and blood segmented neutrophil counts after discontinuation of 5 mg/kg/day cyclophosphamide. Symbols as in Fig. 1

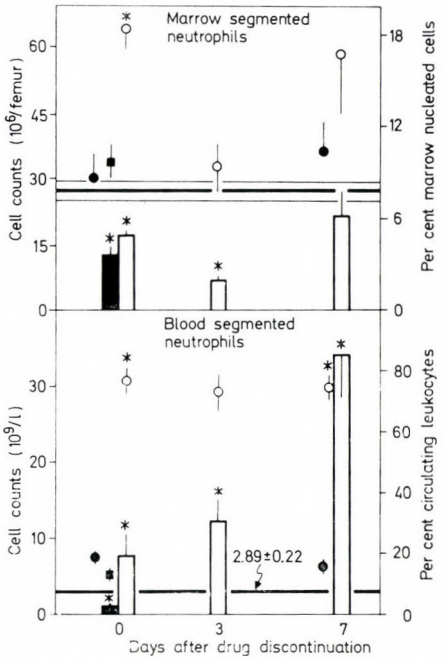

Fig. 8. Marrow and blood segmented neutrophil counts after discontinuation of 15 mg/kg/day cyclophosphamide. Symbols as in Fig. 2

of marrow lymphoid cells but the correlation was not a close one; for example, the decrease of absolute blood lymphocyte counts persisted in the rats treated with 5 mg/kg/day for 7 days of drug withdrawal while the bone marrow showed a full recovery (Table 1).

Discussion

Thus, the relative myelopoietic cell counts increased markedly after repeated intraperitoneal doses of cyclophosphamide, in contrast with the data of Wheeler et al. [20] for rats with oral cyclophosphamide administration but in agreement with their [20] observations in dogs.

Repeated doses of cyclophosphamide had a slight myelotoxic effect in the femoral marrow myeloid mass. These myelotoxic effects of cyclophosphamide were weaker after repeated application than after a single large dose [2]. The higher lethal effect of repeated cyclophosphamide administration may not have been due directly to the haematological injury but to the lowered resistance to infections [12, 17]. Our findings support this suggestion as no increment of absolute counts of circulating segmented neutrophils was observed in the group with restricted food consumption. In this group, the absolute lymphocyte counts and probably

Table 1
Recovery of marrow lymphoid cell and blood lymphocyte counts after repeated administration of 5 mg/kg/day cyclophosphamide

Dose	Characteristic	Days after cylophosphamide withdrawal			
		0	7	14	28
Intact controls	Marrow lymphoid cells $\times 10^5$/femur	305.5± 72.2	542.7± 73.2	332.2± 67.2	504.6± 38.0
	per cent	10.6± 2.2	14.6± 1.5	15.1± 2.9	18.9± 1.4
	Blood lymphocytes $\times 10^9$/l	13.6± 1.0	12.7± 0.8	12.7± 1.5	9.8± 0.7
	per cent	83.8± 1.4	76.1± 4.3	78.1± 2.7	77.0± 2.4
Saline	Marrow lymphoid cells $\times 10^5$/femur	422.9± 37.5			
	per cent	14.8± 2.1			
	Blood lymphocytes $\times 10^9$/l	12.4± 0.9			
	per cent	78.1± 4.0			
Cyclophosphamide 5 mg/kg/day	Marrow lymphoid cells $\times 10^5$/femur	27.0± 14.3*	641.8± 86.1	343.3± 113.0	492.8± 72.5
	per cent	1.9± 1.0*	20.0± 2.7	11.3± 3.0	17.4± 2.5
	Blood lymphocytes $\times 10^9$/l	6.1± 0.6*	7.6± 0.6*	13.0± 2.0	8.3± 0.6
	per cent	57.4± 6.4*	59.7± 5.2	66.7± 3.9*	65.9± 3.6*

* statistically significant *vs* controls ($2\alpha = 0.05$).

Table 2
Recovery of marrow lymphoid cell and blood lymphocyte counts after repeated administration of 15 mg/kg/day of cyclophosphamide

Dose	Characteristic		Days after cyclophosphamide withdrawal		
			0	3	7
Intact controls	Marrow lymphoid cells	$\times 10^5$/femur	334.6± 58.3		305.5± 72.2
		in %	16.3± 2.8		10.6± 2.2
	Blood lymphocytes	$\times 10^9$/l	12.0± 1.7		13.6± 1.0
		in %	79.7± 2.2		83.8± 1.4
Food restriction	Marrow lymphoid cells	$\times 10^5$/femur	232.0± 28.3		
		in %	19.0± 1.4		
	Blood lymphocytes	$\times 10^9$/l	6.8± 0.5*		
		in %	84.6± 1.4		
Cyclophosphamide 15 mg/kg/day	Marrow lymphoid cells	$\times 10^5$/femur	0.0± 0.0*	0.8± 0.8	24.8± 9.1*
		in %	0.0± 0.0*	0.1± 0.1	1.8± 0.6*
	Blood lymphocytes	$\times 10^9$/l	1.6± 0.3*	3.1± 0.5	9.5± 1.1*
		in %	18.9± 3.5*	23.2± 5.4	23.2± 3.8*

* statistically significant *vs* controls ($2\alpha = 0.05$).

the lymphocyte-mediated immunity decreased less than in the groups treated with cyclophosphamide.

With an intermittent cyclophosphamide treatment the therapeutic dose can be increased [19] but the drug will become myelotoxic. Our data also suggest that restriction of food consumption by rats treated with cyclophosphamide may be an important factor in marrow myelo- and lymphosuppression.

The present results have confirmed previous data on circulating hypersegmented neutrophils [20]. The significance of a higher number of lobes of the neutrophil nucleus is not fully clear. As we have observed, no pronounced shift to the right or to the left occurred either in the bone marrow or in the blood, only the circulating segmented neutrophils showed some increment. It seems, therefore, that after repeated doses of cyclophosphamide, the mean age of neutrophils increased, and the many nuclear lobes may have also been a consequence of changes in neutrophil physiology [12].

Nuclear fragments (so-called micronuclei) in neutrophils, which we observed sporadically in the 15 mg/kg/day group, are a consequence of mutagenicity [16]. Similar micronuclei (as Howell–Jolly bodies) were observed in rat marrow polychromatic erythrocytes [3] after administration of a single dose of cyclophosphamide.

Myelograms are a traditional method of bone marrow evaluation. On the other hand, single doses of several cytostatics were tested as to their myelotoxic effect by quantitation of the marrow myeloid mass in small laboratory animals [2, 5, 7]. In the present study we applied the method for the evaluation of the myelotoxic effects of repeated doses of cyclophosphamide. If we compare the relative counts determined by myelograms and the absolute counts determined by femoral cellularity plus myelograms presented in this paper, it is obvious that the quantitative evaluation of myelotoxic effects is the more exact method in chronic studies as well. For example, relative counts of total myeloid cells increased in the 5 mg/kg/day group on the 28th day of cyclophosphamide administration but the absolute total myeloid cell counts did slightly decrease.

The toxicity to myelopoiesis is routinely assessed by following the changes in the absolute circulating segmented neutrophil counts [8]. Our findings, however, suggest that variations in the concentration of circulating segmented neutrophils are not a very sensible index of changes of marrow myelopoiesis after repeated doses of cytotoxic agents: absolute counts of circulating segmented neutrophils of animals treated with the 15 mg/kg/day dose were increased above normal limits of laboratory rats [1] while slight myelosuppression was observed; so a redistribution of circulating neutrophil pools [4] did occur. Thus, a quantitative evaluation of the marrow myeloid mass of laboratory rats following repeated administration of cytostatic agents may be a suitable method for the accurate determination of myelotoxic effects.

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Inhibition of Antibody-Dependent Cellular Cytotoxicity (ADCC) by Normal and Pathological Sera from Patients with Advanced Lung Cancer

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An improved ADCC assay for the detection of cancer-associated serum blocking factors is described. Inhibition of ADCC by sera was performed without preincubation and washing of effector cells. Effects of individual susceptibility of normal effector cells to serum inhibitors and of the background inhibition by normal sera were also avoided. Based on their inhibitory effect on ADCC, normal and tumour sera from lung cancer patients could be distinguished with 81.4 to 100% accuracy, according to effector cell donors and to concentrations of the sera tested.

Introduction

Sera from cancer patients appear to contain factors capable of inhibiting normal leukocyte functions *in vitro* and perhaps also *in vivo* [2, 8, 12, 18]. The inhibition of leukocytes may be responsible for that type of immunosuppression which is closely associated with advanced malignancy in man and animals [1, 8, 18, 21]. It seems likely that important blocking factors found in cancer sera include soluble tumour or viral antigens [20, 21], antigen-antibody complexes [16, 19], immunosuppressive peptides [5], and immunoglobulins [7, 10]. Some of these factors are known to inhibit ADCC activity of leukocytes which are strongly implicated in resistance to malignancy [2, 7, 9].

Inhibition of ADCC is based on the principle that soluble factors able to bind to Fc-receptors can compete with target cell-bound antibody for Fc-receptors on effector cells. The results, however, require caution in interpretation since even normal sera may have inhibitory effects on the assay attributable to non-cancer factors [2, 9, 13].

Thus, experiments in the present report were performed to investigate some of the technical variables interfering with the detection of Fc-receptor blocking activity of tumour sera by the chicken system of Perlmann and Perlmann [15]. Sera from patients with unresectable lung cancer were studied, implicating Fc-receptor blocking factors (possibly immune complexes) in the disease process.

Materials and Methods

Specimens

Blood obtained from 40 normal donors and 37 unresectable lung cancer patients with various tumour histology was allowed to clot at room temperature, centrifuged, inactivated at 56 °C for 1 h, absorbed with packed Chicken Red Blood Cells (CRBC) at 37 °C for 30 min, and kept at −25 °C for maximum 2 weeks.

Cytotoxicity test procedure

Effector cells derived from normal donors were separated by standard Ficoll-hypaque technique [3]. To remove phagocytic cells, whole blood was incubated with carbonyl iron prior to separation [6]. Differential cell counts on interfaced preparations gave values of 95 ±2% lymphocytes, 2 ±2% polymorphonuclear leukocytes and 2 ±1% monocytes.

Target cell damage was assessed by release of ^{51}Cr chromate from CRBC prelabelled with this isotope, essentially as described by Perlmann and Perlmann [15].

