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# HAEMATOLOGIA

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## **Intermittent-flow Versus Continuous-flow Mononuclear Cell Apheresis**

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(Received: July 18, 1990; accepted: August 28, 1990)

An intermittent-flow (Haemonetics V-50) and a continuous-flow (Fenwall CS-3000) blood cell separator were compared regarding mononuclear cell (MNC) collection. 17 evaluable V-50 and 24 CS-3000 aphereses were performed on healthy donors. No significant difference was found in MNC yield (median  $6.4 \times 10^9$  on V-50,  $6.3 \times 10^9$  on CS-3000) or in CFU-GM yield (median  $41 \times 10^3$  versus  $43 \times 10^3$  respectively). Nevertheless, red blood cell as well as polymorphonuclear cell contamination was substantially greater on V-50 than on CS-3000, while platelet contamination was slightly lower on V-50 than on CS-3000. The continuous-flow apheresis system CS-3000 is concluded to be more suitable for MNC harvesting than the intermittent-flow system V-50.

**Keywords:** CFU-GM intermittent-flow apheresis, continuous-flow apheresis, lymphocytapheresis

### **Introduction**

The collection of mononuclear cells (MNC) from peripheral blood by a blood cell separator is currently used for a) obtaining hemopoietic cells for cryopreservation which are reinfused following antineoplastic high dose radio/chemotherapy [1, 2, 3], b) procurement of functional lymphocytes with the aim of reinfusing them after ex vivo activation against malignancy (e. g. by interleukin-2) [4, 5] and c) experimental attempts to deplete patient's blood of either non-functional lymphocytes (e. g. in advanced chronic lymphocytic leukemia) [6] or of lymphocytes presumably causing an autoimmune disorder (e. g. in Chron's disease [7], rheumatoid arthritis [8, 9], aplastic anemia [10], multiple sclerosis [11]).

In this study we compared the MNC harvesting capability of an intermittent-flow separator Haemonetics V-50 with that of a continuous-flow separator Fenwal CS-3000.

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## Donors and Methods

### *Donors*

Healthy apheresis donors (age 21-50 years, male:female ratio 14:1) were included in the study. Informed written consent was obtained from each donor prior to apheresis.

### *Apheresis on Haemonetics V-50 (Haemonetics Corp., Braintree, MA):*

8-cycle Autosurge Lymphocytapheresis I Protocol was used with a slightly earlier start of MNC collection during surge (manually closing valve 3 and opening valve 2, 2-3 seconds after the platelet/white cell jump). (According to our previous experience some MNCs were elutriated into the plasma bag before valves 2 and 3 were automatically switched over.) When the platelet/white cell jump did not take place before the first sign of pink colour was observed in the effluent line, valve 3 was closed and valve 2 was opened immediately. (According to our previous experience in this situation, the collection might fail or be delayed which results in too many erythrocytes and polymorphonuclear cells with relatively few MNCs in the apheresis product). Following apheresis, the product was concentrated by connecting the collection bag to the female connection near the blood pump (instead of the surge line which was no longer needed) and pumping it slowly (40 ml/min) into the bowl; after inverting the plasma bag, the MNC concentrate was pumped back to the collection bag.

### *Apheresis on Fenwal CS-3000 (Fenwal Laboratories, Deerfield, IL):*

Procedure No. 1 modified according to the manufacturer's recommendations for MNC harvesting, was used (GRANULO separation chamber; program step No. 60 parameter changed to 1750, No. 61 to 0000, No. 71 to 1000, No. 78 to 0950 and No. 68 according to the donor's hematocrit; interface detector baseline changed to a new value immediately after the first spillover). 5000 ml blood was processed during each apheresis. No secondary spin for platelet return was carried out.

### *Cell counting*

The apheresis product erythrocytes, leukocytes and thrombocytes were counted in Buckers chamber. For white blood cell differential, the smears were stained according to the May-Grunwald/Giemsa-Romanowski method.



### CFU-GM assay

Triplicates of 3 ml culture medium (Iscove 280 mOsm/L with 20% fetal calf serum, 10% 5637 CM and 0.33% agar) containing  $6 \times 10^5$  leukocytes each were plated on a 60 mm Petri dish and incubated for 7 days at 37°C in a humidified 5% O<sub>2</sub> / 5% CO<sub>2</sub> atmosphere. Aggregates of > 40 cells were counted as colonies.

### Statistical analysis

The differences between V-50 and CS-3000 cell yields were evaluated by the Mann-Whitney-Wilcoxon nonparametric test.

## Results and Discussion

The results are displayed in Table 1. The MNC and CFU-GM yield is similar in both separators. However, with the chosen protocols (supposedly the best currently available) there is much greater contamination of the apheresis product by erythrocytes and polymorphonuclears on V-50 than on CS-3000; at the same time platelet contamination is slightly greater on CS-3000 than on V-50. Purity of the product is desirable especially when used for cryopreservation and later i. v. administration following high-dose chemo/radiotherapy because lysis of erythrocytes during freezing or thawing may cause hemoglobinemia and hemoglobinuria after reinfusion, and because polymorphonuclears cause cell clumping during freezing or thawing as well as febrile and pulmonary reactions after reinfusion.

Even after concentration, the V-50 product volume cannot be reduced under approximately 250 ml (unless the pediatric bowl is used for the concentration which would substantially increase the cost of the procedure). Low volume of the MNC concentrate can save time or reagents: a) time saving: one does not have to spin down the product if higher cell concentration is needed; b) e. g. reagent saving: IL-2 may be saved if lymphocyte stimulation is the next step or dimethylsulfoxide (DMSO) may be saved if cryopreservation and later reinfusion are the subsequent steps (in this case the total amount of DMSO received by the patient during reinfusion is also reduced which helps prevent the DMSO-associated nausea, vomiting and febrile reactions).

V-50 is less noisy, more easily transportable and offers the advantage of one-arm procedure (one-arm procedure on CS-3000 is unreasonably time-consuming). On the other hand, V-50 produces more frequent citrate reactions, every surge must be watched in order to switch the valves in time when the automated MNC collection start fails (this has occurred in at least 1 cycle in every 10 of the 17 completed aphereses) and the risk of temporary hypovolemia is increased (we had to terminate 2 out of 19 MNC aphereses prematurely because of clinically significant hypotension).

In summary, MNC apheresis using the continuous-flow system CS-3000 appears to yield a purer product and to be safer in comparison to the intermittent-flow system V-50. Nevertheless, some donors/patients may prefer the intermittent-flow system mainly due to the convenience of one-arm setting.

Table 1  
Comparison of Haemonetics V-50 and Fenwal CS-3000 mononuclear cell apheresis product

	Fenwal CS-3000 (n=24)	Haemonetics V-50 (n=17)	
		before concentration [difference from CS-3000]	after concentration [difference from CS-3000]
MNC (x 10 <sup>9</sup> )	6.4 (2.0-11.4)*	6.3 (1.5-11.9) [N.S.]	5.6 (2.8-8.4) [N.S.]
PMNC (x 10 <sup>9</sup> )	0 (0-0.7)	0.4 (0.1-1.8) [p<0.001]	0.3 (0.1-3.0) [p<0.001]
RBC (x 10 <sup>9</sup> )	19 (2-527)	303 (56-2193) [p<0.001]	238 (34-2800) [p<0.001]
PLT (x 10 <sup>9</sup> )	210 (8-724)	182 (121-761) [N.S.]	148 (68-440) [p<0.05]
CFU-GM (x 10 <sup>3</sup> )	42 (5-168)	43 (8-290) [N.S.]	38 (7-306) [N.S.]
Volume (ml)	206 (196-212)	481 (440-539) [P<0.001]	254 (241-267) [P<0.001]

MNC = Mononuclear cells, PMNC = Polymorphonuclear cells, RBC = Red blood cells, PLT = Platelets,  
N. S. = not significant, \* Median (range)

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## **Combination Therapy of M2-protocol and Interferon- $\alpha$ as Remission Induction in Refractory Multiple Myeloma**

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(Received: July 24, 1990; accepted: August 31, 1990)

A successful induction of remission in two patients with resistant multiple myeloma using a new combination therapy of modified M-2 protocol and interferon- $\alpha$  (IFN- $\alpha$ ) is described. The first case was a 32-year-old man with K type urinary Bence-Jones protein who became resistant after treatment with melphalan and prednisone (MP protocol). IFN- $\alpha$  alone had a marginal response. The modified M-2 protocol proved insufficient. Therefore, IFN- $\alpha$  was administered in the interval of M-2 protocol. This combination therapy showed remarkable responses. The second case was a 59-year-old woman with advanced IgA myeloma who was treated with M-2 protocol and became resistant. IFN- $\alpha$  alone resulted in a slight response. After addition of IFN- $\alpha$  to the modified M-2 protocol, a rapid reduction in the level of serum IgA was found and clinical symptoms including bone pain, anaemia and so on were dramatically improved. No infection and/or intolerable side effects were observed in either case. This combination treatment appears worthy to try in cases of resistant or relapsing myeloma patients.

**Keywords:** interferon- $\alpha$ , refractory myeloma, M2-protocol

### **Introduction**

The M-2 protocol has improved survival in advanced multiple myeloma [3]. Interferon has also resulted in considerable responses in previously untreated patients [2]. However, the treatment of refractory cases has remained unsatisfactory. The resistant or relapsing patients were submitted to more intensive combination chemotherapies with high doses of glucocorticoid such as VAD protocol [1]. However, intensive chemotherapy is sometimes accompanied by severe infections which are frequently lethal. Therefore, new approaches to the treatment of refractory relapsing cases seem to be justified. This report describes a successful induction of remission in two patients with resistant multiple myeloma using a new combination therapy of modified M-2 protocol and IFN- $\alpha$ .

### **Case reports**

#### *Patient 1*

Case 1 was a 32-year-old man. He had had chest pain and lumbago with multiple pathologic fractures since September, 1986. Examinations showed hypo- $\gamma$ -globulinaemia with K type urinary Bence-Jones protein. Bone marrow puncture showed hypercel-

lular marrow with 80.6% atypical plasmacytosis. Bence-Jones proteinuria was 12-18 g/day. The haemoglobin concentration was 10.9 g/dl and serum calcium level 4.7 mEq/l. The serum levels of creatinine and blood urea nitrogen were within normal limits. Multiple myeloma, Stage III A according to the clinical staging system of Durie and Salmon (1975) [6] was diagnosed. He was first treated with melphalan and prednisone (MP protocol). After two months, myeloma cells in the bone marrow decreased to 48.8% and Bence-Jones proteinuria 8 g/day. The same treatment was continued for one month more. Myeloma cells in the bone marrow, however, did not decrease and proteinuria did not improve. Next, we tried daily intramuscular injection of natural interferon- $\alpha$  (IFN- $\alpha$ ,  $3 \times 10^6$  IU/day), supplied by Sumitomo Pharmacia Co., Japan, for 30 days. A slight response was observed. As the MP protocol and IFN- $\alpha$  were considered insufficient, the modified M-2 protocol (vincristine: 0.03 mg/kg/day iv day 1; Nimustine: 1 mg/kg/day iv day 1; cyclophosphamide: 10 mg/kg/day iv day 1; melphalan: 0.25 mg/kg/day on days 1-4; prednisone: 10 mg/kg/day on days 1-7) was started and given with an interval of 35 days from December, 1986. After two cycles of the modified M-2 protocol, proteinuria (8-9 g/day) still continued with a slight decrease of myeloma cells in the bone marrow. Therefore, a modified M-2 protocol was followed by daily intramuscular injection of IFN- $\alpha$  ( $3 \times 10^6$  IU/day) from day 8 to day 30. After two cycles of this new combination therapy, proteinuria was scarcely found and myeloma cells in the bone marrow decreased to 4.2%. Bone X-ray films in April, 1987 showed formation of fracture callus in the ribs. Performance status also improved. Slight anorexia and headache were observed during this therapy, although they were tolerable. No fever and liver dysfunction were found. The leukocyte count in the peripheral blood fell to  $1.5 \times 10^9/l$  but no infection was observed. He was maintained thereafter with the modified M-2 protocol and IFN- $\alpha$  and has been in good condition till May, 1990.

### *Patient 2*

Case 2 was a 59-year-old woman. She had had lumbago since June, 1988. In October, 1988 multiple myeloma, Stage III A was diagnosed because of monoclonal peak of IgA (7910 mg/dl) in the serum and  $\lambda$ -type urinary Bence-Jones protein with multiple pathologic fracture. The haemoglobin concentration was 8.0 g/dl and serum calcium level was 3.8 mEq/l. The serum levels of creatinine and blood urea nitrogen were within normal limits. Bone marrow puncture showed hypercellular marrow with 19.6% atypical plasmacytosis. The modified M-2 protocol was started in the middle of October. After one cycle of this treatment, the myeloma cells in the bone marrow decreased to 8.4% and the serum IgA level to 7080 mg/dl. However, one more cycle of the modified M-2 protocol proved practically ineffective. At the end of the second cycle, the serum level of IgA was 7130 mg/dl and that of the myeloma cells was 7.6% in the bone marrow. Hereafter, IFN- $\alpha$  ( $3 \times 10^6$  IU/day) alone was injected intramuscularly for 30 days. A marginal response was observed. Therefore, we tried the combination therapy of modified M-2 protocol and IFN- $\alpha$  for next treatment similarly to case 1. After two cycles of this treatment, a rapid reduction in the serum level of IgA (2380 mg/dl) was found. Myeloma cells in the bone marrow decreased to 4.1%. Clinical symptoms and anaemia were dramatically improved by April, 1989. Although the leukocyte count in



the peripheral blood fell to  $1.0 \times 10^9/l$ , no infections were observed. She had no side effects during the treatment. Thereafter, the patient was maintained with this combination protocol and has been in good condition till May, 1990.