Briefly, cultures were set up in triplicate in tubes each containing 50 µl lymphoid cells (1×10^5) with 50 µl target cells (4×10^5) to give an effector-target ratio of 1 : 4, and 10 µl rabbit anti-CRBC (10^{-4} dilution). Human serum albumin (HSA; the same batch), normal and tumour sera were added so as to give final concentrations of 4, 10, 20 and 30%. The total volume of each culture was 500 µl. All dilutions were made in Eagle's medium supplemented with antibiotics. Control tubes without effector cells or sensitizing antiserum, or both, were included in each experiment. Incubation was performed for 4 h at 37 °C in an atmosphere of 5% CO_2 in air. To terminate culture, 1 ml 4 °C Eagle's medium was added to each tube. Aliquots (1000 µl) of each supernatant and the residual supernatant and pellet were counted in a Gamma Automatic Counter (Model NZ 310).

Expression of cytotoxicity data

Percentage cytotoxicity (^{51}Cr release) was calculated from the formula:

$$\frac{\text{radioactivity in supernatant removed } (\times 1.5)}{\text{total radioactivity in supernatant and residue}} \times 100$$

where each tube was corrected for background radioactivity. The percentage inhibition of cytotoxicity was calculated from the formula:

$$\frac{(R_{HSA} - R_0) - (R_{Test} - R_0)}{R_{HSA} - R_0} \times 100$$

where R_{HSA} = percentage of ^{51}Cr release in HSA, R_0 = percentage of ^{51}Cr release in the absence of sensitizing antibody, R_{Test} = percentage of ^{51}Cr release in the presence of tested sera. The percentage of ^{51}Cr release in the presence of effector cells but in the absence of anti-CRBC antibody was normally below 5%.

Statistics

All the computations were performed using the Statistical Package for the Social Sciences (SPSS) on an R-22 computer [14]. To assess the equality of distribution of percentage inhibitions produced by normal and pathological sera, the Kolmogorov–Smirnov test was used. The relationship between the two variables was expressed by means of the Pearson correlation coefficient. To develop the functions of Table 3, a discriminatory analysis was performed [17].

Results

Effects of preincubation by sera and washing of effector cells on the inhibition of ADCC

Experiments were performed to determine the conditions under which blocking sera were inhibitory for ADCC. Data on inhibition of ADCC activity of normal lymphocytes by sera added with or without preincubation as well as washing are presented in Table 1. As can be seen, washing of effector cells preincubated with pathological sera did not affect the level of inhibition of ADCC activity. Alternatively, serum is not necessarily present during the assay period. The ADCC inhibition test proved to be reproducible both with and without preincubation and washing. The inhibition of ADCC by sera was not attributable to a direct cytotoxic effect since no difference in viability of target cells treated with HSA, mixed AB serum or inhibitory sera was observed by trypan blue exclusion.

On the basis of these data, subsequently we used the technically simpler and time-saving procedure: without preincubation of the effector cells, tested sera were present throughout the whole assay period.

Inhibition of ADCC by individual normal and tumour sera

When assaying sera for inhibitory materials, Barkas et al. [2] found that certain serum fractions lysed CRBC in the presence of foetal calf serum. Therefore the base-line cytotoxicity regarded as 100% was defined in Eagle's medium + HSA. Inhibitory activities of normal and tumour sera were measured in two lymphocyte donors. Table 2 illustrates that serum concentration affected the degree of inhibition, but an increase in the efficacy of inhibition attributed to an increase in concentration was statistically linear only in certain combinations. The difference between tumour and control sera was statistically highly significant in all the

Table 1

Effect of washing of effector cells after preincubation with sera on the levels of inhibition of ADCC activity by sera

Sera	Percentage inhibition of ADCC*					
	preincubated, washed**			not preincubated, not washed***		
	April 14	May 20	June 28	April 14	May 20	June 28
Mixed AB serum	24.3	25.5	23.8	22.5	23.7	24.0
Tumour sera						
1	57.1	58.3	54.6	51.3	46.7	47.6
2	50.8	49.2	—	45.8	48.2	—
3	56.3	52.7	53.6	47.5	54.2	50.6
4	58.8	61.3	60.8	56.3	64.8	61.1
5	62.1	62.7	53.7	66.4	67.3	57.6
6	65.2	68.3	57.6	66.5	60.8	61.3
7	51.4	48.5	50.9	47.5	56.4	52.6

* Percentage inhibition was calculated on the basis of cytotoxicity obtained with HSA, as described in Materials and Methods.
** Effector cells after incubation with serum were washed three times with Eagle's medium.
*** Serum was left in the culture with effector cells.

Table 2

Relative inhibition* of ADCC produced by normal and tumour sera at different concentrations

Final concentration of sera, per cent	Percentage inhibition of ADCC by					
	normal sera	tumour sera	P** value	normal sera	tumour sera	P value
	Donor 1			Donor 2		
4	1.25 ± 18.7	41.66 ± 12.5	$p < 0.01$	37.9 ± 2.1	66.1 ± 7.6	$p < 0.01$
10	29.9 ± 17.9†	54.0 ± 10.1†	$p < 0.05$	49.85 ± 15.8	78.05 ± 18.0	$p < 0.05$
20	33.3 ± 16.9†	66.2 ± 7.9†	$p < 0.01$	49.0 ± 12.7	79.2 ± 39.7	$p < 0.05$
30	37.9 ± 22.0†	72.2 ± 7.02	$p < 0.05$	60.4 ± 11.25	90.2 ± 2.64	$p < 0.01$

* Evaluated as a function of ADCC in Eagle's medium + HSA; values quoted are means and ± 1 SD.
** Statistical methods, Kolmogorov–Smirnov two-sample test and *t* test.
† Increasing serum concentrations caused a quasi-linear rise in inhibition of ADCC both with normal sera — $p(PI_{10\%}; PI_{20\%}) = 0.63$; $p(PI_{10\%}; PI_{30\%}) = 0.76$, and with tumour sera: $p(PI_{10\%}; PI_{20\%}) = 0.80$; where PI = percentage inhibition, and p is the Pearson correlation coefficient.

Table 3

Functions discriminating normal from tumour sera on the basis of percentage inhibition of ADCC

Lymphocyte donor	Serum concentration, per cent	Number of		Functions	Number of		Percentage correct classification
		healthy subjects	subjects with tumour		false positive cases	false negative cases	
1	4	20	23	$(PI_{4\%}) \geq 23.1$	3	0	93.02
	10	20	23	$(PI_{10\%}) \geq 43.4$	3	5	81.4
	20	20	23	$(PI_{20\%}) \geq 51.3$	1	4	88.37
	30	16	21	$(PI_{31\%}) \geq 27.6$	2	4	83.78
2	4	20	15	$(PI_{4\%}) \geq 49.4$	0	3	91.43
	10	20	15	$(PI_{10\%}) \geq 63.5$	0	2	92.29
	20	17	15	$(PI_{20\%}) \geq 67.7$	0	0	100.0
	30	14	15	$(PI_{30\%}) \geq 76.2$	1	0	96.55

PI = Percentage inhibition of ADCC produced by individual sera.

experiments. When analyzing the percentage inhibition of ADCC produced by sera, functions able to discriminate between normal and tumour sera could be established (Table 3). The data presented showed that the discriminatory ability of the functions was optimum at a serum concentration of 4% for Donor 2, and at one of 20% for Donor 1. Analyzing the data obtained with serum concentrations of 4% and 20%, respectively, the poor percentage selection on Donor 1's lymphocytes improved, approaching 95%.

Discussion

Recently, a number of sensitive techniques able to detect blocking factors in sera have been described [1, 2, 4, 7, 9, 12]. Among them, ADCC measures the levels of Fc-receptor blocking soluble serum factors [2, 10, 11, 13]. This mechanism is of special importance because these factors may work *in vivo* as well. They bind to leukocyte Fc-receptors and exert effects by inhibiting the cytotoxic response of effector cells to tumour antigens [1, 4, 8].

Our present study demonstrates that both normal and tumour sera from patients with advanced lung cancer contain blocking factors which may be responsible for the impairment of ADCC activity, but the levels of inhibition exerted by tumour sera are significantly higher than those of normal sera. The quantification of the relative inhibition of ADCC by normal and pathological sera, however, is a matter of incertitude since the inhibitory effect depends upon several variables, including the individual capacity of sera to support the cytotoxic reaction *in vitro*, the effects of different concentrations of sera [2, 9] and anti-target antibodies [10, 11], and individual differences in susceptibility to inhibitors of normal lympho-

cytes. The latter technical factor found by us means that follow-up and comparative studies should be performed on lymphocytes of the same origin. In addition, we even found that also the effector-to-target ratio influences the degree of reduction in ADCC produced by sera (Fekete et al., to be published).

In order to develop a reproducible ADCC blocking assay able to discriminate between normal and tumour sera, the effector cells were of the same origin, percentage inhibition of ADCC by normal and tumour sera was expressed as a function of ADCC in Eagle's medium + HSA, and the cancer-associated serum blocking activity was calculated using a single batch of HSA as the reference standard against which were compared the effects upon ADCC of normal and tumour sera. This inhibition was not attributable to a direct cytotoxic effect of sera. Washing of the effector cells after incubation with tested sera did not affect the level of inhibition which might mean an irreversible effect of blocking factors on effector cell activity.

This finding is in agreement with previous reports in that serum blocking factors inhibit murine [2, 13] and human effector cells [7, 9]. On establishing a base-line with which to compare the inhibitory activity of the two groups and by using the functions of Table 3, the sera in question could be classified as normal or pathological. The percentage of correct classifications was between 81.4 and 93.02% for Donor 1, and between 91.43 and 100% for Donor 2. Alternatively, this means that advanced lung cancers are especially associated with high levels of ADCC blocking factors.

The present findings argue that serum blocking factors capable of inhibiting ADCC must be considered in analyzing host reactivity to tumour growth of lung cancer patients. Also, taking into account that the serum levels of the inhibitors may reflect the clinical stage and the tumour burden [8, 16, 18], their serial detection by the ADCC blocking test standardized by us will be useful for monitoring cytoreductive therapy and may have a prognostic value. The question of the exact nature of these inhibitors has not yet been clarified.

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The Results of Prolonged Treatment of Autoimmune Haemolytic Anaemia

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Ninety-seven patients with autoimmune haemolytic anaemia were observed in the period between 1965 and 1979. Corticosteroids were found to be more effective in the acute phase of haemolysis (68.6 %) than in the prolonged treatment (35 %). Favourable results with combined treatment (corticosteroids plus azathioprine or cyclophosphamide) were achieved in 60.5 % of patients, while the therapeutic potency of corticosteroids alone was 18.6 %. Splenectomy was effective in 81 % of the patients. No correlation between postsplenectomy results and spleen to liver radioactivity ratio was observed.

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Corticosteroids and/or cytotoxic drugs as well as splenectomy are the most commonly used in the treatment of autoimmune haemolytic anaemia (AIHA). The effect of corticosteroids is well established and there are no doubts that they should be applied in all cases of AIHA as an initial treatment. The therapeutic value of cytotoxic drugs and splenectomy, however, remains a subject of controversy. This is especially true as far as the indications for such therapy and expected long-term results are concerned. Another problem is the prognostic value of some factors supposed to be useful in prediction of the results of the treatment applied, especially of splenectomy. In an attempt to shed more light on these problems, this work summarizes the results of therapy of patients with AIHA observed in the Institute of Haematology in Warsaw in the years 1965 to 1979.

Materials and Methods

The criteria of diagnosis in 97 patients with AIHA (41 men and 56 women) aged 15 to 80 years, were:

1. The evidence of haemolysis, i.e. elevated reticulocyte count, decreased ^{51}Cr red cell survival, usually anaemia and erythroid hyperplasia of the bone marrow;

2. Positive direct antiglobulin test (DAT) with polyspecific, anti-IgG (KH 16-113-A1), Anti-IgM (KH 15-10-A2), anti-C3 (KH 13-04-A0) sera* and/or

* The polyspecific serum was produced in the Institute of Haematology, Warsaw. Anti-IgG, -IgM and -C3 sera were supplied by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam.

Table 1

Primary and secondary types of AIHA

Autoimmune haemolytic anaemia	No.	Percentage
I Idiopathic (primary type)	41	42.3
II Secondary	56	57.7
Lymphoma non-Hodgkin's	23	41.5*
Hodgkin's	1	1.8
Systematic autoimmune disorders	14	24.5
Rheumatoid arthritis	2	3.6
Chronic hepatitis	6	10.7
Ovarian cyst	2	3.6
Ovarian cancer	1	1.8
Chronic infections		
lymphonodular tuberculosis	1	1.8
other bacterial	3	5.3
Protozoan giardiasis	1	1.8
Sarcoidosis	1	1.8
Carcinoid syndrome	1	1.8

* percentage of secondary AIHA.

Table 2

Serologic results

DAT with antisera	Autoantibodies in serum and/or eluate	AIHA		Total	
		Idiopathic no.	Secondary no.	no.	per cent
IgG	warm	23	27	50	51.6
IgG + C	warm	8	17	25	25.8
IgM + C	cold	5	6	11	11.3
C	—	3	6	9	9.3
—	warm	2	0	2	2.0

the presence of "warm" and "cold" autoantibodies in the serum and/or in the eluate [8].

The percentage of idiopathic and secondary types of AIHA is listed in Table 1, and the results of immunohaematological tests, in Table 2.

The results of treatment were evaluated as follows.

Excellent: Full disappearance of signs of haemolysis with negative or decreased DAT, no relapse for at least one year after cessation of treatment.

Good: Compensation of haemolysis with slight maintenance treatment with daily doses up to 20 mg of prednisone and/or 50 mg of azathioprine or cyclophosphamide.

Fair: Partial compensation of haemolysis on continuous treatment with higher doses of drugs than those in the previous group.

Poor: None or slight control of haemolysis in spite of high doses of corticosteroids (over 60 mg of prednisone) and/or cytotoxic drugs (azathioprine 150 mg or more daily, cyclophosphamide 200 mg or more daily).

Results

The treatment regimens are shown in Fig. 1.

Group A. Out of 80 patients who were given high doses (60 to 80 mg) of corticosteroids as the first treatment, in 55 cases (68.6%) improvement was considerable during three weeks. Prolonged treatment with corticosteroids was much less effective as long lasting favourable results were observed only in 28 patients (35%) (in 19 with idiopathic AIHA). In the remaining 54 patients of this group another treatment had to be introduced: in 22 (27.5%) because of intolerance or complications after corticosteroid therapy and in 32 (40%) due to the insufficient therapeutic response. In 6 patients following an initial response a permanent cure was achieved. Most of them were young (mean age, 21.5 years), the onset of haemolysis was acute and of severe character with fever, abdominal pains, vomiting, diarrhoea and very severe anaemia. In 3 of them a viral infection and in one, exposure to organic solvents was revealed by the history. In 2 patients with severe anaemia a negative DAT was observed in spite of the presence of warm autoantibodies in eluates. All of them responded readily to corticosteroids with disappearance of haemolytic signs and positive serologic tests, within three weeks to four months. None of these patients developed a second relapse of haemolysis during 6 to 9 years of follow-up.

Group B. Among 43 patients who subsequently received a combined treatment consisting of prednisone and cytotoxic drugs (37 azathioprine and cyclo-

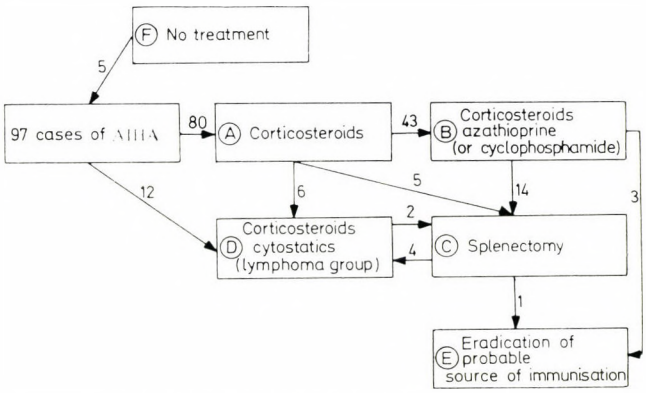

Fig. 1. Schedule of treatment of AIHA

phosphamide) favourable results were obtained in 26 patients (60.5%) with idiopathic AIHA: excellent, 4; good, 10; fair, 12. The main advantage of the combined treatment was the possibility of the reduction of the dose of corticosteroids but better results than with corticosteroids alone were observed in 8 cases only (18.6%). No clinical or serological signs had a prognostic value in prediction of the result of combined treatment.

Two cases with an excellent result of treatment are especially interesting. Both cases were patients with idiopathic AIHA of long duration (8 and 13 years). In the course of combined treatment such a good clinical remission was obtained that complete cessation of treatment was possible. A very weak positive DAT with anti-IgG serum, however, persisted. The patients considered themselves healthy but after one and two years, respectively, a new relapse with accelerated haemolysis occurred. Combined treatment did not provide a satisfactory haemolysis control in 17 patients (39.5%) who required another therapeutic approach (Groups C and D).

Group C. Therapeutic splenectomy was performed in 21 patients. The underlying disease was diagnosed in 9 patients: 6 non-Hodgkin's lymphoma, 2 lupus erythematodes and one ovary cyst (in the last case the diagnosis was made after splenectomy). The pre- and post-splenectomy course of the disease is seen in Fig. 2.

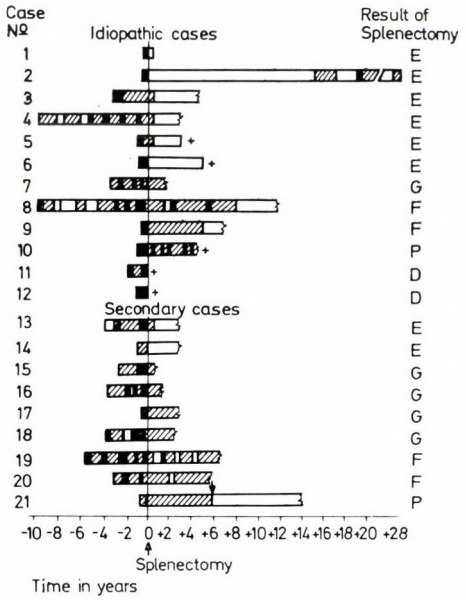

Fig. 2. Clinical course of 21 patients with AIHA and the effect of splenectomy. Duration of the illness before and after splenectomy is indicated by the length of rectangles. ■ represents periods of active, usually decompensated haemolysis; //// less active often compensated haemolysis; □ compensated haemolysis, clinical remission; E — excellent result of splenectomy, G — good, F — fair, P — poor, + death, ↓ surgery of ovary cyst

Splenectomy did not induce a normalization of the DAT in any of the operated patients and only a decrease in some of them was observed.

Excellent and good results were obtained in 13 patients (62%), 7 with idiopathic and 6 with secondary AIHA, and fair results in 4 patients (19%). Among those with idiopathic AIHA some were especially interesting. Patient No. 1 was operated upon during severe haemolysis totally resistant to chemotherapy and an immediate remission was obtained which now has persisted for 11 months without maintenance treatment. Case No. 2 who after splenectomy remained in full clinical remission without any treatment as long as 15 years, developed several relapses in the next 13 years. A weak positive DAT was observed during the remission. Patient No. 4 in spite of considerable haemolysis was not qualified for splenectomy for a long time because he had no splenomegaly and a low spleno-hepatic index (2 : 1). Nevertheless splenectomy resulted in fast and full clinical remission which now has persisted for 3 years. In 4 patients with AIHA (Nos. 13, 14, 17, 18) of obscure origin and considerable splenomegaly (weight 1130, 1350, 3500 and 2800 g) non-Hodgkin's lymphoma was diagnosed at postsurgery examination of the spleen (2 centrocytic, one of lymphoplasmocytoid type, one unclassified). In all of them clinical remission and no sign of progressive lymphoma during a 3 to 4 years follow-up without treatment (except No. 18) was observed. Nevertheless, in each case a positive DAT and in case No. 18 cold agglutinins at a titre of 1 : 500 still persist. Good results were also noted in 2 patients (Nos. 15 and 16) with chronic lymphatic leukaemia, who had been referred for splenectomy due to poor compensation of haemolysis and advanced splenomegaly (1080 and 3500 g).

Fair results were obtained in 4 patients. Two of them (Nos. 19 and 20) had lupus erythematodes. In the remaining 2 with idiopathic AIHA (Nos. 8 and 9) moderate haemolysis persisted after splenectomy but of decreasing intensity and a subsequent full remission with cessation of treatment occurred 5 and 9 years after the splenectomy.

Poor results were observed in 2 patients. In one of them (No. 21) the reason for the AIHA was found later in an ovarian cyst and excellent result was achieved after its surgical removal. In the second patient (No. 10), a 16-year-old girl (spleen weight, 1160 g; spleno-hepatic index, 1.6 : 1) splenectomy did not improve the severe course of the disease. She died 3.5 years later of gonococcal peritonitis during corticosteroid therapy.

No obvious correlation was noticed between the results of splenectomy and the type of autoantibodies (Table 3). Still, excellent results were more frequent in the patients with warm autoantibodies when no complement was detected on red cells. Nevertheless, the number of cases is too small to determine whether the presence of complement on red cells is of prognostic value. The spleno-hepatic index had a doubtful prognostic value in prediction of the results of splenectomy (Fig. 3).