## Discussion

We have demonstrated two resistant cases of multiple myeloma who were successfully treated with combination therapy of modified M-2 protocol and IFN- $\alpha$ . The MP protocol is traditional chemotherapy for multiple myeloma. For advanced or resistant cases, however, a multi-drug combination is becoming popular. Doxorubicin can be used to treat the patients who are refractory to other forms of chemotherapy. Doxorubicin containing regimens with high doses of glucocorticoid like VAD therapy were developed for refractory or resistant myeloma. The main side effects of chemotherapy have been infections, which were frequently lethal. Therefore, an alternative between MP and VAD therapy would be welcome. In in vitro studies, IFN- $\alpha$  showed additive or synergistic effects on myeloma colony cell reduction with combination of melphalan, cyclophosphamide, and/or prednisone. In in vivo studies, IFN- $\alpha$  was reported to increase the response rate in previously untreated patients when it was combined with melphalan and prednisone [5]. IFN- $\alpha$  alone could induce remissions in about 15 percent of the patients with refractory myeloma [4]. Our two cases were previously treated, resistant patients. The effects of therapy appeared to be due not only to IFN- $\alpha$ , as, when given alone, it resulted in marginal responses only. No intolerable side effects were seen in our combination therapy of modified M-2 and IFN- $\alpha$  protocol, therefore, it seems worthy to use this combined protocol for managing resistant or relapsing myeloma prior to more intensive therapy including doxorubicin and high dose glucocorticoids.

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## **Some Changes in Immunity and Blood in Relation to Clinical States of Dengue Hemorrhagic Fever Patients in Vietnam**

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The role of immunological factors in the pathogenesis of Dengue Hemorrhagic Fever (DHF), particularly Dengue Shock Syndrome (DSS) was studied. The complement activity, circulating immune complex (IC), histamine level, platelets and leucocytes were determined in the blood of 30 patients and 43 healthy persons.

The results showed a significant decrease in complement activity, platelet and neutrophil counts, an increase of histamine level ( $P < 0.001$ ), and the presence of circulating IC in 80 per cent of our patients. Furthermore, a marked correlation was found between the changes of the above parameters and the clinical stages of the disease. These parameters can be used in the prognostics of the pre-shock and shock syndrome of DHF.

**Keywords:** DHF/DSS, complement activity, histaminemia, leucocyte-platelet counts, prognostic factors

### **Introduction**

DHF is an acute infectious disease, which appeared after the Second World War and is now circulating in the South and South East Asian countries. In Vietnamese, DHF was first registered in 1960; since then great epidemics have occurred. In past years, the number of hospitalized patients has risen to 45.000. The incidence of pre-shock and shock syndrome (DHF/DSS) is 10–30 per cent. The mortality rate of DHF/DSS is also very high: 30–50 per cent [1–3].

Many authors assume an immunopathological mechanism plays a role in the development of syndromes associated with the disease. It was supposed that immune complex including Dengue virus and Dengue virus antibody or Dengue virus particles alone could directly activate the complement system, hemocoagulation system, aggregate the platelets and liberate the vasoactive substances resulting in the development of shock [4, 5, 6].

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The purpose of our present study was to determine the complement activity, histamine level, presence of immune complex, platelet and leucocyte count in the blood of DHF patients, and to follow up these changes in the course of the disease.

## Materials and Methods

### *Patients*

30 DHF patients in Hanoi between 3 and 27 years of age were included in the study; 60% of the patients were under 14 years, the male to female ratio was 1:3. In all cases, the diagnostic criteria for the disease were: sudden high fever, headache, pain in different parts of the body, symptoms of haemorrhage, haemorrhagic spots or plaques, haemorrhage from the digestive system, nose and dental bleeding, hypotension. At the same time, serological tests to confirm the Dengue virus types were also performed.

We registered the course of the disease at two different times by taking blood samples first at the acute period of the disease (3-6 days after the first fever) and second 10-15 days later (the period of remission).

In parallel, a control study on 43 normal healthy persons, including blood donors and medical students of Hanoi University, was carried out.

### *Methods*

The activity of the classical complement pathway was determined in the serum, with the 50% hemolytic unit technique of Mayer [7]. Titrations were kindly performed by Nguyen Ngoc Lan [8].

The level of the C<sub>3</sub> was determined by the technique of Mancini [9] using anti-C<sub>3</sub> serum prepared according to the technique of Hudson et al. [10].

Circulating immune complexes were determined by the technique described by Santoro et al. [11]. This method is based on the ability of the immune complex to consume the complement activity of normal guinea pig blood by the activation of the complement system.

The level of histamine in the serum was determined by the biological method, based on the principle of histaminopexie described by Parrot [12] and applied by other authors [13, 14] as well.

Platelet and leucocyte counts were also determined in these patients. The results were statistically analysed and compared with Student's *t*-test (mean  $\pm$  SD).

## Results

Comparing the 30 DHF patients to 43 normal persons, the following results were found:

### 1. Changes of complement levels in DHF patients

Complement activity was determined in 30 patients. The results calculated in CH<sub>50</sub>/ml unit were  $23.3 \pm 1.9$ , whereas in the 43 normal persons the values were  $36.0 \pm 1.4$ . Similarly, in the patients a C<sub>3</sub> level of  $37.0 \pm 3.1$  mg/100 ml, while in the control donors a C<sub>3</sub> level of  $55.4 \pm 2.5$  mg/100 ml was measured. Comparing the two groups, a marked decrease of complement levels in the group of DHF patients was found,  $P < 0.001$  (Table 1).

Table 1  
Complement levels in sera of DHF patients

Experiment*	n	Complement activity	
		C' total/CH <sub>50</sub> /ml	C <sub>3</sub> /mg/100 ml
DHF patients	30	$23.3 \pm 1.9$	$37.0 \pm 3.1$
Normal persons	43	$36.0 \pm 1.4$	$55.4 \pm 2.5$
P value		<0.001	<0.0001

\*Total C' and C'<sub>3</sub> levels of blood in two groups (Mean  $\pm$  SD), significant difference ( $P < 0.001$ ).

### 2. Immune complex in the blood of the DHF patients

We determined immune complexes in the blood of 30 DHF patients. Consumption exceeding the upper limit of the normal values (mean =  $0.97 \pm 0.39 = 1.4$  CH<sub>50</sub>/ml unit) was found in 80% of the DHF patients. In contrast, positive IC values were observed in only 3/17 (17.6%) of the controls (Table 2).

Table 2  
Immune complex in the blood of DHF patients

	n	No positive	% positive
DHF patients	30	24	80.0
Normal persons	17	3	17.6

### 3. Changes of histamine level in the blood of DHF patients

Investigating 30 DHF patients we found an increased blood histamine level in 29 cases (96.6%). A high histamine level (3+ and 4+) was observed in almost 50% of the patients. In contrast, in controls, only 5/43 cases showed a low level (11.5%). Compared to the controls, the increase of histamine level in DHF patients was highly significant,  $P < 0.001$  (Table 3).

Table 3  
Histamine level in blood of DHF patients

	n	% positive	Degree of positivity			
			1+	2+	3+	4+
DHF patients	30	96.6	3.4	48.3	17.2	31.0
Normal subjects	43	11.5	9.2	2.3	0	0

#### 4. Changes in the counts and quality of platelets in the blood of DHF patients

Parallel to the above investigation, we determined the number and aggregability of platelets in DHF patients. The result shows that in the DHF patients, the number of platelets was significantly decreased, and the aggregability of platelets was also decreased (Table 4).

Table 4  
Changes of platelets in the DHF patients

	n	No. of platelets/mm <sup>3</sup>	Aggregability
DHF patients	30	134.000 ± 19.100	Marked decrease
Normal subjects		200.000 - 300.000	Normal

#### 5. Changes in complement, histamine level and leucocytes in the course of the disease

In 18 patients, the three above-mentioned parameters were registered according to the course of the disease. The results were as follows:

Table 5  
Changes of complement activity in the course of the DHF disease

Phase of disease		Complement activity	
		C' total (u. CH <sub>50</sub> )	C'3 level mg/100 ml
Acute	18	24.0 ± 1.5	38.6 ± 1.7
Remission	18	28.6 ± 1.5	49.2 ± 4.1
Normal subjects	43	36.0 ± 1.4	55.4 ± 2.5

\*Total C' and C'3 levels in the serum of patients in two phases of disease (mean ± SD) are listed.  
± significant difference (P < 0.001).



- Complement: We determined the complement level twice: First, when the disease was in the acute phase 3-6 days after the first fever: the total complement was  $24.0 \pm 1.5$  (u.CH<sub>50</sub>) and the C<sub>3</sub> level  $38.6 \pm 1.7$  mg/100 ml. Secondly at the time of remission (10-15 days after the first fever): C' total was  $28.6 \pm 3.2$  (u.CH<sub>50</sub>) and C<sub>3</sub> level  $49.2 \pm 4.1$  mg/100 ml. This difference is statistically significant,  $P < 0.001$  (Table 5).

- Histamine: The result shows that in the phase of the remission, the histamine level of the patients also decreased markedly, the percentage of cases with increased blood histamine level decreased from 100% to 77.7%. The degree of positivity also decreased significantly. In the phase of remission (Table 6) no 4+ case was found.

Table 6  
Changes of blood histamine in the course of the disease

Phase of disease	n	% positive	Degree of positivity			
			1+	2+	3+	4+
Acute	18	100	5.5	50.5	16.6	27.7
Remission	18	77.7	28.4	57.1	14.3	0
Normal subjects	43	11.5	9.2	2.3	0	0

\*Histamine level in serum of patients in two phases of the disease are listed.

- Leucocytes: We followed up changes in the leucocytes of the above patients. Accordingly, in the acute stage the neutrophils were  $39 \pm 19\%$  absolute number  $2513 \pm 1123$  mm<sup>3</sup> of blood; lymphocytes  $57.8 \pm 25\%$ ; absolute number  $3435 \pm 1650$  mm<sup>3</sup>. In the phase of remission the neutrophils were  $51 \pm 16\%$  absolute number  $3166 \pm 1558$  mm<sup>3</sup> of blood; lymphocytes were  $45 \pm 21\%$  absolute number  $2516 \pm 1126$  mm<sup>3</sup> of blood (Table 7).

Table 7  
Changes of peripheral leucocytes in the course of disease

Phase of disease	n	Neutrophils		Lymphocytes	
		%	No./mm <sup>3</sup>	%	No./mm <sup>3</sup>
Acute	18	$39 \pm 19^{**}$	$2515 \pm 1923$	$57 \pm 25^{**}$	$3435 \pm 1650$
Remission	18	$51 \pm 16^{**}$	$3166 \pm 1558$	$45 \pm 21^{**}$	$2516 \pm 1126$
Normal subjects	26	$69 \pm 11$	$5209 \pm 1605$	$29 \pm 7$	$2002 \pm 850$

\*The percentages and absolute numbers of neutrophils and lymphocytes in three groups (mean  $\pm$  SD) are listed.

\*\*Significant difference ( $P < 0.01$ ).

**Table 8**  
Relationship between complement activity, histamine level, platelet count and clinical states of DHF patients

Groups*	n	Hista- mine level	Complement activity	Platelet Number/mm <sup>3</sup>	Conc.	Clinical states
I	3	/-/	35.6 ± 1.0	183000 ± 22500	nor.	Haemorr.** /-/
II	4	/1+/	28.8 ± 2.2	160000 ± 15000	dec.	Haemorr. /+/
III	12	/2+/	27.0 ± 1.7	130000 ± 24600	dec.	Haemorr. /+/
IV	3	/3+/	23.3 ± 2.3	110000 ± 22500	dec.	Haemorr. /++/
V	8	/4+/	19.0 ± 1.3	91000 ± 21300	dec.	Haemorr. /++++/

\*Histamine level, complement activity, platelet count in 5 groups (mean ± SD) were listed. Conc.= concentration, nor.= normal, dec.= decrease.

\*\*Haemorrhagic levels.

#### *6. Relationship complement activity, histamine level, platelet count and clinical course in DHF patients*

The 30 DHF patients studied were divided into 5 groups based on the level of histamine in their blood. Changes of complement activity, number of platelets and their aggregate level and clinical states of these patients were compared. The results show that in the groups (groups III, IV, V) with high blood histamine level, markedly decreased complement activity and low platelet counts were found. In addition the most severe cases were observed in these groups (Table 8).

### **Discussion**

In DHF patients the decrease in complement activity is a frequent phenomenon. This decrease appears to be due to in vivo complement activation, and some authors regard it as the primary factor causing haemocoagulation and increase of capillar permeability [5, 15, 16]. In all investigated DHF patients we noticed a highly significant decrease in total complement activity as well as in the C<sub>3</sub> concentration (Table 1).

Parallel to decrease of complement activity, a high level of immune complex can be found in the 70% of the DHF patients [17, 18].

In the 30 patients studied, we observed circulating immune complex in 24 cases (80%) (Table 2). This result seems to support the hypothesis on the role of the immune complex in the pathogenesis of DHF, particularly DHF/DSS [6, 16, 17].



The changes of blood cell counts in DHF patients have also been studied. Nelson et al. [19] and Halstead et al. [20] observed a decrease in platelet count in all the examined patients, and a decrease of leucocytes in 58% of the cases. Present findings show a significant decrease of the platelet and leucocyte counts in all patients. We found a marked decrease in neutrophils and an increase in lymphocytes (Table 7). Decrease of the neutrophils may be one of the causes which contribute to the pathogenesis of the DHF/DSS by the release of their lysosomal enzymes [29].

The role of a humoral amplifying system involving haemocoagulating, fibrinolytic, plasma-kinin, vaso-active, complement in the mechanism of capillar permeability in DHF have been discussed [23-27]. In the present study, a significant increase of the histamine level in the blood of DHF patients was found (Table 6).

Investigations of the above described parameters in the acute phase (4-6 days after the first fever) have shown a marked decrease in total complement and C<sub>3</sub>, significant diminution of platelet and neutrophil counts, and increase of the blood histamine level. In the remission phase (10-15 days after the first fever), these parameters have been gradually restored to their normal levels (Table 8). In severe cases where complement activity, particularly C<sub>3</sub> decreased markedly, blood histamine level increased, platelets diminished significantly. Manifestations such as haemorrhage in the nose and digestive system, decrease of blood tension were frequent. A number of patients had threatening shock or suffered a real shock, one died of shock (Table 8).

Many authors have discussed the pathogenesis of DHF and DHF/DSS. The role of the immune complex has been emphasized. Several factors are assumed to play a role: immune complex activated platelets and release of vaso-active mediators [23], immune complex activated complement system and released chemotactic factors which attract polymorphonuclear leucocytes, the leucocytes phagocytose immune complex and release of the lysosomal enzymes into surrounding tissue [24]. These enzymes continue activation of the other enzyme-systems in the plasma. Finally, a humoral amplification system, involving complement, haemocoagulating, fibrinolytic, plasma-kinin systems are involved [25-27].

Recently, Halstead [28] once again emphasized the role of immune complex in the pathogenesis of DHF and DHF/DSS, first, immune complex grasped to the surface of the cells, which have surface Fc-receptors. The author has demonstrated that the first condition for shock in DHF is the presence of anti-Dengue virus antibody which activates macrophages. These cells will phagocytose a great number of Dengue virus, they then will be lysed and liberate lysosomal enzymes. C<sub>3</sub> activator, vaso-active mediators and other factors will cause vascular permeability, hemorrhage, hypotension and shock [4, 6, 20, 28, 29].

In conclusion, the presence of immune complexes, the marked decrease of platelets and neutrophils, activation of complement system, the high level of blood histamine, and the correlation of these parameters with the clinical states such as haemorrhage, sudden hypotension and shock in the DHF supported the hypothesis that immune complexes may be an important factor in the pathogenesis of DHF/DSS. On the other hand, the present results will also contribute to investigations of the prognostic factors of the pre-shock syndrome in DHF patients.



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## Blood Count Changes Due to Mononuclear Cell Apheresis

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Complete blood count and CFU-GM count of 41 healthy donors was determined immediately before and immediately after mononuclear cell (MNC) apheresis. Red blood cell count dropped from a mean of  $5.2$  to  $4.9 \times 10^{12}/l$ , the difference being significant ( $p < 0.001$ ). Platelet count was reduced from a mean of  $219$  to  $187 \times 10^9/l$ , the difference was once again significant ( $p < 0.005$ ). Polymorphonuclear cell count did not change significantly, while MNC count was reduced substantially (from mean  $3.1$  to  $2.4 \times 10^9/l$ ,  $p < 0.001$ ). The difference between pre- and postapheresis CFU-GM count (median  $11.3$  versus  $8.8 \times 10^3/l$  respectively) was on the borderline of statistical significance ( $p = 0.05$ ).

**Keywords:** CFU-GM, complete blood count, lymphocytapheresis, mononuclear cell apheresis

### Introduction

The collection of mononuclear cells (MNC) from peripheral blood by a blood cell separator is currently used for a) obtaining hemopoietic cells for cryopreservation with the goal of reinfusing them following antineoplastic high dose radio/chemotherapy [1-3], b) procurement of functional lymphocytes with the aim of reinfusing them after ex vivo activation against malignancy (e. g. by interleukin-2) [4, 5] and c) attempts to deplete patient's blood of either non-functional lymphocytes (e. g. in advanced chronic lymphocytic leukemia) [6], or of lymphocytes presumably causing an autoimmune disorder (e. g. in Crohn's disease [7], rheumatoid arthritis [8, 9], aplastic anemia [10], multiple sclerosis [11]).

The patients undergoing MNC apheresis as well as the physicians indicating the procedure frequently ask what changes in blood count to expect as a result of the apheresis. We try to answer the question in this brief report.

### Materials and Methods

#### *Donors*

Healthy apheresis donors, age 21-50 years, male: female ratio 14:1.

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### *Aphereses*

24 MNC aphereses were performed on Fenwal CS 3000, using modified Procedure No 1 (GRANULO separation chamber; parameter of program step No. 60 changed to 1750, No. 61 to 0000, No. 71 to 1000, No. 78 to 0950 and No. 68 according to donor's hematocrit; interface detector baseline changed to a new value immediately after the first spillover; 5000 ml blood processed; no secondary spin for platelet return). 17 aphereses were completed on Haemonetics V 50, using 8-cycle modified Autosurge Lymphocytapheresis I Protocol (manually closing valve 3 and opening valve 2 2-3 seconds after the platelet/white cell jump or when the first pink had appeared in the effluent line).

### *Cell counting*

Blood samples were drawn immediately before and immediately after the apheresis. Erythrocytes, leukocytes and thrombocytes were counted in a Burkert's chamber. At least 100 white blood cells were differentiated per smear stained according to May-Grunwald/Giemsa-Romanowski. (Only 24 successive pre- and postapheresis smears were differentiated into the whole spectrum of morphological leukocyte subgroups). CFU-GM count was determined in a 7-day culture (10% 5637 CM as a source of colony-stimulating activity,  $2 \times 10^5$ /ml leukocytes plated, humidified 5% CO<sub>2</sub>/5% O<sub>2</sub>/37°C atmosphere).

### *Statistical analysis*

Pre- and postapheresis cell counts were compared by the paired t-test.

## **Results and Discussion**

The results are displayed in Tables 1 and 2.

The statistically significant red blood cell count decrease ( $0.3 \times 10^{12}/l$ ) cannot be explained only by erythrocyte loss into the MNC concentrate as the median red blood cell content of the MNC concentrate in our experiments was only  $0.045 \times 10^{12}$ . Hemodilution by saline or citrate solution during apheresis and/or partial hemolysis in the apheresis kit may play a role. The presumed platelet loss counted as platelet count decrease  $\times$  estimated average donor blood volume (i. e.  $32 \times 10^9 \times 6 = 192 \times 10^9$ ) roughly equals the collected number of thrombocytes (median  $203 \times 10^9$ ). The non-significant change in polymorphonuclear cell count corresponds to the unremarkable polymorphonuclear cell content of apheresis product (median  $0.2 \times 10^9$ ).

The presumed MNC loss (MNC count decrease  $\times$  estimated average donor blood volume, i. e.  $0.7 \times 10^9 \times 6 = 4.2 \times 10^9$ ) seems to be lower than the collected number of MNCs (median  $6.4 \times 10^9$ ), maybe due to perapheresis blood enrichment by MNCs from extravascular sites. Likewise, the presumed CFU-GM loss ( $2.5 \times 10^3 \times 6 = 15.0 \times 10^3$ ) is lower than the collected number of CFU-GMs (median  $42.7 \times 10^3$ ), perhaps because

Table 1  
Complete blood count of healthy donors immediately before and immediately after mononuclear cell (MNC) apheresis (n=41)

	Before apheresis	After apheresis	Difference
RBC <sup>a/</sup> ( $\times 10^{12}/l$ )	5.2 $\pm$ 0.8 <sup>d/</sup>	4.9 $\pm$ 0.8	p < 0.01
PLT <sup>b/</sup> ( $\times 10^9/l$ )	219 $\pm$ 135	187 $\pm$ 131	p < 0.005
PMNC <sup>c/</sup> ( $\times 10^9/l$ )	3.9 $\pm$ 1.5	3.7 $\pm$ 1.3	N.S. <sup>f/</sup>
MNC ( $\times 10^9/l$ )	3.1 $\pm$ 1.7	2.4 $\pm$ 1.7	p < 0.001
CFU-GM ( $\times 10^3/l$ )	11.3 (0-49.9) <sup>e/</sup>	8.8 (2.3-54.0)	p = 0.05

a/ red blood cells, b/ platelets, c/ polymorphonuclear cells, d/ average $\pm$ standard deviation (normal distribution of the variable), e/ median (range) (other than normal distribution of the variable), f/ not significant

of perapheresis CFU-GM migration from bone marrow to peripheral blood. This hypothetical supplementation of peripheral blood by MNCs and CFU-GMs during separation may account for the good collection efficiency of prolonged MNC aphereses [12].

Table 2  
Leukocyte subgroup counts of healthy donors immediately before and immediately after mononuclear cell apheresis (n = 24).

	Before apheresis	After apheresis	Difference
Segments ( $\times 10^9/l$ )	3.45 $\pm$ 1.51 <sup>a/</sup>	3.66 $\pm$ 2.01	N.S. <sup>c/</sup>
Bands ( $\times 10^9/l$ )	0.03 (0-0.29) <sup>b/</sup>	0.05 (0-0.83)	N.S.
Eosinophils ( $\times 10^9/l$ )	0.13 $\pm$ 0.09	0.10 $\pm$ 0.07	N.S.
Basophils ( $\times 10^9/l$ )	0(0-0.10)	0(0-0.08)	N.E. <sup>d/</sup>
Monocytes ( $\times 10^9/l$ )	0.35 $\pm$ 0.21	0.27 $\pm$ 0.16	p=0.06
Lymphocytes ( $\times 10^9/l$ )	2.36 $\pm$ 0.90	1.64 $\pm$ 0.63	p<0.005

a/ average  $\pm$  standard deviation (normal distribution of the variable), b/ median (range) (other than normal distribution of the variable), c/ not significant, d/ not evaluable by paired t-test (the distribution of pre/postapheresis differences is not normal)



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## **Enzyme Negative Blastic Transformation of Chronic Myeloproliferative Disorders: Immunophenotyping of the Blastic Cell Population**

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Enzyme negative blast cells from 27 patients with chronic myeloproliferative disorders (CMPDs) in blastic transformation were analysed with a panel of monoclonal antibodies (MoAbs). According to morphologic features of the bone marrow and laboratory data, the 27 cases were divided into 8 cases of myelofibrosis (MF), 3 cases of chronic megakaryocytic granulocytic myelosis (CMGM) and 16 cases of chronic myeloid leukaemia (CML).

Of the 27 cases, 23 showed a positive reaction with myeloid MoAbs, but in 12 cases expressing myeloid markers, megakaryocytic, monocytic or lymphoid cell features were also detected. In 7 cases of MF, 1 case of CMGM and 1 case of CML a bilineage, myelo-megakaryocytoid immunophenotype of peripheral blast cells was seen.

Of the 4 patients with CML expressing lymphoid markers, 2 showed early B-cell, 1 T-cell surface antigens, and 1 both myeloid and early B-cell features.

In this group of cytochemically immature blastic transformation of CMPD, only 1 case was termed "undifferentiated" blastic transformation.

**Keywords:** blastic transformation, chronic myeloproliferative disorder, immunophenotyping, monoclonal antibodies

### **Introduction**

Chronic myeloproliferative disorders (CMPDs) are regarded as a neoplastic transformation of haemopoietic stem cells producing one or more cell line proliferations in the bone marrow as well as in the foetal haemopoietic organs [1, 2]. The well defined CMPD types, such as chronic myeloid leukaemia (CML), polycythaemia vera (PV), chronic megakaryocytic granulocytic myelosis (CMGM) and primary thrombocythaemia (PT), may change from one type into another [3], but more frequently they transform into blastic proliferation or myelofibrosis [4, 5, 6, 7]. Although "blast crisis" represents an immature transformation of the disease, the cells may retain their lineage specificity, and most of the blasts may be identified by cytochemical methods [8]. In the blastic transformation of CML, myeloblasts are predominant in about 60%, and lymphoblasts in about 20% of the cases. The lymphoblastic and other uncommon types of transformation cannot be identified by cytochemical methods alone [9].

Table 1  
The clinical and histological findings of 27 CMPD patients in blastic transformation

No.	Age/sex	Clinical signs	WBC	Therapy	Duration	Bone marrow findings	Final pathological diagnosis
1.	40/F	splenomegaly, fever	20	Busulphan	16 months	myelofibrosis with increased blast cells	myelofibrosis
2.	67/M	hepatosplenomegaly, loss of weight	122	Busulphan	24 months	myelofibrosis with atypical megakaryocytes	myelofibrosis
3.	60/M	splenomegaly	100	Busulphan splenectomy	23 months	myelofibrosis with atypical megakaryocytes	myelofibrosis
4.	66/M	hepatosplenomegaly	32	Cyt-Arab	7 months	myelofibrosis with mononuclear megakaryocytes	myelofibrosis
5.	60/F	splenomegaly	82	Cyt-Arab	10 years	myelofibrosis with hypocellularity	myelofibrosis
6.	38/M	hepatosplenomegaly	18	Busulphan lien CO irradi.	5 years	myelofibrosis with increased blast cells	myelofibrosis
7.	67/F	fever	24	Melphalan	5 years	myelofibrosis with increased blast cells	myelofibrosis
8.	56/M	hepatosplenomegaly	38	Busulphan Prednisolon	3 years	myelofibrosis with atypical megakaryocytes	myelofibrosis
9.	40/F	painful splenomegaly	12	Busulphan splenectomy	15 months	myelofibrosis with atypical megakaryocytes	CMGM
10.	60/M	hepatomegaly, anaemia	26	Busulphan	24 months	replaced by blast cells and atypical megakaryocytes	CMGM
11.	50/M	splenomegaly	12	Busulphan splenectomy	6 years	replaced by blast cells and atypical megakaryocytes	CMGM



12.	32/F	hepatomegaly	42	Cyt-Arab	3 years	replaced by blast cells, focal fibrosis	CML
13.	63/F	hepatosplenomegaly	112	Busulphan	14 months	ND	CML
14.	65/M	hepatosplenomegaly	11	Busulphan	24 months	ND	CML
15.	60/F	fever	50	Cyt-Arab	11 months	replaced by blast cells	CML
16.	45/F	splenomegaly	25	splenectomy	6 years	replaced by blast cells	CML
17.	52/F	splenomegaly	48	Busulphan splenectomy	3 years	ND	CML
18.	54/F	hepatosplenomegaly anaemia	54	Busulphan	23 months	replaced by blast cells	CML
19.	46/M	mediastinum enlargement adenomegaly	20	Busulphan Biosuppressin	14 months	replaced by blast cells and mononuclear megakaryocytes	CML
20.	44/M	painful hepatomegaly	150	Busulphan	20 months	replaced by blast cells and mononuclear megakaryocytes	CML
21.	64/M	hepatosplenomegaly	36	Busulphan	12 months	ND	CML
22.	25/M	hepatomegaly	95	Busulphan Biosuppressin	24 months	ND	CML
23.	52/F	anaemia	3	Busulphan	16 months	replaced by blast cells	CML
24.	59/F	splenomegaly	20	Busulphan lien Co irradi.	4 years	replaced by blast cells	CML
25.	50/M	hepatosplenomegaly painful bones	10	Merkaptopurin	12 months	ND	CML
26.	42/F	hepatosplenomegaly skin bleeding	20	Busulphan lien Co irradi.	3 years	replaced by blast cells	CML
27.	48/F	hepatosplenomegaly loss of weight	10	Busulphan	5 years	replaced by blast cells	CML

Distinction between the myeloid, lymphoid, megakaryocytoid and erythroid acute phase is important because of the therapeutic and prognostic consequences [10, 11]. The combined use of morphological, cytochemical, immunological and cytogenetical studies has led to a decrease in the number of cases called "undifferentiated blastic transformation of CMPD".