Figure 3 illustrates in addition the results of splenectomy in relation to the weight of the spleen. In all patients with considerable splenomegaly (spleen weight over 2000 g) excellent and good responses were observed and in the spleen non-

Table 3

Results of splenectomy in dependence on serologic tests

DAT with antisera	Autoantibodies in serum and/or eluate	Results of splenectomy				
		excellent	good	fair	poor	death
IgG	warm	6	1	—	1	1
IgG + C	warm	2	1	3	1	1
IgM + C	cold	—	1	—	—	—
C	—	1	2	1	—	—

Hodgkin's lymphoma was found; this was probably limited to the spleen and not diagnosed preoperatively. Excellent and good results were also achieved in patients with smaller spleens.

Group D. Eighteen patients received combined steroid and cytostatic therapy from the very beginning or after a short course of corticoids, for AIHA in the course of lymphoma (usually chronic lymphatic leukaemia). The precise evaluation of this group was difficult because of the progressive character of the underlying disease. Nevertheless, the haemolytic process itself was usually well controlled by the applied chemotherapy (patients with chronic lymphatic leukaemia and poorly controlled haemolysis were discussed in the group of splenectomy). Four additional patients from Group C were ranged into this group after splenectomy, because the underlying disease necessitated a continuation of chemotherapy.

Group E. This group consisted of 4 patients who required a special approach due to the failure of previous treatment; in one case even splenectomy was ineffective. In three, excellent results were achieved and even complete cure is likely after

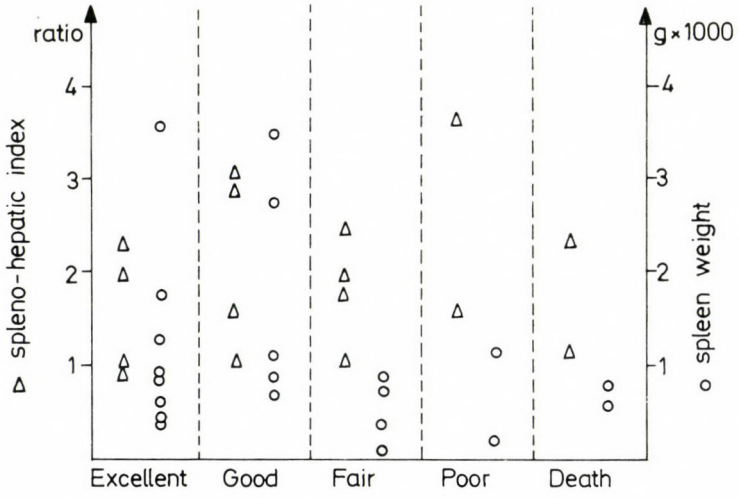

Fig. 3. Results of splenectomy in comparison with spleno-hepatic index and spleen weight

a surgical removal of the probable source of immunization which was discovered later or was initially neglected (bronchiectatic lobe of the lung and two ovarian cysts). The last patient with poor response to treatment and large splenomegaly has refused to be splenectomized but consented to deep X-ray therapy of the spleen (3000 r) which resulted in an excellent effect with long remission of haemolysis and involution of the splenomegaly. In all the patients of Group E, remission has been lasting since over 10 years and, except in the last one, is associated with normalization of a positive DAT.

Group F. In 5 patients out of 97, haemolysis was mild, compensated and required no treatment. This group consisted of 3 patients with chronic lymphatic leukaemia, one with Hodgkin's disease (in remission) and one with giardiasis cured by drugs.

Side effects were most frequently in Groups A and B. Thirty-two patients developed side effects after corticotherapy (12 diabetes, 8 Cushing syndrome, 10 infections, one gastric ulcer, one hypertension). Cytotoxic drugs in the doses applied were generally well tolerated except by one patient who after cyclophosphamide developed haemorrhagic cystitis. In a few patients temporary leukopenia was observed but in no case was a permanent discontinuation of cytotoxic drugs necessary. Eight patients are not being followed up and it is assumed that some of them are no longer alive. Twenty other patients died: because of progress of lymphoma, 8; infections complicating the course of corticotherapy, 5; ovarian cancer, one; lung cancer, one; cirrhosis of the liver, one; chronic circulatory failure, one; carcinoid, one; and post-splenectomy complications: pulmonary embolism, acute pancreatitis, 2 cases.

Discussion

The observation that corticosteroids are effective in the acute phase of haemolysis (68.6%) confirm the results reported by Murphy and Lo Buglio [5]. This concerns idiopathic as well as secondary AIHA. In some, especially in young patients with idiopathic AIHA, a complete cure can be expected after the first haemolytic crisis; a spontaneous remission cannot, however, be excluded [2]. The clinical picture in these cases is similar to that observed in postinfectious AIHA of childhood [4, 10].

A precise evaluation of the results of prolonged corticotherapy creates many difficulties especially because AIHA itself has a tendency to alternate remissions and relapses. The low percentage of favourable results in our material (35%) in comparison with those reported in the literature (40 to 60%) [5], is probably due to the fact that in our study the periods of observation were long enough to note intolerances or serious side effects of corticosteroid therapy. Prolonged corticotherapy was somewhat more effective in idiopathic than in secondary AIHA but the number of patients is too small to draw final conclusions.

According to the literature cytotoxic drugs alone or combined with corticosteroids give positive results in 47 to 64% [5, 9]. In our material favourable results were observed in 60.5%. The therapeutic potency of combined treatment was, however, greater than of corticosteroids alone in 18.6% of cases. In the remaining patients the major advantage was the possibility of dose reduction of poorly tolerated corticosteroids without losing the control of haemolysis. Unfortunately, neither clinical nor serological signs have been found to be of prognostic value in prediction of the late results of combined treatment. The results were favourable in idiopathic and in secondary AIHA.

Splenectomy was found to be an effective therapy of AIHA, with 62% excellent and good results, and 19% fair results. This is at the upper limit of the favourable results of Bowdler's survey where the range varied from 30 to 75% [3]. The prognostic significance of the analyzed factors for the prediction of late results of splenectomy was of limited value. As it was suggested by Bowdler [3], in our material also the complement on erythrocytes seemed to diminish the response to splenectomy in IgG-mediated haemolysis, but from the small number of cases no final conclusion can be drawn. Despite previous reports about a high correlation between spleen to liver radioactivity ratio and the late results after splenectomy [1], this factor too was of limited value in our material. Its doubtful usefulness has been reported recently also by other authors [6]. Our observation suggests that splenectomy may be indicated not only in idiopathic AIHA but in selected cases of secondary AIHA. It was beneficial in 4 patients with splenic localization of non-Hodgkin's lymphoma. These patients remain disease-free for 3 to 5 years of follow-up. Distinct alleviation (fair results) of the haemolytic process was achieved also in patients with disseminated lupus erythematodes without acceleration of the underlying disease which was sometimes suggested [7]. Our observation indicates also that in selected cases of severe, life-threatening haemolysis resistant to drug therapy, splenectomy may cure the haemolytic process.

When should the patient with AIHA be considered to be cured? It seems that only a stable normalization of positive DAT and disappearance of autoantibodies might be signs suggesting a final cure. Severe bouts of haemolysis after 15 years of full clinical remission (with a very weak persisting positive DAT) in one of our patients showed that as long as autoantibodies are present, there is a high risk of relapse. Nevertheless, an attempt at suspending treatment should be undertaken in patients who are in prolonged clinical remission. This is especially true in splenectomized patients in clinical remission even when the DAT is still positive.

None of the patients died directly from AIHA. Deaths occurred from the underlying disease and less frequently from the side effects and complications of treatment (severe infections in the course of corticotherapy, post-splenectomy complications).

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ABSTRACTS OF SELECTED PAPERS PRESENTED AT THE XIth CONFERENCE ON MEMBRANE TRANSPORT

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Surface and Functional Characteristics of Lymphocyte and Monocyte Subpopulations

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Ontogenesis of lymphocytes and monocytes as well as the stages of differentiation characteristic of subpopulation formation are described together with the most important membrane markers and receptors of T lymphocyte subpopulations (T-helper, T-supressor, killer, T-effector cells), and the differentiation forms of B cell lines.

The differentiation markers, histocompatibility antigens and receptor structures of basic importance in cell cooperation are described in detail. The function and the genetic control of the cell populations are discussed.

The functions related to the structure of biomolecules embedded into the cell membrane surface are still under investigation. A major topic of our present interest is the connection between basic immunological functions and the membrane-bound acceptor–receptor–effector structures.

Investigation of the Mobility of Cell Surface Receptors in Mouse Lymphocytes

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Every cell has connection with its environment through its cell membrane. This connection is a dynamic one and, as a result, is manifold. The isolating and at the same time the information transfer and metabolism intervening role of the cytoplasmatic membrane is ensured by its appropriate structure.

After a survey of the Singer and Nicolson fluid mosaic membrane model, the most widely accepted understanding of the structural and functional properties of cell membranes, the latest experimental methods for the investigation of the dynamic properties of membranes are reviewed. Within these methods a detailed analysis of the depolarization technique carried out with triplet probes

is followed by the description of the FPR (fluorescence photobleaching recovery) method used to examine the lateral mobility of integral membrane proteins.

An account is given on our own receptor mobility investigations in mouse lymphocytes.

Acetylcholinesterase Activity of Lymphocytes from Normal and Leukaemic Blood

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Indirect immunological and histochemical data suggested the presence of acetylcholinesterase (AchE) on normal human lymphocytes. Adaptation of the colorimetric technique of Ellman and the radiometric technique of Fonnum, developed for nerve and/or muscle cells, rendered it possible quantitatively to determine the AchE activity of lymphocytes of different origin. Comparing the AchE activity of normal lymphocytes and that of chronic lymphoid leukaemic (CLL) and acute lymphoid leukaemic (ALL) cells, we have demonstrated a significantly lower activity of CLL lymphocytes as compared to their normal counterparts. The enzyme activity seemed to be correlated to the number of T lymphocytes in the blood. To decide whether only the T-cells possess AchE activity, the lymphocyte subpopulations were separated on Leukopac filter. B lymphocytes had no measurable enzyme activity, while the T lymphocyte fraction represented the total activity demonstrated in the unseparated sample.

On further separation of T-cells on Percoll density gradient, the majority of AchE activity could be demonstrated in the middle region, associated to the T_1 subpopulation.

Since AchE is a lipid-dependent peripheral membrane enzyme, its activity may be affected by the composition of the surrounding lipids and proteins. Although the role of AchE on the plasma membrane of various blood cells is not known, it is a good marker of the integrity and functional state of the membrane. The described difference in AchE activity of the lymphocyte subpopulations seems to be suitable for use as a marker enzyme.

Topo-optical Reactions of the Membrane of Human Lymphocytes

J. MAKOVITZKY

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Polarization microscopic studies combined with topo-optical reactions provided evidence of the molecular construction of the membrane as well as of the conformation of its constituents. The lymphocytes stained with thiazine exhibited a positive topo-optical reaction, the cells an optically positive polarization cross. Lymphocytes stained with quinoline were radially negative birefringent so that it has to be assumed that the dye molecules are deposited parallel to the membrane. In all cases, the topo-optical reaction was affected by a pretreatment with neuraminidase or by acid or alkaline hydrolysis.

The sialic acid component of the membrane of human lymphocytes is not uniform. In collaboration with a group of biochemists we have demonstrated on the surface of lymphocytes a large amount of 9-O-acyl-sialic acid in well-arranged form, besides sialic acid. The positive topo-optical reaction with 1 : 9 dimethyl methylene blue (pH 1–2) can be explained by the occurrence of oriented SO_4 groups on the surface of human lymphocytes.

We selectively demonstrated the RNA component associated with the membrane of human lymphocytes by a specific topo-optical reaction (combined alkaline hydrolysis and/or RNAse digestion). Our histochemical and biochemical results suggest a much more complex membrane composition in the case of human lymphocytes than in that of other cells, for example human erythrocytes.

Active Calcium Transport in Human Peripheral Blood Lymphocytes

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Active calcium transport has a basic function in ensuring a low intracellular calcium concentration in most mammalian cells. Specific changes in cytoplasmic calcium concentration play an essential role in the activation of lymphocytes.

In our experiments lymphocytes, separated from freshly drawn human blood, were loaded with calcium by using the ionophore A23187. The cells were incubated in the presence of A23187 + calcium (+^{45}Ca tracer) at 37 °C for 60–80 seconds,

and thereafter the ionophore was eliminated by repeated washings in cold, albumin-containing media. By using this technique intracellular calcium concentration can be varied between 10–2000 μM and, as checked by various methods, elimination of the ionophore is complete under these conditions.

According to our results, the calcium-loaded intact lymphocytes possess an ATP-dependent rapid calcium extrusion which is activated by intracellular calcium and is practically insensitive to changes in calcium concentration in the media. The calcium pump in lymphocytes is insensitive to ouabain, oligomycin or 2-4-dinitrophenol (DNP). There is no measurable Na-sensitive fraction of the calcium efflux from lymphocytes, while external lanthanum completely blocks calcium pumping. Part of the cellular calcium is sequestered into organelles and can only be liberated by the addition of A23187 ionophore. Our aim is to further investigate the regulation and characteristics of active calcium transport in intact lymphocytes and in lymphocyte membrane preparations.

Demonstration of the Ca-dependent K(Rb)-transport in Human Lymphocytes

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Activation of the K, Na-pump on the stimulation of lymphocytes by concanavalin A or phytohaemagglutinin has been shown by several teams. In addition, Negendank and Shaller in 1977 reported a net K-efflux in the early phase of lymphocyte stimulation, without significant changes in cell Na. This K-efflux may play a role in the activation of the K, Na-pump. In 1979, Freedman demonstrated an early Ca-influx in spleen lymphocytes of the mouse on concanavalin A treatment. These findings urged us to check whether lymphocytes possess the Ca_i-induced K-transport system (= Gárdos-effect), well known in the red cells of various species and detected also in some other tissues.

Lymphocytes from fresh human blood were prepared by Ficoll-Uromiro gradient centrifugation after iron powder treatment and were separated from platelets by washings at low speed centrifugation. Cells were either loaded with ^{86}Rb and Rb-efflux (with and without ouabain) was measured or ^{86}Rb-uptake was followed in the presence of ouabain (i.e. with an inhibited K, Na-pump). Using the Ca-ionophore A23187 or acetylcholine + neostigmine as stimuli, ouabain-insensitive Rb-transport rate increased rapidly with no similar change in Na-transport. In short-term experiments the process was dependent on external Ca, and resulted in a 12–15% reduction of the cell size. The inhibitors of the Gárdos-effect (0.5 mM quinine, 3 mM chlorobutanol, 50 μM chloropromazine) significantly inhibited the rapid ouabain-insensitive Rb-transport. Rb-transport

rate correlated with the ^{45}Ca-uptake measurements, and was reduced after the Ca-pump mechanism had been activated by norepinephrine. On the other hand Ca-uptake was accelerated by hyperpolarization of the cell membrane due to the selectively increased K(Rb) permeability and was reduced by inhibiting the Ca-sensitive K-efflux by quinine or chlorobutanol. Ca-sensitive K-transport in lymphocytes might play a physiological role by a) increasing the rate of the early Ca-influx on stimulation and b) activating the K, Na-pump. Further experimentation is needed to decide whether or not this regulatory effect represents an obligatory step in the sequence of events in lymphocyte stimulation.

Preparation and Characterization of Membrane Subfractions of Purified Lymphocyte Plasma Membranes

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Institute of Virology, German Cancer Research Centre, Heidelberg, FRG and Institute of Biochemistry, Department 1, Semmelweis University Medical School, Budapest, Hungary

A method is described for the subfractionation of plasma membrane vesicles from thymus lymphocytes by means of affinity chromatography on concanavalin A sepharose.

Thymus lymphocytes were disrupted by nitrogen cavitation, microsomal membranes were isolated by differential centrifugation and plasma membranes were purified from microsomes by sucrose gradient ultracentrifugation. Plasma membranes were highly purified as indicated by marker enzyme activities and chemical analysis. To obtain membrane preparations suited for lectin-dependent affinity-chromatography, sucrose was removed slowly by gradient dialysis. Plasma membranes were then equilibrated for 20 min at 4 °C with conA-sepharose which allowed the separation of membranes into a fraction eluting freely (MF_1) and a second fraction binding to the affinity absorbent (MF_2) with a total recovery of about 90%. Increasing the temperature or binding time did not alter the amount of the plasma membrane subfraction bound to conA-sepharose.

Fractionation required the binding of matrix bound conA to plasma membrane binding sites as in the presence of α-methyl-mannoside no separation occurred. Both plasma membrane subfractions proved to have preserved their original orientation (right side out) as revealed by sialidase treatment.

The plasma membrane fraction specifically bound to the affinity absorbent and recovered after α-methyl-mannoside treatment (MF_2) contained specific activities of ($Na^+ + K^+$)-ATPase and lysolecithin acyltransferase several-fold higher than in the non-adherent fraction (MF_1). When the non-adherent fraction was

rechromatographed on conA-sepharose it was nearly completely depleted of these enzymes. In contrast, other plasma membrane bound enzymes (Mg^{2+}-ATPase, alkaline phosphatase and γ-glutamyl-transferase) showed a random distribution in the plasma membrane subfractions. After conA stimulation for 1 hour the activity of ($Na^+ + K^+$)-ATPase and lysolecithin acyltransferase was increased by about 90 and 200%, respectively, in the membrane subfraction specifically bound to conA-sepharose (MF_2). In contrast Mg^{2+}-ATPase, alkaline phosphatase and γ-glutamyl-transferase showed decreased activity in both plasma membrane subfractions.

Our results suggest a close association of receptors with high affinity for conA and certain membrane bound enzymes, representing a functional mosaicism in the vicinity of the high affinity mitogen receptors.

The Role of the Plasma Membrane in the Regulation of Lymphocyte Stimulation

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A great number of membrane bound enzymes has been found to be affected after the binding of mitogens and antigens to the high-affinity receptors of the lymphocyte plasma membrane. Previously, in experiments analyzing the receptor-associated functions an intimate linkage of at least two enzymes emerged, which respond immediately to the binding of mitogens: ($Na^+ + K^+$)-ATPase and lysolecithin acyltransferase. The close anatomical association of both enzymes supported the idea of a possible functional coupling between ($Na^+ + K^+$)-ATPase and lysolecithin acyltransferase, the latter being responsible for the elevated phospholipid turnover in stimulated lymphocytes. We have suggested that the increase in the degree of fatty acid unsaturation due to activated acyltransferases underlies the changes of plasma membrane enzymes in stimulated lymphocytes. To prove this, isolated plasma membranes were incubated with increasing concentrations of the coenzyme A derivatives of long-chain fatty acids in the presence of lysolecithin. Control experiments showed that added acyl CoA was nearly completely converted into membrane associated lecithin. Lysolecithin alone or incorporation of palmitoyl CoA or oleioyl CoA had no effect on the specific activity of any of the plasma membrane bound enzymes tested. Incorporation of linoleyl CoA and arachidonoyl CoA modulated these enzymes dose dependently: the activity of the ($Na^+ + K^+$)-ATPase and lysolecithin acyltransferase itself were increased, maximally when 150–200 nmol polyunsaturated fatty acids were incorporated by mg membrane protein. At higher

uptake, enzyme activity returned to the control level. Concomitantly, the activity of Mg^{2+}-ATPase and γ-glutamyl transferase decreased, depending on the degree of fatty acid substitution. Inhibition of acyltransferase by merthiolate during incubation of membranes with polyunsaturated fatty acids left all enzyme activities unchanged indicating that the transfer of these fatty acids to phospholipid is required for modulating the activity of membrane associated enzymes. The changes observed in the specific activity of plasma membrane enzymes after increasing the content of polyunsaturated fatty acids strikingly resembled the changes observed in the membranes of stimulated lymphocytes. Investigating the role of $(Na^+ + K^+)$-ATPase we made use of the fact that ouabain, the specific inhibitor of $(Na^+ + K^+)$-ATPase, prevented lymphocyte stimulation most probably by a mechanism different from the inhibition of cation transport activity of the enzyme. The time kinetics of the inhibition of RNA synthesis by ouabain indicated that ouabain interfered with a membrane associated event immediately following the binding of the mitogen. Ouabain exactly at the concentrations being effective in suppressing macromolecular synthesis inhibited one of the earliest changes occurring in stimulated cells, e.g. the increased incorporation of oleate into lecithin. A similar inhibition of oleate uptake occurred in isolated microsomes following ouabain treatment. After conA stimulation for 1 hour the activity of $(Na^+ + K^+)$-ATPase and lysolecithin acyltransferase was increased in isolated plasma membranes by about 80 and 200%, respectively. Pre-incubation with ouabain before conA stimulation prevented the activation of both enzymes. Lysolecithin acyltransferase was unaffected by monovalent cations over a wide range, suggesting that the inhibition of its activation was not due to cation shifts. Binding of ouabain as well as the concomitant suppression of conA induced activation of macromolecular synthesis and oleate incorporation could be reversed by elevating the extracellular K^+ concentration.