In our Department, twenty-seven cases of blastic transformation of CMPD, unidentifiable by cytochemical analysis, were studied by a panel of monoclonal antibodies (MoAbs).

### Materials and Methods

*Patients:* Twenty-seven patients with the diagnosis of CMPD in enzyme negative blastic transformation were studied between 1983 and 1988. Eight cases of MF, 16 cases of CML and 3 cases of CMGM were diagnosed according to recently proposed criteria

Table 2  
Monoclonal antibodies used in this study

CD	Antibody	Specificity	Source and references
	VID-1	HLA-DR	(15)
CD 71	VIP-1	transferin receptor	(16)
CD 3	VIT-3b	pan-T lymphocytes	
CD 4	VIT-4	T-helper lymphocytes	
CD 8	VIT-8	T-suppressor lymphocytes	
CD 1a	VIT-6	thymocytes	
	VIT-12	pan-T-cells	
CD 10	VIL-A1	common ALL antigen	(17)
	VIB-C5	(pre-B)-cells	(18)
CD 24	VIB-E3	B-cells	(19)
	VI-FCR2	Fc-receptor	
CD 14	VIM-13	myelomonocytic cells	(20)
	VIM-12	myelomonocytic cells	
CDw65	VIM-2	myeloid	(21)
CD 15	VIM-D5	granulocytic cells	(22)
CD 41	VI-PL1	platelet (GpIIb-IIIa)	(16)
CD 61	VI-PL2	platelet (GpIIIa)	(16)
	VIE-G4	(glycophorin A)	(23)

The CD nomenclature is used according to the Fourth International Workshop on Human Leukocyte Differentiation, Vienna, February 1989.

All the monoclonal antibodies originated from Prof. Walter Knapp, Immunological Institute, Vienna.

[12, 13]. The diagnosis of blastic transformation was based on the presence of more than 30% of blast cells in the peripheral blood or bone marrow. The clinical data of the patients are presented in Table 1.

**Cytochemistry:** May-Grünwald-Giemsa (MGG), myeloperoxidase (MPO), Sudan Black B (SBB), periodic acid-Schiff (PAS), acid-phosphatase (AP) and alpha-naphthyl acetate esterase (ANAE) reactions were performed using standard methods [14].

**Monoclonal antibodies (MoAb):** A panel of 18 MoAbs was selected for this study. The specificities are summarized in Table 2.

**Immunophenotyping of blast cells:** Mononuclear cells from heparinized peripheral blood were removed by density gradient sedimentation in Ficoll-Hypaque. Interface cells were washed twice in PBS. Immunological labelling was performed using the alkaline phosphatase-antialkaline phosphatase (APAAP) technique, the indirect immunoperoxidase (IPO) method on cytospin preparations, and the immunofluorescence (IF) method on cell suspensions [24, 25, 26].

## Results

**Clinical and histological observations:** Table 1 summarizes the clinical and histological bone marrow findings of 27 CMPD patients in blastic transformation. On the basis of clinical data and histological findings of the bone marrow, 8 cases were diagnosed as MF, 3 as CMGM, and 16 as CML.

**Cytochemical findings:** In 21 of the 27 patients the five cytochemical reactions used (PO, SBB, ANAE, AP and PAS) were completely negative. In three cases (Nos 8, 10 and 21) the AP, and in 4 (Nos 11, 13, 17 and 22) the ANAE reactions were weak positive.

**Immunophenotype of blast cells:** In order to study the differentiation antigens of blast cells, we used a panel of MoAbs with restricted specificity to myeloid, lymphoid, monocytoid, megakaryocytoid and erythroid lineages. Positive immune reactions were determined by the IF, IPO or APAAP methods. In some cases, two or three different methods were employed simultaneously to exclude possible cross reactions between residual mature cells and blast cells. The percentage of positive blasts is shown in Table 3.

In 14 out of 27 patients the phenotype of the blast cells was unilinear. In 11 cases, the blast cells expressed myeloid differentiation antigens (VIM-D5 and/or VIM-2). A positive reaction was obtained with both antibodies in 9 patients, and in 2 (Nos 23, 27) only with VIM-D5. Three cases expressed lymphoid differentiation antigens. The blast cells of patients 15 and 24 showed B-cell differentiation antigens (VIL-A1, VIB-C5), and those of patient 21 T-cell differentiation antigens (VIT-3b, VIT-4, VIT-8). In these 3 cases, Ia antigen (VID-1) was detected.

In 12 cases (45%), the reaction patterns of blast cells showed a bilinear, mixed immunophenotype. Within this subcategory, the myeloblastic-megakaryoblastic types were the most frequent. The myeloid lineage was VIM-D5 and VIM-2 positive, and the cells of megakaryoblastic origin were VI-PL1 and VI-PL2 positive. In these mixed cases of blastic transformation, the megakaryoblasts showed a relatively low percentage of positivity with anti-platelet antibodies (15%-30%). The IPO and APAAP methods



Table 3  
Monoclonal antibody positivity of blast cells of 27 patients in blastic transformation of CMPD

case	VID-1	VIP-1	VIL-AL	VIB-CS	VIB-E3	VIT-3b	VIT-4	VIT-6	VIT-8	VIT-12	VI-FeR2	VIM-D5	VIM-2	VIM-12	VIM-13	VI-PL1	VI-PL2	VIE-G4	Immunophenotype of the blasts
1.	+++	-	-	-	-	-	-	-	-	-	-	++	++	-	-	+	+	-	myeloid, megakaryocyte
2.	-	-	-	ND	ND	ND	-	ND	-	ND	ND	+	+	-	-	+	+	-	myeloid, megakaryocyte
3.	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	+	+	-	myeloid, megakaryocyte
4.	ND	ND	-	-	-	ND	-	ND	-	ND	-	+	+	-	-	+	+	-	myeloid, megakaryocyte
5.	++	-	-	-	-	-	-	-	-	-	-	+	++	-	-	-	+	-	myeloid, megakaryocyte
6.	++	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-	myeloid
7.	-	-	-	-	-	-	-	-	-	-	-	+++	+++	-	-	-	-	-	myeloid
8.	+	-	-	-	-	-	-	ND	-	ND	ND	+	+	-	-	+	+	-	myeloid, megakaryocyte
9.	++	-	-	-	-	-	-	-	-	-	-	++	++	-	-	+	+	-	myeloid, megakaryocyte
10.	++	-	-	-	-	ND	-	ND	-	ND	ND	+	++	-	-	-	-	-	myeloid
11.	ND	ND	-	-	-	ND	-	ND	-	ND	ND	+	+	-	-	-	-	-	myeloid
12.	+	-	-	-	-	-	-	-	-	-	-	++	+	-	-	+	+	-	myeloid, megakaryocyte
13.	ND	ND	-	-	-	-	-	-	-	ND	ND	+++	+++	-	-	-	-	-	myeloid
14.	+	-	-	-	-	-	-	-	-	ND	ND	+	++	-	-	-	-	-	myeloid
15.	+	-	+++	+++	ND	-	-	ND	-	-	-	-	-	-	-	-	-	-	lymphoid (B-cell)
16.	-	-	-	-	ND	-	-	ND	-	ND	ND	+++	+	+	-	-	-	-	myeloid, monocyte

17.	+	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	myeloid, lymphoid
18.	+	-	-	-	-	-	-	-	-	-	-	+++	+	+	-	-	-	-	myeloid, monocyte
19.	-	-	-	-	-	-	-	ND	-	-	-	++	+++	-	-	-	-	-	myeloid
20.	-	-	-	-	-	-	-	-	-	-	-	+++	+++	-	-	-	-	-	myeloid
21.	++	-	-	-	-	++	+	-	+	-	-	-	-	-	-	-	-	-	lymphoid (T-cell)
22.	+	-	-	-	-	-	-	-	-	-	-	++	++	+	-	-	-	-	myeloid, monocyte
23.	++	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	myeloid
24.	ND	ND	+++	+++	ND	-	-	-	-	-	ND	-	-	-	-	-	-	-	lymphoid (B-cell)
25.	ND	ND	-	-	ND	-	-	-	-	-	ND	+	+	-	-	-	-	-	myeloid
26.	ND	ND	-	-	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	undifferentiated
27.	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	myeloid

ND – not detected

-- the number of the positive cells < 25%

+ – the number of the positive cells between 25% and 50%

++ – the number of the positive cells between 50 % and 75%

+++ – the number of the positive cells > 75%

revealed that the VI-PL1 and VI-PL2 positive cells were small lymphocyte like mononuclear cells. In three cases (Nos 16, 18, 22), the blast cells expressed myeloid and monocytoid (VIM-12) antigens. Only one case (No. 17) showed early B-cell character (VIB-E3) besides myeloid antigens (VIM-D5 and VIM-2).

The blast cells of one patient (No. 26) could not be identified; however, this case was not detected by VID-1, VIP-1, VIP-2b and VIB-E3 MoAbs.

A comparison of the immunophenotype and histological findings of the bone marrow revealed that megakaryoblastic transformation was accompanied with MF in 8 cases.

## Discussion

After various periods of time, CMPDs frequently undergo terminal blastic transformation or the myelofibrotic stage called "burned out" stage [12]. In the course of blastic transformation, the blast cells might be of myeloid, lymphoid, monocytoid, megakaryocytoid and erythroid character, supporting the theory of "pluripotent stem cell" origin of the disease [27, 28]. The detection of lineage specific differentiation markers (cytoplasmatic enzymes, terminal deoxynucleotidyl transferase, membrane antigens) helps to distinguish between blast cell types [29].

This is a report on a group of patients in blastic transformation of CMPD. The blast cells in these cases were enzyme negative and represented a relatively undifferentiated population. With a panel of MoAbs against haemopoietic differentiation antigens, it was possible to classify all of the 27 cases except one. Despite PO negativity of the cases, the blast cells most frequently (85%) expressed myeloid antigens (CD-15, CDw-65), but no more than 40% showed only myeloid lineage. Rosenthal and Moloney [5] also found a myeloid cell dominance among 306 cases of CMPD in blastic transformation, but other authors reported that monocytoid, lymphoid and megakaryocytoid cell populations might also be accompanied by myeloblastic components [30, 31, 32].

The presence of circulating micromegakaryocytes has been reported in CMPD, but recent literature suggests that megakaryoblastic transformation may be a more frequent event in CMPDs than previously believed [33, 34, 35]. In the diagnosis of megakaryoblastic transformation of CMPD the electron microscopic platelet peroxidase reaction (PPO), and antibodies against platelet glycoproteins and against factor VIII antigens may be of help. With the help of these methods, the number of cases with megakaryoblastic proliferation has increased substantially in the past few years [36, 37, 38]. According to San Miguel et al. [38], the frequency of megakaryoblastic transformation exceeds 30% within the poorly differentiated types of blastic transformation of CML. In our study, more than 29% of the cases showed megakaryocytic involvement, but the number of Gp IIIa positive cells did not exceed the 25% of blast cells. Using the APAAP method it was demonstrated that most of the Gp IIIa (CD 61) positive cells were small "lymphocyte like" mononuclear cells; however some had a wide and budding cytoplasm. This morphological heterogeneity of the megakaryoblasts suggests a tendency to mature in the blood [39]. Histological examination of the bone marrow revealed that the myelo-megakaryoblastic cases were affected by myelofibrosis. The atypical prolifera-



ration and disturbed maturation of megakaryocytes and the abnormal release of biogenic amines from the specific granules of megakaryocytes may play a role in myelofibrosis [7, 40].

In four patients, lymphoblastic involvement was detected. In 2 cases, the high percentage of positivity of the blast cells with VIL-A1 (CD 10) and VIB-C5 MoAbs supported their early B-cell origin. In one case, the blood cells were positive with anti-T-cell MoAb (CD 3). Griffin et al. [41] reported eleven cases of lymphoblastic transformation of CML, where most of the cells displayed B-cell differentiation markers (CD 10, CD 20 and TdT). The B-cell nature of the blasts in these lymphoblastic cells was proven by immunoglobulin-gene rearrangement studies [42, 43].

In 1 case (No. 17), a mixed myeloid and B-cell phenotype was demonstrated confirming that in CML B cells share a common stem cell with myeloid cells [1].

A few cases of T-lymphoblastic transformation have also been reported [44]. The much lower frequency of T-blastic transformation suggested the possibility of a simultaneous occurrence of two distinct diseases (CML and T cell leukaemia) or abnormal, promiscuous expression of T-cell antigens on myeloid cells [45]. However, Griffin et al. [27] reported a case where the isolated T-blast cells were Ph1 chromosome positive.

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## **Immunohistochemical Evaluation of Bone Marrow Biopsies in Myelodysplastic Syndromes**

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Immunohistochemical studies were performed with monoclonal antibodies (MAbs) reactive on paraffin embedded bone marrow biopsies in 19 patients with myelodysplastic syndromes, 8 of them during  $\gamma$ -interferon treatment. CD15 MAbs stained mature myeloid cells predominantly located close to the bone marrow trabeculae. Anti-gpIIIa MAbs permitted precise identification of megakaryocytic cells including precursors and dysplastic megakaryocytes. Labelling with CD45 and CD68 MAbs, recognizing lymphocytes and macrophages respectively, was intense in patients in steady state, but progressively decreased during leukemic transformation. Increase in CD45<sup>+</sup> and/or CD68<sup>+</sup> cells was also observed in most bone marrow biopsies after 3 months of  $\gamma$ -interferon therapy.

**Keywords:** bone marrow biopsies, immunohistochemistry, interferon, monoclonal antibodies, myelodysplastic syndromes

### **Introduction**

Myelodysplastic Syndromes (MDS) are a heterogeneous group of disorders, usually affecting subjects over 50 years of age and are characterised by dysplasia of one or more hematopoietic lineages and a variable tendency to evolve into acute leukemia [1]. Cytopenia may be single or multiple, with normo- or hypercellular bone marrow due to the ineffective production of red cells, platelets and/or granulocytes.

The FAB classification has subdivided these diseases into different types according to the criteria suggested by Bennett et al. [2]: Refractory Anemia (RA), Refractory Anemia with Ring Sideroblasts (RARS), Refractory Anemia with Excess of Blasts (RAEB), Chronic Myelomonocytic leukemia (CMML) and RAEB in transformation (RAEBt).

While the usefulness of light microscopy examination of bone marrow biopsies has been established for the diagnosis and follow up of these diseases for many years [3, 4, 5], immunohistochemical studies have been limited by the poor technical quality of frozen undecalcified bone marrow biopsies [6].

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Table 1  
Clinical and laboratory characteristics of 19 patients with MDS included in the study

Pts	Age	Sex	Diagnosis	% blasts*	Cytogenetics	Therapy	Evolution**
1	50	F	RAEB	10.00%	Monosomy 7	Y-IFN	Transf
2	66	M	RAEB	6.00%	Normal karyotype	Y-IFN	No transf
3	63	M	RAEB	18.50%	not done	Y-IFN	No transf
4	65	M	RAEB	16.15%	Trisomy 8	Y-IFN	Transf
5	50	F	RAEB	12.25%	Normal karyotype	Y-IFN	No transf
6	58	M	RAEB-t	21.50%	not done	Y-IFN	Transf
7	63	M	RAEB	9.20%	Trisomy 8	Y-IFN	No transf
8	62	M	RAEB	10.00%	not done	Y-IFN	No transf
9	56	M	RAEB	9.00%	not done	Steroid	No transf
10	50	M	RAEB-t	21.25%	not done	Steroid	No transf
11	60	F	RAEB	15.25%	Normal karyotype	Steroid	Transf
12	52	M	RAEB	6.20%	not done	Steroid	Transf
13	63	F	RAEB-t	25.25%	Normal karyotype	Ara-C (low doses)	Transf
14	71	M	RAEB	17.26%	Normal karyotype	Ara-C (low doses)	Transf
15	65	F	RAEB	8.00%	Del. long arm Chr. 5	Ara-C (low doses)	Transf
16	64	F	RAEB-t	23.00%	not done	Ara-C (low doses)	Transf
17	62	F	RAEB	17.15%	Trisomy 8	Ara-C (low doses)	Transf
18	58	M	RAEB	9.00%	Monosomy 7	no therapy	No transf
19	52	M	RAEB	6.25%	not done	no therapy	Transf

\* % of bone marrow blasts

\*\* transformed into overt leukemia; no transformed



However, since monoclonal antibodies (MAbs) reactive on routine bone marrow sections (formalin or Bouin's fixed and paraffin embedded) are now available [7], we evaluated the diagnostic and prognostic significance of a small panel of antibodies in the study of bone marrow samples from MDS patients.

In addition, the use of the same MAbs was extended to the study of marrow biopsies of a small group of patients treated with recombinant gamma interferon ( $\gamma$ -IFN).

### Patients and Methods

Nineteen patients affected by myelodysplastic syndromes (fifteen RAEB and four RAEBt) were evaluated (see Table 1). Diagnosis was established by light microscopy and cytochemistry on bone marrow smears according to the FAB criteria [2]. Eight of these patients were also treated with low doses of  $\gamma$ -IFN ( $0.2 \text{ MU/m}^2/3 \times$  week subcutaneously) for up to 6 months and a bone marrow biopsy was regularly taken every three months during therapy.

Bone marrow biopsies were fixed with Bouin's fixative, decalcified with nitric acid and paraffin embedded. Sections were deparaffinized in xylene and graded ethanols, transferred, without prior enzymatic treatment, to Tris buffered saline (TBS), pH 7.4, and incubated for 30 min with the primary monoclonal antibodies.

The following MAbs were used:

- Leu-M1 (CD15; Becton Dickinson, Mountain View, CA) which recognizes the X-aptin [8]. In routine bone marrow samples, this MAb reacts with increasing intensity with intermediate to late stage myeloid cells [9].

- DakoLC (PD7/26 and 2B11, CD45; Dakopatts, Glostrup, Denmark) which recognizes normal and neoplastic lymphoid cells in paraffin embedded tissues [10, 11].

- Kim-6, (H. J. Radzun; obtained from the IIIrd International Workshop on Leucocyte Antigens, Oxford 1987) is a CD68 MAb reactive with tissue macrophages [12].

- VIPL2 (W. Knapp; obtained from the IIIrd International Workshop on Leucocyte Antigens, Oxford 1987) is a CD61 MAb which binds to platelet glycoprotein gpIIIa, expressed in all stages of megakaryocytic differentiation up to the promegakaryoblast [13].

Negative controls were performed using normal mouse serum instead of the primary monoclonal antibody. Immunocytochemical labelling was performed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [14] according to Cordell et al., using commercially available reagents (Dakopatts). Sections were counterstained with hematoxylin and observed with a Leitz Laborlux K microscope. For semi-qualitative evaluations, immunohistochemically stained cells were counted at  $400 \times$  magnification with a  $10 \times 10 \text{ mm}$  grid ocular in 20 randomly selected fields, equalling  $2 \text{ cm}^2$  of hemopoietic tissue.

## Results

In all examined cases, the CD15 MAbs intensely labelled myelocytes and more mature myeloid cells; these cells often showed a characteristic tendency to gather close to the bone marrow trabeculae. As expected, a variable but clearcut reduction in the number of CD15<sup>+</sup> cells was observed in patients undergoing leukemic transformation.

In all 19 patients, evident cytoplasmic and membrane staining of pleomorphic bone marrow megakaryocytes was obtained with the anti-gpIIb MAb. Furthermore, occasionally smaller mononucleated cells, probably representing early megakaryocytic precursors, were also intensely reactive with this MAb (Fig. 1).

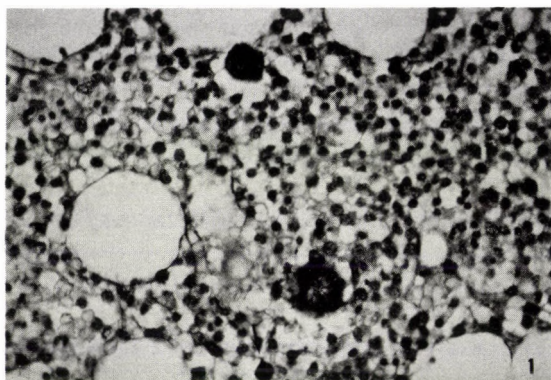


Fig. 1. Bone marrow biopsy in a case of MDS. Labelling with CD61 MAb; APAAP method. Two multinucleated megakaryocytes are intensely stained, but staining of a mononuclear megakaryocyte (arrow) is also visible. ( $\times 400$ )

CD68 MAbs labelled elongated, dendritic, highly reactive cells, homogeneously admixed with the hemopoietic tissue (Fig 2); lymphoid cells, recognized by the CD45 MAbs, appeared as small round cells with intense cytoplasmic and membrane reactivity

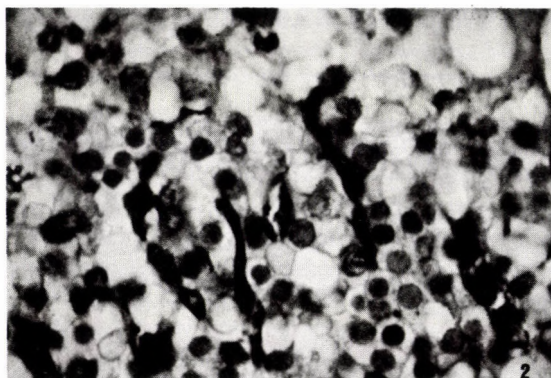


Fig. 2. Bone marrow biopsy showing numerous CD68<sup>+</sup> cells with a dendritic morphology admixed with different hemopoietic cells. ( $\times 400$ )



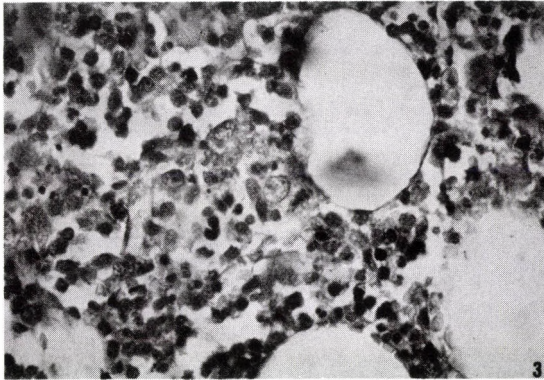


Fig. 3. Bone marrow biopsy of a patient with MDS. Isolated CD45<sup>+</sup> lymphoid cells are well visible. ( $\times 600$ )

(Fig. 3). In 9 of the 19 patients, the number of both CD68<sup>+</sup> and CD45<sup>+</sup> cells did not differ from that observed in normal bone marrow biopsies. In the other 10 patients, a significant reduction in the number of CD68<sup>+</sup> and CD45<sup>+</sup> cells ( $\leq 30\%$ ) was seen, which was inversely proportional to the percentage of blast cells present in the biopsy. Occasionally, intensely labelled lymphoid nodules were also observed.

Table 2

Reactivity of CD45 and CD68 MAbs on bone marrow biopsies of 8 patients with MDS after three months of therapy with gamma-Interferon

pt	CD45*	CD68*
1	increased %	increased %
2	increased %	no changes
3	increased %	no changes
4	increased %	increased %
5	increased %	no changes
6	no changes	increased %
7	no changes	no changes
8	no changes	no changes

\* number of CD45 and CD68 positive cells: a significant increase was arbitrarily considered  $\geq 30\%$ .

In comparison with pre-treatment samples, no qualitative nor quantitative changes in CD15 or CD61 staining were observed in the bone marrow biopsies of eight patients after 3 months of  $\gamma$ -IFN therapy. Furthermore, in these patients a rough estimate of the lymphoid compartment (CD45<sup>+</sup> cells) and of tissue histiocytes (CD68<sup>+</sup> cells) was made, as shown in Table 2; an increased percentage of CD45<sup>+</sup> cells ( $\geq 30\%$ ) in 3 patients and of CD68<sup>+</sup> cells in 2 patients was observed (Fig. 4). In the bone marrow of another patient, we found a higher number of both CD45<sup>+</sup> and CD68<sup>+</sup> cells, while the labelling pattern



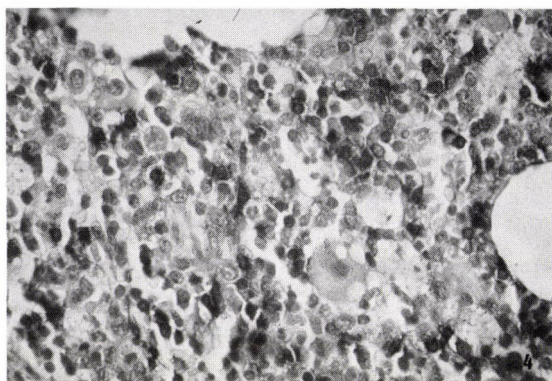


Fig. 4. Bone marrow biopsy of the same patients after 3 months of therapy with  $\gamma$ -IFN showing a clearcut increase of CD45<sup>+</sup> lymphoid cells. ( $\times 600$ )

was unchanged in the remaining 2 patients. While no correlation was found between increase of CD45<sup>+</sup> cells and further clinical course, all cases (3/3) with increased CD68<sup>+</sup> cells subsequently evolved into overt leukemia.

### Discussion

The importance of bone marrow trephine biopsy in the diagnosis of MDS has been clearly established [3, 4, 5, 15]. However, numerous problems still persist, such as the precise assessment of bone marrow blasts and thus of the time of transformation or the distinction between MDS with marrow fibrosis and idiopathic myelofibrosis [16, 17].

Additional information can be provided, as shown in our study, by immunohistochemical studies employing a small panel of MAbs reactive on Bouin's fixed, paraffin embedded bone marrow biopsies. The ideal immunohistochemical reagent for the myeloid lineage, which is predominantly involved in MDS, is still lacking; this should recognise, on routine biopsies, a granulocytic-specific antigen expressed by both immature and mature cells. The numerous antibodies available are either cross-reactive with other lineages [18] or stain also monocytic cells, including macrophages [7]. Up to now CD15 MAbs, although negative on immature myeloid cells, are still the best immunohistochemical marker for the granulocytic lineage. In our study, as expected, a clearcut reduction of bone marrow CD15<sup>+</sup> cells was observed in patients undergoing leukemic transformation. Furthermore, staining with CD15 antibodies revealed that the maturation gradient from the periphery to the center of the hemopoietic trabeculae, which is present in normal bone marrow [5], was completely lost. The finding of the reverse arrangement of differentiating myeloid cells in MDS bone marrow (more mature cells at the periphery of the intratrabecular space and immature cells in the center) suggests a profound perturbation of myelopoiesis.

In routine hematoxylin-eosin preparations, early megakaryocytic cells cannot be differentiated from other immature cells and sometimes the same problem applies to dysplastic megakaryocytes, which are difficult to detect because of their various morphological anomalies. We demonstrate that anti-gpIIb MAb may specifically label these cells in routine paraffin-embedded biopsies, this allowing a much more precise morphological evaluation of the megakaryocytic compartment; immunohistochemistry should therefore be useful for the differential diagnosis of a heterogeneous group of disorders including MDS, MDS with fibrosis and myeloproliferative syndromes [16, 17].