Our results suggest a functional interrelationship between $(Na^+ + K^+)$-f ATPase and lysolecithin acyltransferase specifically implicated in the activation of lymphocytes and thus involved in the plasma membrane dependent regulation of cell growth and division.

The Application of Fluorescent Methods in Surface Receptor Studies on Rat Peritoneal Macrophages

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The Fc and C3b receptors of macrophages play an important role in phagocytosis. Fluorescent methods offer new possibilities in the study of the ingestion mediated by both types of receptors. A quantitative spectrofluorimetric

method has been elaborated (VRAY et al., *Scand. J. Immunol. 11*, 147, 1980) to determine the ingestion of FITC-labelled bacteria. The results obtained by this method combined with simultaneous microscopic observations provide important data about the phagocytic process.

FITC-labelled, heat aggregated IgG was applied to study the Fc receptors. Using the fluorescent method, the number and saturation of Fc receptors and other binding properties were determined on rat peritoneal macrophages elicited by thioglycolate. The proportion of FITC-labelled aggregate binding cells was estimated by fluorescent microscopy. The effect of this IgG-aggregate binding was studied on the phagocytes by both types of receptors. Inhibition could not be observed in any case but binding and ingestion were exclusive in a given cell using FITC-labelled aggregate and RITC-labelled *Micrococcus lysodeikticus* bacteria.

The dependence of ingestion upon time and the number of bacteria were measured. The C3b-mediated phagocytosis – on elicited macrophages – exceeded the Fc-mediated process. Enhancement of the ingestion is due to the increase of the number of ingested bacteria without a considerable change in the proportion of ingesting phagocytes.

It was shown that a N-formylated tripeptide having chemotactic properties caused a considerable increase in the ingestion of non-stimulated macrophages while it had no effect on elicited cells. Comparing these results to other investigations, one may conclude that some relation must exist between chemotaxis and the stimulation of phagocytosis.

^{86}Rb Uptake by Various Populations of Mouse Splenocytes

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A number of data show that lymphocyte activation is closely combined with relevant alterations of K^+ transport. Moreover, evidence has been reported suggesting a correlation between changes of DNA synthesis and those of K^+ transport in lymphocytes under activation. These phenomena have been extensively studied in the course of mitogenic stimulation of lymphocytes.

Few studies were devoted to related phenomena taking place under allogeneic stimulation of lymphocytes. The present work was undertaken to throw light upon the changes in K^+ transport of lymphocytes under allogeneic stimulation.

Splenic lymphocytes were obtained from different strains (CBA, A, B_{10}) of inbred mice and incubated in RPMI 1640 medium supplemented with 10% fetal calf serum in a CO_2 thermostat. The K^+ transport of splenocyte populations from single strains as well as that of mixed populations from two strains was analyzed

for 6 days by determining the time course of ^{86}Rb uptake each day for 4 hours. K^+ content of the cells was simultaneously measured by atomic absorption spectrometry.

Both K^+ content and ^{86}Rb uptake rates of the splenocyte populations from the single strains showed a significant decrease during the 6-day-period of incubation. The expression of these phenomena in the above 3 strains was different and the variations suggested a correlation between immune-reactivity of splenocytes and features of K^+ transport. In contrast, mixed populations of the splenocytes from two of the above strains lost significantly less K^+ as compared to those lost by single strains. The decrease of ^{86}Rb uptake rate in the course of 6-day incubation was also insignificant in the mixed populations of splenocytes.

Our findings suggest that K^+ transport is somehow involved in the set of events leading to allogeneic stimulation.

Malondialdehyde Production Influenced by Cytochrome C and its Haemnonapeptide in Human Platelets

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Cytochrome c ("Cytomack") is used parenterally as a drug in hypoxic conditions. Some authors reported that it might also reduce lipid peroxidation and atherosclerosis. Others demonstrated the uptake and perinuclear localization of cytochrome c in hepatocytes. Oral administration was found effective, although the alkaline protein (MW 11 800) was cleft proteolytically. Based on these data cytochrome c was digested with trypsin and a haemnonapeptide fragment (MW 1779) was purified by ion-exchange chromatography.

The effect on lipid peroxidation of the intact cytochrome c molecule was compared with that of the haemnonapeptide fragment in human platelets by measuring malondialdehyde (MDA)-formation.

It was demonstrated that both cytochrome c and the haemnonapeptide fragment after a temporary slight reduction increased MDA-production with a maximum at 30–60 minutes. The dose dependence of the two compounds was similar. Cytochrome c at therapeutic concentrations exerted a half maximum effect. Extracellular calcium was needed to increase MDA-production, which did not surpass 50% of that achieved by treatment with 10 mM N-ethylmaleimide. The increased MDA formation could be inhibited almost completely by acetylsalicylate. Haem, ferri-ions or protoporphyrin were without any effect.

From the above data we concluded that the intact cytochrome c molecule and its haemnonapeptide fragment affect platelets basically in the same way. They increase the Ca-permeability of the plasma membrane and induce lipid peroxidation at the acetylsalicylate sensitive (cyclooxigenase) pathway. In therapeutic doses one has to consider the side-effects of cytochrome c on platelets.

Vesiculation and Reversible Intramembrane Particle Aggregation of Nonhaemolyzed Human Erythrocytes

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By means of freeze-fracture electron microscopy we detected that acridine orange in 10–15 mmol concentration caused vesiculation of nonhaemolyzed human erythrocytes. The vesicles were empty, mostly unilamellar, free of intramembrane particles (IMP) and could be separated easily from the erythrocytes. During vesiculation, the IMP-s of the nonhaemolyzed red cells showed strong aggregation in the presence of the dye. Removal of the dye resulted in rapid and complete disaggregation of the IMP-s, in addition an increased particle density was observed in a considerable number of erythrocytes. Brillantcresyl blue caused similar alterations, while rivanol, quinacrine and orange G had no such effects. By thin layer chromatography the ratio of the different phospholipid classes of the vesicle-depleted red cell membranes showed no significant alteration relative to the control, similarly, the cholesterol to phospholipid ratio was also equal to that of the control indicating no selective lipid segregation into the vesicles. The ultrastructural localization of the cholesterol in the vesicles and red cell membrane was detected with filipin. The filipin-sterol complexes showed highest density in the particle-free vesicles, they were numerous in the particle-depleted areas of membranes showing IMP aggregation, while the IMP clusters completely lacked these complexes. These results are discussed in terms of the haemoglobin-membrane interaction, the translational mobility of the IMP-s and the ultrastructural detectability of the cholesterol within the plasma membrane.

Book Review

Progress in Haemostasis and Thrombosis. Vol. 5. Ed. by Theodore H. Spaet. Grune and Stratton, New York, London, Toronto, Sydney, San Francisco 1980. 298 pages. Price: $ 39.50.

The 5th volume of *Progress in Haemostasis and Thrombosis*, edited by T. H. Spaet, deals with a series of subjects connected in some way with haemostasis, where many new data have been collected in recent years. Uncovering the relationships in these fields may be valuable to specialists and researchers of haemostasis.

The role of the condition of the vessel wall in the process of haemostasis and thrombosis is well known, however handbooks on haemostasis treat this subject, as well as questions of bleeding of vascular origin, rather scantily. Two chapters in this book elaborate on these problems. T. N. Wight discusses the role of vessel proteoglycans in the elasticity and permeability of vessel walls and in the development of atherosclerosis. C. S. Kitchens writes in his chapter "The Anatomic Basis of Purpura" about the pathophysiology of purpura accompanying various diseases.

The reactions of blood coagulation are often regarded as completed after fibrin and the blood clot have been formed. Still, as is revealed in D. F. Mosher's chapter on fibronectin, important processes occur following fibrin formation where fibronectin plays a central role. This chapter also discusses the role of fibronectin in fibrinolysis, in cell migration, in the opsonization of bacteria and in various other processes. In the events that follow the polymerization of fibrin, Factor XIII can be considered a key enzyme. L. Loránd, M. S. Losowsky and K. Miloszewski discuss the role of Factor XIII in the stabilization of fibrin, as well as the various Factor XIII deficiency conditions.

In recent years great progress has been made in exploring the relationship between platelet membrane glycoproteins and various platelet reactions. D. R. Phillips gives a good summary of the role of membrane glycoproteins in the adhesion and aggregation reaction of thrombocytes.

The most practical chapter in the volume, "The Significance of Abnormal Preoperative Hemostatic Tests" by E. J. Walter Bowie and C. A. Owen, evaluates the interpretation of laboratory tests from the clinician's point of view. This study is valuable not only to the surgeon, but also physicians who treat patients prone to bleeding or thrombosis.

Susan Elődi

Abstracts

The isolation and characterization of 60 nm vesicles ("nanovesicles") produced during ionophore A23187-induced budding of human erythrocytes. D. Allan, P. Thomas and A. R. Limbrick (Department of Experimental Pathology, School of Medicine, University College London, and Department of Biophysics, University College London, London). *Biochem. J. 188*, 881 (1980).

In addition to the microvesicles released during the treatment of human erythrocytes with Ca^{2+} and ionophore A23187, a new subpopulation of still smaller dense vesicles ("nanovesicles") has now been identified. Nanovesicles are about 60 nm in diameter, have an acetylcholinesterase activity higher than that of microvesicles and appear to be rich in sphingomyelin and correspondingly depleted of phosphatidylethanolamine. They have a polypeptide composition quite different from those of erythrocyte membranes or microvesicles, consisting largely of components of mol.wt. 60 000 and 26 000 in addition to haemoglobin. These two major polypeptides do not appear to represent contaminating cytoplasmic proteins or proteolytic subfragments of a larger protein.

G. Gárdos

A re-evaluation of the surface complexity of the intact erythrocyte. S. Thompson, C. M. Rennie and A. H. Maddy (Department of Zoology, University of Edinburgh, Edinburgh, U.K.). *Biochim. biophys. Acta 600*, 756 (1980).

Surface proteins and glycoproteins on intact human red blood cells were labelled with ^{125}I by the lactoperoxidase method. The radioactive proteins were then separated in each of the Fairbanks and Laemmli one-dimensional polyacrylamide gel electrophoresis systems. The radioactive polypeptides had different mobilities in the two systems, due largely to the anomalous migration of glycoproteins in polyacrylamide gel. A two-dimensional system was therefore developed using the Fairbanks and Laemmli buffer systems to explore these anomalies. This procedure clearly resolved radioactive glycoproteins and proteins and allowed the identification of many more surface components than had previously been possible.