In previous publications [19, 20] we demonstrated that immunohistology could be usefully applied both in the detection of hairy cell leukemia bone marrow involvements and in the follow up of patients treated with interferon for this disease.

In the present study we used immunohistochemistry for evaluating bone marrow biopsies from a small group of patients undergoing  $\gamma$ -IFN therapy. Due to the lack of more specific markers for the different lympho-hemopoietic subpopulations on paraffin embedded sections, only a rough morphological evaluation of lymphoid and monocytic compartments was possible. The observed increased expression of CD45<sup>+</sup> cells in 5/8 patients undergoing IFN-therapy may reflect an increase of helper/cytotoxic T cells, as observed by other authors [21], but did not correlate with further clinical course. On the other hand an increase of CD68<sup>+</sup> cells after IFN therapy was observed only in the three patients who subsequently developed acute leukemia. This finding, which may be related to some peculiar bone marrow changes taking place in IFN-treated patients, needs further investigation in a larger population of patients.

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## **Is Dehydroepiandrosterone-sulphate a Possible Pathogenetic Factor in Osteopenia of Patients with Iron Overload?**

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Bone metabolism studies were performed on 5 patients with transfusional haemosiderosis (2 male, 3 female; mean age: 30.8 years) and 5 patients with idiopathic haemochromatosis (2 male, 3 female; mean age: 48.8 years). In the majority of the patients, the modern osteodensitometric method showed a significant decrease of the mineral content of the trabecular and cortical bones. Low calcitonin and dehydroepiandrosterone-sulphate concentrations were measured in the majority of the patients with calcipenic osteopathy. Besides marked bone loss, hypogonadotropic hypogonadism was found in 3 male patients, while normal LH, FSH concentration values were measured in 3 female patients. The prolactin concentration was normal in all 6 patients. The authors suppose that besides the already known pathogenetic factors, insufficient calcitonin effect and especially the partial lack of dehydroepiandrosterone-sulphate can play a role in the development of bone loss connected with iron overload. Further study with a greater number of patients is required to support the above findings.

**Keywords:** haemochromatosis, haemosiderosis, osteopenia, hypogonadism, osteodensitometry

### **Introduction**

In idiopathic haemochromatosis (IHC) and in transfusional haemosiderosis (THS), cirrhosis, cardiomyopathy, diabetes mellitus and other endocrine hypofunctions develop due to iron overload [1, 2]. Osteoporosis, osteomalacia, and osteoarthropathy have also been observed [3, 4, 5, 6, 7]. Osteoporosis disclosed previously by radiologic methods and recently by osteodensitometry was found in 15-66% of IHC cases. Hypogonadism appears to be an important pathogenetic factor of calcipenic osteopathy [3, 5, 8]. Many authors considered increased erythropoiesis accompanied by local bone marrow proliferation to be responsible for bone deformation in thalassemia major [4, 9, 10, 16]. At the same time, changes in calcium and phosphorous metabolism have been observed in patients with iron overload [3, 11, 12, 13]. Iron associated osteopathy has also been claimed to be associated with vitamin C deficiency [7] and with iron overload in the

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\* now working in Yemen

bone [10, 14]. The life expectancy and life quality of the patients with iron overload has improved [1, 15], thus it has now become necessary to extend our knowledge on the osteopenia and arthropathy in these diseases [3, 10, 16].

This paper presents a calcium and bone metabolism study performed on 10 patients with transfusional haemosiderosis and idiopathic haemochromatosis.

### Materials and Methods

Five patients with transfusional haemosiderosis (2 males and 3 females, mean age: 30.8 years - from 25 to 38) and five patients with idiopathic haemochromatosis (2 males and 3 females, mean age: 48.8 years - from 35 to 60) were studied (Table 1). They gave their informed consent to be included in the study. The diagnosis of idiopathic haemochromatosis was based on clinical, laboratory and histochemical data. Patients with THS were given desferrioxamine treatment, and patient 4 both desferrioxamine and prednisone. In patient 10 (IHC) virilizing suprarenal adenoma was also present. The geometrical average value of the serum ferritin concentration in the THS group was 3419 ng/ml (min: 960, max: 8100) and 1288 ng/ml (min: 660, max: 3000) in the IHC group.

Table 1

Clinical, biochemical and histological data of patients with transfusional haemosiderosis (No. 1-5) and idiopathic haemochromatosis (No. 6-10)

<i>A. Transfusional haemosiderosis</i>						
Serial no.	Sex	Age (year)	Diagnosis	Serum ferritin ng/ml	Transf. Fe (gr)	Liver damage
1.	male	25	CHA	6300	147	cirrhosis
2.	male	35	PNH	8100	257	cirrhosis
3.	female	38	CHA	6800	60	cirrhosis
4.	female	30	AIHA	960	13	hepatic laesion
5.	female	26	CHA	1400	51	hepatomegaly, normal values of liver enzymes
<i>B. Idiopathic haemochromatosis</i>						
Serial no.	Sex	Age (year)	Diagnosis	Serum ferritin ng/ml	Prussian-blue reaction	Liver damage
6.	male	35	IHC	1260	++++	cirrhosis
7.	female	41	IHC	660	++++	cirrhosis
8.	female	59	IHC	660	+++	cirrhosis
9.	male	49	IHC	2200	++++	cirrhosis
10.	female	60	IHC	3000	+++	fibrosis

CHA - congenital (non spherocytic) haemolytic anaemia, PNH - paroxysmal nocturnal haemoglobinuria, AIHA - autoimmune haemolytic anaemia, IHC - idiopathic haemochromatosis, Trans. Fe (gr) - amount of iron administered by transfusion



Serum ferritin was measured by radioimmunoassay [17]. Sections of liver specimens were stained with Perls's Prussian blue for histochemical evaluation; the amount of stainable iron in hepatocytes was scored from 0 to 4+ [18]. In patient 1, the clinical studies indicated liver cirrhosis. In patients 2, 3 and 4 - now deceased - the histological study in the liver showed strongly positive Prussian blue reaction, and in patients 2 and 3 it also indicated pigment cirrhosis. The liver biopsy performed in the IHC group supported cirrhosis in four cases and fibrosis in one.

The mineral content of the radius diaphysis was measured using a Norland Cameron single photon absorptiometer. The results are expressed as a percentage of the value in the age- and sex-matched healthy Hungarian population [19]. The mineral density of the femoral neck and the lumbar spine was studied by a dual photon absorptiometer type NOVO BMC Lab 22a. The results are presented in the form of Z-score i. e. the distance between the normal and patient values in the standard deviations of the normal American white population's data. A Z-score below -2 SD means significantly decreased bone density, while over -1 SD means normal value. Hand and wrist X-rays were taken in all patients, and X-rays of the shoulder and hip joints were also done in the IHC group.

The studies on bone metabolism were performed without any dietetic restriction in the IHC group prior to the venesection therapy. 24 hour urine sample was collected and blood was drawn after a 12 hour fast. Serum and urine calcium (Se Ca, U Ca) concentrations were measured by atomic absorption spectrophotometry and serum and urine phosphorous (Se P, U P) by modified Fischke-Subarow method [20]. Routine laboratory examination showed normal creatinine and protein concentrations in the

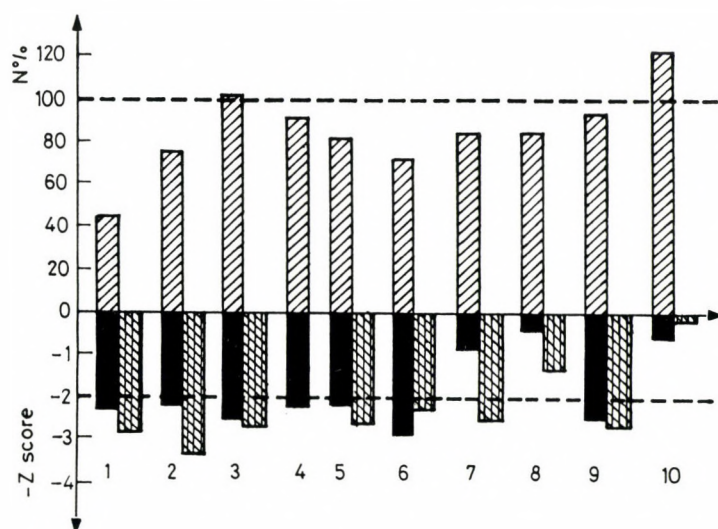


Fig. 1. The mineral content of the radius diaphysis, femoral neck and lumbar spine in patients with transfusional haemosiderosis (No. 1-5) and with idiopathic haemochromatosis (No. 6-10).

▨ radius  
 ■ lumbar spine  
 ▩ femur neck



blood of all but patient 1 (serum creatinine over 200  $\mu\text{mol/l}$ ). The C-terminal parathormone (PTH) and calcitonin (CT) content of the blood was determined by Byk-Mallinckrodt RIA kit. The serum 25 hydroxyvitamin D concentration (25-OHD3) was measured with Bodrogi's method [21]. The determination of dehydroepiandrosterone (DEA), dehydroepiandrosterone-sulphate (DEA-s) [22], testosterone (T) [23], luteinizing hormone, follicle stimulating hormone and prolactin [24] were performed on six (still living) osteopenic patients. The data of the THS and IHC patient groups were analyzed by the paired t-test [25].

Table 2  
Serum and urine biochemistry in patients with transfusional haemosiderosis (No. 1-5) and idiopathic haemochromatosis (No. 6-10)

<i>A. Transfusional haemosiderosis</i>							
Serial no.	Norm. values (SI)	Se Ca 2.25-2.61 mmol/l	Se P 0.97-1.13 mmol/l	U Ca 3.5-5.0 mmol/l	U P 20-44 mmol/l	PTH 0.2-0.6 ng/ml	25-OH D3 60-200 mmol/l
1.		—	—	0.84	23.2	0.81	143
2.		2.7	1.0	—	—	0.80	137
3.		2.38	1.2	0.12	27.4	0.96	80
4.		2.53	1.04	2.06	6.44	0.82	54
5.		2.44	1.41	1.39	35.42	0.15	125
	x:	2.51	1.16	1.1	23.1	0.71	107.8
	SD:	0.13	0.18	0.82	12.2	0.32	38.8
<i>B. Idiopathic haemochromatosis</i>							
Serial no.	Norm. values (SI)	Se Ca 2.25-2.61 mmol/l	Se P 0.97-1.13 mmol/l	U Ca 3.5-5.0 mmol/l	U P 20-44 mmol/l	PTH 0.2-0.6 ng/ml	25-OH D3 60-200 mmol/l
6.		2.57	1.36	3.31	27.36	0.31	213
7.		2.49	1.08	3.50	24.9	0.23	99
8.		2.26	1.22	3.30	15.0	0.47	221
9.		2.44	0.89	—	—	0.12	55
10.		2.55	0.97	2.5	10.0	1.3 x	155
	x:	2.46	1.1	3.15	19.3	0.48	148.6
	SD:	0.11	0.18	0.44	8.1	0.47	71.8
p++0.01						p++0.05 without x	

## Results

The results of osteodensitometric studies are shown in Fig. 1. A decrease of mineral content on one or more bone was observed in all but one patient. The mineral content of the radius was  $x(\text{SD}) = 78.8 \ 20.6\%$  and  $91.2 \ 16.9\%$  of the normal value in the THS and IHC patient's group, respectively. Significantly decreased mineral content of the lumbar spine and femoral neck was found in the THS group: the Z-score was  $x(\text{SD}) = -2.26 \ 0.14$  and  $-2.85 \ 0.27$ . The decrease of mineral content in the IHC group was less

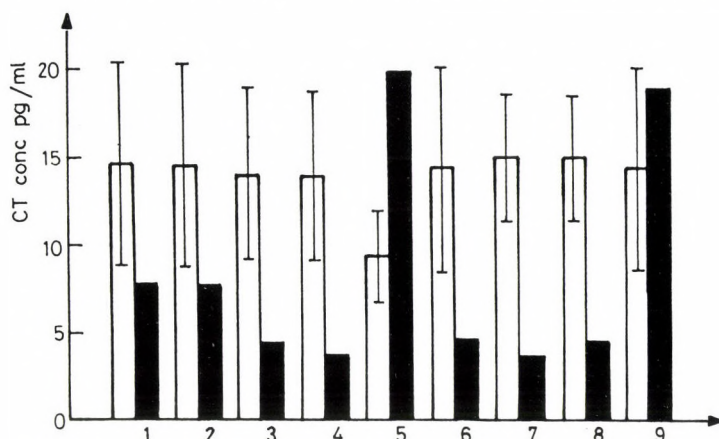


Fig. 2. Calcitonin (CT) concentrations in osteopenic patients with transfusional haemosiderosis (No. 1-5) and with idiopathic haemochromatosis (No. 6-9).

□ normal values according to age and sex

■ patients' values

marked: the Z score was  $X(\text{SD}) = -1.42 \ 0.98$  on the lumbar spine and  $-1.76 \ 0.95$  on the femoral neck, but no significant difference was observed between the two groups.

In the THS patient group no radiological signs indicating arthropathy were found. In the IHC group the I-IV and II-III metacarpophalangeal joints on both hands of patients 9 and 10 showed typical haemochromatic arthropathy. In patient 10 subchondral cyst was also found with markedly elevated PTH (1.3 ng/ml), but normal Se Ca and Se P concentrations. Normal Se Ca, Se P, serum alkaline phosphatase, 25-OHD3 and uric phosphorous concentrations were measured in all patients.

The mean U Ca concentration was low and the mean PTH concentration was in the upper normal range in the THS patient group (see Table 2). These results differ statistically from those of the IHC group in spite of the small number of cases studied:  $p < 0.01$  and  $p < 0.05$ , respectively.

We found the calcitonin concentration to be lower than normal in patients with osteopenia in both groups (Fig. 2). Figure 3 shows the serum androgen concentrations of two patients with THS and four with IHC. The level of dehydroepiandrosterone-sulphate and testosterone was found to be low in almost all the patients. In two male patients a number of clinical symptoms, in one, decreased libido pointed to hypogonadism. Besides the low testosterone concentrations, we measured in all three patients (1, 6, 9)

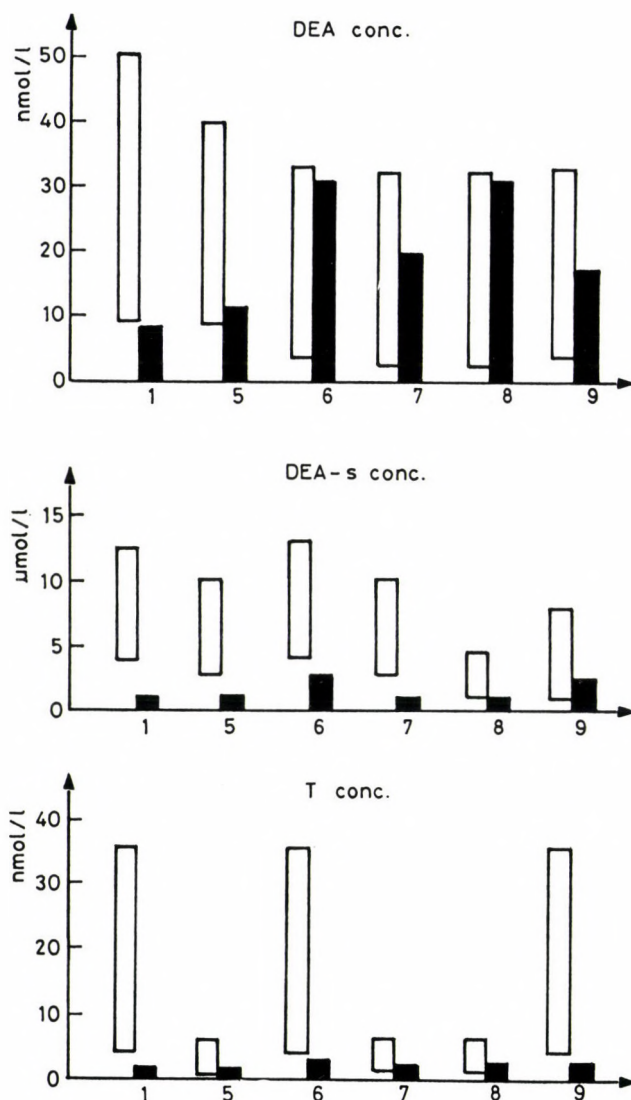


Fig. 3. Dehydroepiandrosterone (DEA), dehydroepiandrosterone-sulphate (DEA-s) and testosterone (T) concentrations in patients with transfusional haemosiderosis (No. 1-5) and idiopathic haemochromatosis (No. 6-9).

□ normal values according to age and sex

■ patients' values

low luteinizing hormone (6.6, 3.2 and 8.8 U/l - norm. value: 8-30 U/l-) and follicle stimulating hormone (1.8, 3.3 and 1.55 U/l - norm. value: 5-20 U/l) values. In three female patients (5, 7, 8) the blood's dehydroepiandrosterone-sulphate content was markedly below normal, while the testosterone content was in the lower range of the normal value. At the same time the levels of luteinizing hormone (6.7, 20.4, 73 U/l -



norm. value: 5-20 U/l) and the follicle stimulating hormone (5.4, 12.7, 34 U/l - norm. value: 5-20 U/l) were found to be in the normal range or above. The serum prolactin content was in the normal range in all the patients.

## Discussion

A study on bone metabolism was performed on patients with severe transfusional haemosiderosis and with idiopathic haemochromatosis. The mineral content of the bones - measured by the photonabsorption method and compared to the values of age and sex matched people - was significantly low in practically all the patients. Osteopenia was present on both the radius composed primarily of cortical bone (about 95%) and on the markedly trabecular femoral neck (about 25%) and the lumbar vertebrae (60%). The mineral content of bones measured in the THS group was lower than in the IHC group, although this difference was not reflected mathematically significant. The difference between the two groups can be attributed to the varying degrees of iron overload.

The cause of osteopenia observed in iron overload is not as yet clear. In thalassemia major, the significance of bone resorption resulting from bone marrow expansion accompanying the increased erythropoiesis has been emphasized [16]. We, for our part, found low bone density in our non thalassemic patients with transfusional haemosiderosis not only on the primarily trabecular bones, but also on the practically solely cortical radius, too.

Hypoparathyroidism in THS and IHC has been reported in the literature [2, 5, 26, 27]; we did not observe this in our patient groups. At the same time in THS group we found the PTH concentrations to be elevated besides normal Se Ca and pathologically low uric calcium discharge. Decreased 25-OHD3 level did not explain hypocalciuria, while real hypoparathyroidism was contradicted by normal Se Ca and Se P levels. We believe it possible - in connection with the course of the disease in the bone marrow - that the parathormone does not exert its influence (sufficiently) on the bone tissue, while its influence on the kidneys is unchanged. This hypothesis needs further study. We would like to emphasize the fact that - in contrast with the IHC group - no joint deformation was observed in any of the patients with THS.

In man, calcitonin is the most important agent that protects bones against resorption. The role of calcitonin deficiency has been shown in physiological and pathological menopausal osteoporosis [28, 29], just as in bone loss following total thyroidectomy [30]. Thus we determined the basal calcitonin content of our osteopenic patients and found it to be significantly decreased in both the THS and IHC patient groups. Others found normal [10] or elevated [16] calcitonin levels in thalassemia major and considered the latter to be a compensational reaction accompanying bone resorption. To our knowledge calcitonin production in IHC accompanied by osteopenia has not as yet been studied.

One of the generally accepted hypotheses on the development of osteoporosis in the menopause is the lack of the sexual hormones which leads to insufficient calcitonin secretion and to its decreased biological efficacy. Such effects of the lack of dehydroepiandrosterone-sulphate and oestrogens are especially well documented [28, 31, 32]. A similar connection between the testosterone and calcitonin secretion was found in young



male patients with hypogonadotropic hypogonadism [33]. Most recently, a significant connection between the testosterone concentration and the rate of bone loss was found in male patients with IHC [3]. The most frequent endocrine dysfunction of male patients with IHC is hypogonadotropic hypogonadism [34], but it has also been well documented in thalassemia major and adult patients with THS [35]. In 3 male patients (1 with THS, 2 with IHC) we found hypogonadotropic hypogonadism besides a marked osteopenia. In 3 female patients (1 with THS, 2 with IHC) we found normal testosterone and gonadotropin levels besides marked bone loss. According to literary data [36], hypogonadotropic hypogonadism is less frequent in women because of the smaller degree of iron overload. It is striking, that in the above mentioned 6 patients we found markedly decreased dehydroepiandrosterone-sulphate concentrations in spite of the fact that the group was so far from homogenous (1 postmenopausal, 2 premenopausal women and 3 young and middle-aged men). Our observations are of interest because in THS and in IHC the marked iron overload is in the zona glomerulosa of adrenal glands and the zona reticularis and zona fasciculata practically are spared [1, 37]. Recently authors studying patient groups with thalassemia major found significantly lower dehydroepiandrosterone and dehydroepiandrosterone-sulphate concentrations [38]. These data were attributed more to the regulatory disturbance of hypothalamus-hypophysis than to the decreased synthesis of these steroids in adrenal glands.

Based on our data we suspect (though due to small number of cases studied it is not possible to prove it mathematically) that the insufficient effect of calcitonin may also be in the background of osteopenia accompanying iron overload. This may be explained by the injured calcitonin secretion due to cell damage because of the iron overload, or by the hypogonadism accompanying iron overload, especially by the lack of dehydroepiandrosterone-sulphate. We think the data of patient 10 to be an indirect evidence of our hypothesis: she has a virilizing suprarenal adenoma with high serum DEA and DEA-s concentration and her bone mineral content was normal as was her calcitonin level.

Besides this, we have raised the possibility of partial resistance to the PTH in patients group with THS, which can be a further pathogenetic factor in the greater degree of osteopenia as compared to the IHC group.

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## Abstracts

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*Ca<sup>2+</sup>-activated K<sup>+</sup> channel from human erythrocyte membranes: single channel rectification and selectivity.* P. Christophersen (August Krogh Institute, Copenhagen, Denmark). *J. Memb. Biol.* 119, 75 (1991).

The Ca<sup>2+</sup>-activated K<sup>+</sup> channel of the human red cell membrane was characterized with respect to rectification and selectivity using the patch-clamp technique. In inside-out patches exposed to symmetric solutions of K<sup>+</sup>, Rb<sup>+</sup>, and NH<sub>4</sub><sup>+</sup>, respectively, inward rectifying i-V curves were obtained. The zero current conductances were: K<sup>+</sup> (23.5 pS ± 3.2) > NH<sub>4</sub><sup>+</sup> (14.2 pS ± 1.2) > Rb<sup>+</sup> (11.4 pS ± 1.8). With low extracellular K<sup>+</sup> concentrations (substitution with Na<sup>+</sup>) the current fluctuations reversed close to the Nernst potential for the K ion and the rectification as well as the i-V slopes decreased. With mixed intracellular solutions of K<sup>+</sup> and Na<sup>+</sup> enhanced rectification was observed due to a Na<sup>+</sup> block of outward currents. From bi-ionic reversal potentials the following permeability sequence (P<sub>K</sub>/P<sub>X</sub>) was calculated: K<sup>+</sup> (1.0) > Rb<sup>+</sup> (1.4 ± 0.1) > NH<sub>4</sub><sup>+</sup> (8.5 ± 1.3) > Li<sup>+</sup> (> 50); Na<sup>+</sup> (> 110); Cs<sup>+</sup> (>> 5). Li<sup>+</sup>, Na<sup>+</sup>, and Cs<sup>+</sup> were not found to carry any current, and only minimum values of the permeability ratios were estimated. Tl<sup>+</sup> was permeant, but the permeability and conductance were difficult to quantify, since with this ion the single channel activity was extremely low and the channels seemed to inactivate. The inward rectification in symmetric solutions indicate an asymmetric open channel structure, and the different selectivity

sequences based on conductances and permeabilities reflect interionic interactions in the permeation process.

B. Sarkadi

*Kinetics of Cl-dependent K fluxes in hyposmotically swollen low K sheep erythrocytes.* E. Delpire and P. K. Lauf (Department of Physiology and Biophysics, Wright State University, School of Medicine, Dayton, Ohio, USA). *J. Gen. Physiol.* 97, 173 (1991).

A detailed kinetic study of K:Cl cotransport in hyposmotically swollen low K sheep red blood cells was carried out to characterize the nature of the outwardly poised carrier. The kinetic parameters were determined from the rate of K efflux and influx under zero-K-trans conditions in red cells with cellular K altered by the nystatin method and with different extracellular K or Rb concentrations. Although apparent affinities for efflux and influx were quite similar, the maximal velocity for K efflux was approximately two times greater than for influx. Furthermore, at thermodynamic equilibrium (i. e., when the ion product of K and Cl within the cell was equal to that outside) a temperature-dependent net K efflux was observed, approaching zero only when the external product reached approximately two times the internal product. The binding order of the ions to the transporter was asymmetric, being ordered outside (Cl binding first, followed by K) and random inside. K efflux but not influx was

transinhibited by KCl. Trans inhibition of K efflux was used to verify the order of binding outside: trans inhibition by external Cl occurred in the absence of external K, but not vice versa. Thus K:Cl cotransport is kinetically asymmetric in hyposmotically swollen low K sheep red cells.

B. Sarkadi

*Characteristics of  $^{86}\text{Rb}^+$  transport in human erythrocytes infected with Plasmodium falciparum.* K. Kirk, K. J. Ashworth, B. C. Elford, R. A. Pinches and J. C. Ellory (University Laboratory of Physiology, Oxford, U. K.). *Biochim. Biophys. Acta* 1061, 305 (1991).

Human red cells infected in vitro with *Plasmodium falciparum* showed a significant increase in the rate of both ouabain-sensitive and ouabain-insensitive  $^{86}\text{Rb}^+$  influx. The increase in ouabain-insensitive  $^{86}\text{Rb}^+$  influx was due, in part, to increased transport via a bumetanide-sensitive system and, in part to transport via a pathway that was absent (or at least inactive) in uninfected cells. The parasite-induced pathway was inhibited by piperine and had a dose response very similar to that of the Gárdos channel of uninfected cells but was less sensitive than the Gárdos channel to inhibition by quinine.

B. Sarkadi

*Surface exposure of phosphatidylserine is associated with the swelling and osmotically-induced fusion of human erythrocytes in the presence of  $\text{Ca}^{2+}$ .* J. M. Baldwin, R. O'Reilly, M. Whitney and J. A. Lucy (Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine London, UK). *Biochim. Biophys. Acta* 1028, 14 (1990).

An assay for procoagulant activity has been used to investigate the  $\text{Ca}^{2+}$ -dependent exposure of phosphatidylserine at the surface of human erythrocytes that were induced to swell and to fuse osmotically. Since the phosphatidylserine of human erythrocytes is located in

the inner leaflet of the plasma membrane, it is inaccessible in intact cells which therefore had no procoagulant activity in an isotonic solution of sucrose. The procoagulant activity of erythrocytes incubated in increasingly hypotonic, sucrose solutions containing  $\text{Mg}^{2+}$  paralleled the percentage haemolysis, reflecting the accessibility of phosphatidylserine in an increasing number of lysed cells. However, cells in mildly hypotonic sucrose solutions containing  $\text{Ca}^{2+}$  had an abnormally high procoagulant activity indicating that phosphatidylserine was exposed in intact cells under these conditions. Erythrocytes that were subjected to continuous swelling at  $37^\circ\text{C}$ , which was induced by entry of the permeant molecule poly(ethylene glycol) 400 (PEG 400) developed procoagulant activity in the presence of  $\text{Ca}^{2+}$  prior to extensive lysis. Cells treated in this way also fused. With  $\text{Mg}^{2+}$ , PEG 400-treated erythrocytes lysed without fusing, and the development of procoagulant activity paralleled the rate of lysis. Erythrocytes incubated with ionophore A23187, subtilisin, and  $\text{Ca}^{2+}$  developed procoagulant activity (with < 20% lysis), and they fused on subsequent exposure to a hypotonic medium. The procoagulant activity reached its maximum before fusion could be induced in the hypotonic medium. It is concluded that the entry of  $\text{Ca}^{2+}$  facilitates a translocation of phosphatidylserine to the outer leaflet of the erythrocyte plasma membrane that plays an important role in fusion protocols that involve cell swelling. It is also suggested that transbilayer movements of phosphatidylserine could be an important control factor in the cell biology of membrane fusion phenomena.