G. Gárdos

The monosaccharide transport system of the human erythrocyte. Orientation upon reconstitution. J. M. Baldwin, G. E. Lienhard and S. A. Baldwin (Department of Biochemistry Dartmouth Medical School, Hanover, N.H.). *Biochim. biophys. Acta 599*, 699 (1980).

Treatment of intact human erythrocytes with trypsin had no effect upon either the rate of hexose transport or the binding of cytochalasin B to the transport system. In contrast, proteolysis of inside-out vesicles prepared from human erythrocyte membranes inactivated both hexose transport and cytochalasin B binding. When purified hexose

transporter, reconstituted into phospholipid vesicles of undetermined size, was treated with trypsin, approx. 50% of the cytochalasin B activity was lost. This loss correlated with a decrease in the amount of the transporter polypeptide, as assayed by gel electrophoresis. These results show that the orientation of the transporter can be established through trypsin treatment in conjunction with cytochalasin B binding. Small unilamellar vesicles containing transporter were prepared by sonication of larger species and by a cycle of cholate solubilization and removal of the detergent. In the former case, the transporter orients almost randomly, whereas in the latter approx. 75% of the transporters have the cytoplasmic domain external.

G. Gárdos

Association of glyceraldehyde-3-phosphate dehydrogenase with the human red cell membrane. A kinetic analysis. H. J. Kliman and T. L. Steck (Department of Biochemistry, The University of Chicago, Chicago, Ill.). *J. biol. Chem. 255*, 6314 (1980).

A rapid filtration technique was used to analyze the kinetics of the binding reaction between glyceraldehyde-3-P dehydrogenase and the band 3 protein of the human erythrocyte membrane. Saponin was used to eliminate the membrane as a rate-limiting barrier. Re-equilibration of the enzyme following dilution of membranes in buffer took only a few seconds. Dissociation rates were greatly stimulated and association rates were reduced by increasing ionic strength. A Brönsted–Bjerrum analysis suggested that two or three charges of opposite sign on each protein were involved in binding. NADH appeared to elute the enzyme by interaction with its catalytic site, but the band 3 binding site extended beyond the NADH site, permitting the formation of a ternary NADH–enzyme–membrane complex. Release of the enzyme from fresh erythrocytes immediately following saponin lysis showed kinetics similar to the release of enzyme from ghosts. The extrapolated zero time intercepts of these reactions suggested that two-thirds of cellular glyceraldehyde-3-P dehydrogenase was membrane bound prior to hemolysis. This value is similar to that calculated for the association of the enzyme with band 3 in the intact red cell when the high ionic strength and enzyme-eluting compounds in the cytoplasm are taken into account.

G. Gárdos

The location of a disulfonic stilbene binding site in band 3, the anion transport protein of the red blood cell membrane. M. Ramjeesingh, A. Gaarn and A. Rothstein (Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada). *Biochim. biophys. Acta 599*, 127 (1980).

The binding site for 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid, a specific, potent, irreversible inhibitor of anion transport in red blood cells, is located in a 15 000 dalton transmembrane segment of band 3, produced by chymotrypsin treatment of ghosts stripped of extrinsic proteins. The segment was cleaved by CNBr into three fragments of 7000, 4000 and 4000 daltons. The C-terminal of the segment is located in the 7000 dalton fragment; the N-terminal in one of the 4000 dalton fragments; and the binding site for 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid in the middle 4000 dalton fragment. This last fragment was cleaved by N-bromosuccinimide into two fragments of 2000 daltons. The binding site for 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid was located on the fragment containing the newly formed N-terminal. It is concluded that the binding site is located about 9000 daltons from the C-terminal (at the outside face of the membrane) and 6000 daltons from the N-terminal (at the cytoplasmic face). In view of the existing evidence, the binding site may be located near the outside face of the membrane, it is suggested that the 15 000 dalton is folded, so that it crosses the bilayer three times.

G. Gárdos

Studies on a soluble human erythrocyte protein kinase. K. W. Simkowski and M. Tao (Department of Biological Chemistry, University of Illinois at the Medical Center, Chicago, Ill.). *J. biol. Chem. 255*, 6456 (1980).

A cyclic AMP-independent protein kinase has been isolated from human erythrocyte

cytosol and purified about 28 000-fold. The enzyme uses ATP as the phosphoryl donor and exhibits a high activity towards casein and phosvitin. The kinase has a molecular weight of about 30 000 to 32 000 as estimated by sucrose density gradient centrifugation and Sephacryl S-200 gel filtration. Upon isoelectrofocussing, two kinase activities are resolved. The major component, which comprises about 85% of the activities, focusses at a pH of about 9.6 and the minor component, at about 4.6. The relationship between these two kinase activities is not clear. NaF, ADP and, to a lesser extent, AMP, all inhibit the kinase activity. 2,3-Diphosphoglyceric acid, on the other hand, has no effect. The kinase also catalyses the phosphorylation of a number of erythrocyte membrane proteins including spectrin, band 3, and the sialoglycoproteins.

Ilma Szász

The interaction of deoxyhemoglobin with the red cell membrane. N. Shaklai and H. Abrahami (Department of Chemical Pathology, Sackler School of Medicine, Tel Aviv University, Israel). *Biochem. biophys. Res. Commun. 95*, 1105 (1980).

The interaction of deoxyhemoglobin with the red cell membrane is characterized by comparing the affinity of deoxyhemoglobin for the membrane with that of oxyhemoglobin. The two techniques used, namely light scattering induced changes and quenching of the fluorescence intensity of a membrane embedded probe, demonstrate that deoxyhemoglobin has a much lower affinity with the membrane than has oxyhemoglobin. The binding constant of 2×10^6 M^{-1} calculated for deoxyhemoglobin at 5 mM phosphate buffer and pH = 6.0 is two orders of magnitude lower than the one calculated for oxyhemoglobin. It seems that under physiological conditions the only species capable of interacting with the membrane is the oxyhemoglobin.

Ilma Szász

Is "deformability" a parameter for the rate of elimination of erythrocytes from the circulation? C. W. M. Haest, G. K. Driessen, D. Kamp, H. Heidtmann, T. M. Fischer and M. Stöhr-Liesen (Abteilung Physiologie, Medizinische Fakultät, Rheinisch-Westfälische Technische Hochschule, D-5100 Aachen, BRD). *Pflügers Arch. 388*, 69 (1980).

The "deformability" of rat erythrocytes can gradually be decreased by treatment *in vitro* with the SH-oxidizing agent diamide. Despite this reduced deformability, the cells are retained in the circulation for many hours when reinfused into the rat. Cells made rigid by glutaraldehyde are even less deformable than diamide treated cells, but also survive for many hours. In contrast to rigidified cells of normal volumes, swollen rigidified cells obtained by a heat treatment of erythrocytes are rapidly eliminated. The results support the view that the "recognition" preceding the elimination of senescent and damaged cells of normal volumes by the reticulo-endothelial system is not solely based on their diminished deformability.

Ilma Szász

Effect of reduced red cell "deformability" on flow velocity in capillaries of rat mesentery. G. K. Driessen, C. W. M. Haest, H. Heidtmann, D. Kamp and H. Schmid-Schönbein (Abteilung Physiologie, RWTH Aachen, D-5100 Aachen, BRD) *Pflügers Arch. 388*, 75 (1980).

A graded reduction of "deformability" of rat red blood cells (RBC) was obtained by treatment with the SH-oxidizing agent, diamide. Rigidified RBC's were injected into rats by isovolaemic exchange against 60% of the native RBC, and RBC flow velocities in capillaries of the rat mesentery were measured. At normal mean arterial pressure, RBC flow velocity decreased by 29% in rats receiving cells rigidified with 0.5 mmol $\cdot$ l^{-1} diamide. Surprisingly, a further rigidification of erythrocytes by 1.5 mmol $\cdot$ l^{-1} diamide resulted in a decrease of flow by only 15%. During hypotension RBC flow velocities dropped precipitously to 8 ± 15% for the 0.5 nmol $\cdot$ l^{-1}, and to 2 ± 6% for the 1.5 mmol $\cdot$ l^{-1} diamide group as compared to velocities during normotension. Microscopy revealed a stop of flow in many vessels. This result outlines the importance of a norma

red cell "deformability" for the maintenance of sufficient perfusion of the microcirculation, in particular at low blood pressure gradients.

Ilma Szász

Stoichiometry of wheat germ agglutinin as a morphology controlling agent and as a morphology protective agent for the human erythrocyte. R. E. Lovrien and R. A. Anderson (Biochemistry Department, Gortner Laboratory, Biological Sciences, University of Minnesota, St. Paul, Minn.). *J. Cell Biol. 85*, 534 (1980).

The lectin wheat germ agglutinin (WGA) is an unusually effective agent in controlling both the forward and reverse reactions of the reversible morphology conversion discocyte ⇄ echinocyte for the human erythrocyte. Under conditions severe enough to drive the reactions to completion in either direction without the lectin, WGA is able to stabilize both these morphologies and to fully prevent conversion of either morphology. The lectin can quantitatively block both reactions. The ability of WGA to carry out these functions has no obvious rate limitation. Its effectiveness depends mainly on its binding stoichiometry, particularly toward the transmembrane glycoprotein, glycophorin. The critical binding stoichiometries for both the lectin and echinocytic agent were determined in relation to the binding isotherms using ^{125}I-labelled WGA and ^{35}S-labelled dodecyl sulphate. There appear to be two principal stoichiometries for WGA binding that are important in its control of erythrocyte morphology. The first stoichiometry marks the threshold of obvious protection of the discocyte against strong echinocytic agents such as detergents and, likely, is simply a 1 : 1 stoichiometry of WGA : glycophorin, assuming currently recognized values of $3–5 \times 10^5$ copies of glycophorin per cell. The second important stoichiometry, whereby the cell's morphology is protected against extremely severe stress, involves binding of ~4–5 WGA molecules per glycophorin. The controls that WGA exerts can be instantly abolished by adding N-acetylglucosamine. N-acetylglucosamine ligands on the erythrocyte are, however, of less importance than membrane neuraminic acid residues in enabling WGA to control the cell's morphology, as is shown by comparing intact cells with completely desialated cells. WGA can also be used to produce elliptocytes *in vitro*, but it does this at levels approaching monolayer coverage of the cell with WGA.

Ilma Szász

Acknowledgement to Reviewers

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5th EUROPEAN IMMUNOLOGY MEETING
Istanbul, 1–4, June 1982

CONGRESS ORGANIZING COMMITTEE

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the Turkish Society of Immunology

VENUE

The 5th European Immunology Meeting will take place at the Atatürk Cultural Centre at Taksim Square, Istanbul, Turkey. 1–4 June 1982.

LANGUAGE

The official language of the Meeting is English. There will be no simultaneous translations.

REGISTRATION FEES

Deadline: 31st January 1982.
Participation in the Meeting is subject to payment of the following registration fees:

	Before 31. 1. 1982 (Postmark)	*After* 31. 1. 1982 (Postmark)
Member (IUIS or EFIS)	US $ 100.–	US $ 120.–
Non-Member	US $ 120.–	US $ 140.–
Social Member	US $ 50.–	US $ 60.–

SCIENTIFIC PROGRAMME

Preliminary Scientific Programme in outline presented below:

Main Lectures

K. Eichmann: Idiotypic network
T. Tada: Antigen specific T-cell factors, their roles in the immunological circuit
R. M. Zinkernagel: MHC-restriction of cytotoxic T-cells
M. F. Greaves: Cell differentiation and membrane glycoproteins of normal and leukaemic lymphoid cells
E. Kabat: Immunoglobulin structure

Symposia

1 – Complement genetics (P. Lachmann)
2 – Pathophysiological mechanisms of immediate-type hypersensitivity reactions (A. de Weck)
3 – Auto-immunity (R. S. Schwartz)
4 – Immunomodulation (L. Chedid)
5 – Natural immunity and macrophage activities (M-L. Lohmann-Matthes)
6 – Immunity in infectious diseases (A. C. Allison)
7 – Cellular cooperation and lymphocyte receptors (H. Wigzell)
8 – Recent aspects of immunodeficiency (C. Griscelli)
9 – Hybridomas (G. Köhler)
10 – MHC and immunity (H. Festenstein)
11 – Amyloidosis (J. B. Natvig)
12 – Veterinary immunology (Ch. Pilet)
13 – Molecular biology: Ig genes (T. Rabbitts)

In addition there will be 20 workshops in several interesting fields of immunology.

FOR FURTHER SCIENTIFIC INFORMATION

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LADY TATA MEMORIAL TRUST INTERNATIONAL AWARDS FOR RESEARCH ON LEUKAEMIA AND ALLIED CONDITIONS

ACADEMIC YEAR 1982—1983

The Trustees of the Lady Tata Memorial Trust invite applications for Awards for personal support for research on leukaemia, in the Academic Year beginning 1st October, 1982. In view of the affinity between leukaemia and other forms of neoplastic disease, candidates with programmes of research on any aspect of malignant diseases which may throw light on problems of leukaemia will be eligible for consideration. The Trustees specially wish to encourage studies of the leukaemogenic viruses in animals, the epidemiology, pathogenesis, and immunology of leukaemia.

Awards are open to suitably qualified investigators of any nationality, working either in their own institutions or in other centres abroad.

Stipends will be adjusted according to the place and Institution at which the work will be done, with provision for annual increments and superannuation if required (usually in recent years between £ 4—8000).

Awards are tenable for 1 year in the first instance, renewable up to a maximum of 3 years.

Further particulars and forms of application may be obtained by writing to the Secretary of the European Scientific Advisory Committee, Lady Tata Memorial Trust, Mrs. MIGLIERINA, Unité 108 INSERM, Hôpital Saint-Louis, 2, place du Dr. Fournier. 75475 PARIS CEDEX 10, France. Applications must be submitted before 1st March, 1982 and awards will be announced by the Trustees in June.

Errata

E. Carapella-De Luca, M. Purpura, I., I. Coghi, M. Nicotra, E. Bottini: Blood groups and histocompatibility antigens in habitual abortion. *Haematologia 13*, 105 – 111 (1980).

Page 108, Table 2, column 7, last line should read 0.140.
Page 108, last line should read $p < 0.05$.

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R. Machovich, I. Horváth

Thrombin and haemostasis: regulation of the biological functions of thrombin. Haematologia *14*, 339 (1981).

Thrombin plays an essential role in the maintenance of haemostatic balance through several biochemical reactions. Its functions as well as the regulation of its activities seem to be decisive in controlling thrombus formation. The present paper endeavours to give a brief summary of the control of thrombin activity and the regulation of biological actions of thrombin. As to the control of the amount of the active enzyme, three possible ways are discussed, i.e. prothrombin synthesis, activation of prothrombin, and inactivation of thrombin by plasma proteinase inhibitors (antithrombin III, α-2-macroglobulin, α-1-proteinase inhibitor). Regarding the regulation of the various actions of thrombin, the most important biological targets of this enzyme are summarized, namely interaction with fibrinogen, activation of Factor XIII, Factor IX, Factor VIII, Factor V and prothrombin, and the binding to platelets, fibroblasts and endothelial cells. The limits of our present knowledge about the determination of the possible reaction routes catalyzed by thrombin are also considered. Finally, a tentative hypothesis is put forward to explain how endogenous modulators like heparin determine the biological functions of thrombin.

A. Aviel, E. Nir

Cytomorphology and lysosomal enzyme activity of the haemopoietic and reticuloendothelial system of the SJL/J mouse. Haematologia *14*, 361 (1981).

The cytomorphology and lysosomal enzyme activity of the haemopoietic and reticuloendothelial system (RES) of SJL/J mice, which develop a spontaneous reticulum cell neoplasm type B (RCN-B), were studied. The cytological findings during development of the disease were a predominance of medium-sized lymphocytes and an increase in the number of hyperbasophilic cells and plasma cells in the RES. These pathological findings were compared to the changes due to ageing in control groups of healthy C3H/eB mice. Acid phosphatase activity increased in the RES, whereas that of β-D-glucuronidase remained unchanged during development of the disease. These findings indicate that the RCN-B of the SJL/J mice bear a certain resemblance to Hodgkin disease in man.

B. Kotelba-Witkowska, B. Żupańska, E. Brojer, J. Daszyński, A. Gaczkowski

Predictive value of lymphocytotoxic test and platelet aggregometry for the effect of transfused platelet concentrates. Haematologia *14*, 375 (1981).

Sera of patients with thrombocytopenic diathesis before platelet transfusion were evaluated using donor's platelets in the aggregometry test and donor's lymphocytes in the lymphocytotoxicity test. In most cases the results of both tests were in agreement, although sometimes, despite strongly positive results of LCTT, the Agr was negative.

Both tests showed a similar value for the prediction of the effectiveness of platelet concentrates: Agr in 80% and LCTT in 71% of cases.

O. F. Ramos, E. C. Morón, R. De Castro Arenas

Platelet function abnormalities in acute leukaemia. Haematologia *14*, 383 (1981).

Studies on platelet function *in vitro* were done in fourteen patients with different kinds of acute leukaemia. Quantitative clot retraction was abnormal in all patients. Aggregation was different with the various agents tested. Availability of platelet factor 3 was abnormal in six patients. All patients were studied prior to chemotherapy. Two patients were studied in complete remission, as well; they showed an almost normal platelet function. Another patient without remission after induction treatment displayed a platelet function worse than in the initial study.

The results suggest that the defect in leukaemic platelets is due to the leukaemic process itself.

S. Głowiński, K. Worowski

The haemostatic system components of neointima forming in polyester aortic grafts in the early postoperative period. Haematologia *14*, 393 (1981).

The aim of the studies was to determine the content of certain components of the haemostatic system in the neointima of a polyester graft implanted in the dog abdominal aorta over a 7-day period of observation.

The neointima of an aortic polyester graft in the early postoperative period possesses a high thromboplastic activity, greater than that of the artery or vein, and containing prothrombin, active thrombin, platelet aggregating substances and heparin neutralizing substances. The plasminogen activator activity in the neointima of the polyester graft is also considerably higher than in the intima of arteries and veins.

Yaroslava I. Vygovskaya, V. E. Loginsky, Anisia V. Vorobel, Natalia A. Kondratieva, Annah A. Mazurok, V. V. Korotky, B. R. Kotsay

Lycurium (R-74) in the therapy of chronic lymphocytic leukaemia. Haematologia *14*, 399 (1981).

Lycurim was used in the therapy of 41 CLL patients, in 34 a positive clinical effect was found. The effect was marked in the cases of CLL associated with autoimmune haemolytic anaemia.

The immunodepressive property of Lycurim was confirmed by immunological investigation. It has been shown that the levels of IgG, IgA and IgM fall after Lycurim treatment of CLL patients. At the same time the T-lymphocyte count decreases and a tendency for EAC rosette-forming B-lymphocyte decrease is marked.

J. Berger

Changes in marrow myelopoietic and lymphoid cell counts after repeated cyclophosphamide administration in the rat. Haematologia *14*, 407 (1981).

Rat marrow myeloid and lymphoid counts were estimated after repeated doses of cyclophosphamide injected intraperitoneally. It was found that in order to evaluate the bone marrow response, a quantitative approach is a necessity. Myelotoxic and lymphotoxic effects in the bone marrow following repeated doses of cyclophosphamide were markedly similar to suppression which followed the restriction of food consumption. Relationships between rat white blood and marrow counts were analyzed. Convenience of determination of marrow cellularity for measurement of myelotoxic effects is discussed with respect to studies with repeated administration of cytostatic agents.

B. Fekete, L. Sachno, K. Tamássy, A. Pál, I. Angyal, I. Forrai

Inhibition of antibody-dependent cellular cytotoxicity (ADCC) by normal and pathological sera from patients with advanced lung cancer. Haematologia 14, 417 (1981).

An improved ADCC assay for the detection of cancer-associated serum blocking factors is described. Inhibition of ADCC by sera was performed without preincubation and washing of effector cells. Effects of individual susceptibility of normal effector cells to serum inhibitors and of the background inhibition by normal sera were also avoided. Based on their inhibitory effect on ADCC, normal and tumour sera from lung cancer patients could be distinguished with 81.4 to 100% accuracy, according to effector cell donors and to concentrations of the sera tested.

B. Żupańska, T. Sylwestrowicz, S. Pawelski

The results of prolonged treatment of autoimmune haemolytic anaemia. Haematologia *14*, 425 (1981).

Ninety-seven patients with autoimmune haemolytic anaemia were observed in the period between 1965 and 1979. Corticosteroids were found to be more effective in the acute phase of haemolysis (68.6%) than in the prolonged treatment (35%). Favourable results with combined treatment (corticosteroids plus azathioprine or cyclophosphamide) were achieved in 60.5% of patients, while the therapeutic potency of corticosteroids alone was 18.6%. Splenectomy was effective in 81% of the patients. No correlation between postsplenectomy results and spleen to liver radioactivity ratio was observed.

INSTRUCTIONS TO CONTRIBUTORS

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

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

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
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