G. Gárdos

*Interactions of glycolytic enzymes with erythrocyte membranes.* S. J. Harris and D. J. Winzor (Department of Biochemistry, University of Queensland, St. Lucia, Australia). *Biochim. Biophys. Acta* 1038, 306 (1990).

Partition equilibrium experiments have been used to characterize the interactions of erythrocyte ghosts with four glycolytic enzymes,



namely aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase and lactate dehydrogenase, in 5 mM sodium phosphate buffer (pH 7.4). For each of these tetrameric enzymes a single intrinsic association constant sufficed to describe its interaction with erythrocyte matrix sites, the membrane capacity for the first three enzymes coinciding with the band 3 protein content. For lactate dehydrogenase the erythrocyte membrane capacity was twice as great. The membrane interactions of aldolase and glyceraldehyde-3-phosphate dehydrogenase were mutually inhibitory, as were those involving either of these enzymes and lactate dehydrogenase. Although the binding of phosphofructokinase to erythrocyte membranes was inhibited by aldolase, there was a transient concentration range of aldolase for which its interaction with matrix sites was enhanced by the presence of phosphofructokinase. In the presence of a moderate concentration of bovine serum albumin (15 mg/ml) the binding of aldolase to erythrocyte ghosts was enhanced in accordance with the prediction of thermodynamic nonideality based on excluded volume. At higher concentrations of albumin, however, the measured association constant decreased due to very weak binding of the space-filling protein to either the enzyme or the erythrocyte membrane. The implications of these findings are discussed in relation to the likely subcellular distribution of glycolytic enzymes in the red blood cell.

G. Gárdos

*Membrane alterations in G6PD- and PK-deficient erythrocytes exposed to oxidizing agents.* P. Caprari, A. Bozzi, L. Ferroni, A. Giuliani, B. Furciniti la Chiusa, R. Strom, and A. M. Salvati. (Laboratori di Biochimica Clinica e Immunologia, Istituto Superiore di Sanita, Rome, Italy). *Biochem. Med. Metab. Biol.* 45, 16 (1991).

After *in vitro* treatment of normal, glucose-6-phosphate dehydrogenase-deficient or pyruvate kinase-deficient human erythrocytes with three different oxidizing agents, the extent of

lipid peroxidative degradation and the alterations of membrane proteins were evaluated. Exposure to tert-butylhydroperoxide induced, most markedly in G6PD- and PK-deficient erythrocytes, a reduction of protein bands 1, 2, 2.1, 3, 4.1, 4.2 and 5, with the appearance of high-molecular-weight aggregates and of "new" polypeptide components in the 29 - to 23-kDa region and with a marked increase of membrane-bound globin. Malonyldialdehyde production was highest in G6PD-deficient cells and relatively low in PK-deficient ones. Methylene blue, which had similar but less relevant effects on lipid peroxidation, in G6PD-deficient erythrocytes caused a conspicuous appearance of high-molecular-weight aggregates and a simultaneous relevant decrease of bands 1 and 2 and of membrane-bound globin: it brought about an almost opposite effect in PK-deficient red cells. Acetylphenylhydrazine, which under these conditions appeared the mildest agent, failed, in normal and PK-deficient erythrocytes, to increase malonyldialdehyde production or to alter membrane proteins, whereas it caused, in G6PD-deficient cells, a slight decrease of bands 1 and 2, a more pronounced decrease of band 3, and a marked increase of band 4.5 and 4.9.

G. Gárdos

*Electroinsertion of full length recombinant CD4 into red blood cell membrane.* Y. Mounenimne, P-F. Tosi, R. Barhoumi and C. Nicolau (Cell Biology Section, Institute of Biosciences and Technology, Tamu Research Park, College Station, TX, USA). *Biochim. Biophys. Acta* 1027, 53 (1990).

Electroinsertion is a novel technique of protein implantation in cell membranes using electrical pulses, of field strength between 1.3 kV/cm and 2.1 kV/cm and up to 1 ms duration. The full length recombinant CD4 receptor could thus be inserted in human and murine red blood cell (RBC) membranes. 100% of the RBC subjected to this procedure were shown to expose different CD4 epitopes after electroinsertion. An average of 5000 epitopes per cell



has been detected by immunofluorescence assay using flow cytometry and whole cell ELISA. CD4 electroinserted in red blood cell membranes showed upon reaction with monoclonal antibody significant patching similar to that observed in T4 cells expressing CD4. Furthermore, the fluorescent enhancement coming from accumulation of immune complex phycoerythrin-antiphycoerythrin was similar for both native CD4 on T4 cells or CD4 electroinserted into erythrocyte membrane. Attempts to electroinsert proteins without a membrane spanning sequence have consistently failed, suggesting that adsorption is not responsible for the observed phenomena.

G. Gárdos

*Long-term intercalation of residual hemin in erythrocyte membranes distorts the cell.* I. Solar, U. Muller-Eberhard, Y. Shviro and N. Shaklai (Sackler Institute of Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel). *Biochim. Biophys. Acta* 1062, 51 (1991).

The effect of long-term incubation of residual globin-free hemin on whole red blood cell and isolated cytoskeletal proteins was studied. Hemin at concentrations found in pathological red cells was inserted to fresh erythrocytes. Increased hemolysis developed in the hemin-containing cells after a few days at 37°C and after about four weeks at 4°C. Since lipid and hemoglobin peroxidation did not depend on the presence of hemin, time-dependent effects on the cytoskeleton proteins were studied. Observations were: (1) spectrin and protein 4.1 exhibited a time-dependent increasing tendency to undergo hemin-induced peroxidative crosslinking. (2) The ability of the serum proteins, albumin and hemopexin, to draw hemin from spectrin, actin and protein 4.1 decreased with time of incubation with hemin. These results were attributed to time-dependent hemin-induced denaturation of the cytoskeletal proteins. Albumin taken as a control for physiological hemin trap was unaffected by hemin. Small amounts of hemo-spectrin (2-5%) were analy-

zed in circulating normal cells, and this in vivo hemo-spectrin also failed to release hemin. It was concluded that slow accumulation of hemin, a phenomenon increased in pathological cells, is a toxic event causing erythrocyte destruction.

Ágnes Enyedi

*Phosphoinositide-protein interactions of the plasma-membrane  $\text{Ca}^{2+}$ -transport ATPase as revealed by fluorescence energy transfer.* J. Verbist, T. W. J. Gadella, Jr., L. Raeymaekers, F. Wuytack, K. W. A. Wirtz and R. Casteels (Physiological Laboratory, Catholic University of Leuven, Campus Gasthuisberg, Leuven, Belgium). *Biochim. Biophys. Acta* 1063, 1 (1991).

Fluorescence energy transfer has been used to study the interaction of various phospholipids with the erythrocyte  $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase. The fluorescence energy transfer between tryptophan residues of the  $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase purified from erythrocytes and pyrene-labelled analogues of phosphatidylcholine (Pyr-PC), phosphatidyl-inositol (Pyr-PI), phosphatidylinositol 4-phosphate (Pyr-PIP), phosphatidylinositol 4,5-bisphosphate (Pyr-PIP<sub>2</sub>), phosphatidylglycerol (Pyr-PG) and phosphatidic acid (Pyr-PA) was measured. A positive correlation was found between the number of negative charges on the phospholipids (PIP<sub>2</sub> > PIP > PA > PI = PG > PC) and the potency of their pyrene-labelled analogues to act as quantum acceptors in fluorescence energy transfer from the tryptophan residues of the  $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase. This is the first time that a physical interaction between PIP/PIP<sub>2</sub> and an intrinsic membrane protein has been demonstrated. The dependence of the energy transfer on the number of negative charges of the phospholipids closely resembles the previously demonstrated charge dependence of the enzymatic activity of the  $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase. It is concluded that the stimulation of the  $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase activity by negatively charged phospholipids is based on a binding of



these lipids to the  $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase and that the negative charges are a major modulatory factor for this interaction.

Ágnes Enyedi

*Inhibition of the phosphatase activity of the red cell membrane  $\text{Ca}^{2+}$  pump by acidic phospholipids.* J. P. F. C. Rossi and A. J. Caride (Instituto de Química Físicoquímica Biológicas [UBA-CONICET], Facultad de Farmacia y Bioquímica, Buenos Aires, Argentina). *Biochim. Biophys. Acta* 1061, 49 (1991).

The effect of phospholipids was tested on the p-nitrophenylphosphatase activity of the  $\text{Ca}^{2+}$  pump. Acidic phospholipids like phosphatidylserine and phosphatidylinositol inhibited the phosphatase activity, while neutral phospholipids like phosphatidylcholine did not. This result contrasts sharply with the known activating effect of acidic phospholipids on the  $\text{Ca}^{2+}$ -ATPase activity of the pump. It is known that the phosphatase activity of the  $\text{Ca}^{2+}$  pump can be elicited either by calmodulin and  $\text{Ca}^{2+}$  or by ATP and  $\text{Ca}^{2+}$ . Unlike calmodulin, acidic phospholipids failed to stimulate the phosphatase activity. Furthermore, calmodulin-activated phosphatase was completely inhibited by acidic phospholipids. Maximal inhibition of the ATP-activated phosphatase was only 70%. Inhibition by acidic phospholipids was non-competitive regarding to calmodulin, suggesting that acidic phospholipids and calmodulin do not bind to the same domain of the pump. The presence of  $\text{Ca}^{2+}$  was essential for the inhibition, and the apparent affinity for  $\text{Ca}^{2+}$  for this effect was increased by acidic phospholipids. Results are consistent with the idea that acidic phospholipids stabilize an enzyme-Ca complex lacking phosphatase activity.

Ágnes Enyedi

*Differential effect of extracellular calcium on the  $\text{Na}^{+}$ - $\text{K}^{+}$  pump activity in intact polymorphonuclear leucocytes and erythrocytes.* R. H. Petersen, T. Knudsen and T. Johansen (Depart-

ment of Pharmacology, Institute of Medical Biology, Odense University, Odense, Denmark). *FEBS Letters* 279, 153 (1991).

The effect of extracellular calcium on the  $\text{Na}^{+}$ - $\text{K}^{+}$  pump activity in human polymorphonuclear leucocytes and erythrocytes was studied and compared with the activity in mixed peritoneal leucocytes from rats. While there was maximal decrease in the pump activity (25-30%) of leucocytes from both rat and human by calcium 0.6 mM, a concentration of 0.1 mM caused a substantial decrease indicating a high sensitivity for extracellular calcium. In contrast, calcium had no effect on the pump activity in erythrocytes. The effect of calcium on the pump activity in leucocytes may be due to regulation of the influx of sodium across the plasma membrane, since in human leucocytes calcium had no effect on the pump activity if the cells were loaded with sodium.

G. Gárdos

*Characterization of high affinity binding sites for charybdotoxin in human T lymphocytes. Evidence for association with the voltage-gated  $\text{K}^{+}$  channel.* C. Deutsch, M. Price, S. Lee, V. F. King and M. L. Garcia (Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania, USA). *J. Biol. Chem.* 266, 3668 (1991).

Charybdotoxin (ChTX) inhibits with high affinity a voltage-gated  $\text{K}^{+}$  channel that is present in human T lymphocytes. In this system,  $^{125}\text{I}$ -ChTX binds specifically and reversibly to a single class of sites which display a  $K_d$  of 8-14 pM, as measured by either equilibrium or kinetic binding protocols. The maximum density of sites, 542 sites/cell, correlates well with the density of  $\text{K}^{+}$  channel as determined by electrophysiological experiments. Binding of  $^{125}\text{I}$ -ChTX is modulated by the ionic strength of the incubation media and by  $\text{Ca}^{2+}$ . Increasing concentrations of either  $\text{K}^{+}$ ,  $\text{Na}^{+}$ , or  $\text{Ca}^{2+}$  cause inhibition of toxin binding. Inhibition of binding by  $\text{Ca}^{2+}$  is due, primarily, to an effect on toxin dissociation rates. Increasing the pH of



the external media from 6.8 to 8.5 enhances toxin binding, due to an increase in affinity with no significant effect on the maximum density of receptor sites. Different agents that block the voltage-gated  $K^+$  channel in human T lymphocytes, inhibit toxin binding. Mitogen-stimulated T cells display 2.5-3-fold increase in toxin binding as compared with unstimulated control cells. These data, taken together, sug-

gest that  $^{125}I$ -ChTX binding sites identified in this study, represent the predominant voltage-gated  $K^+$  channel present in peripheral human T lymphocytes. Therefore,  $^{125}I$ -ChTX is a useful probe for elucidating the physiological role of this type of  $K^+$  channel.

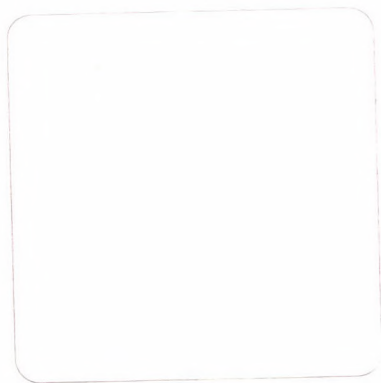
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## Correction

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Reference 17 in S. Hollán "How T Cells Talk to Target Cells" (Volume 23, Number 3, pp: 129–144) should read:

Szelényi, J. G., Bartha, E., Hollán, S. R.: Acetylcholinesterase activity of lymphocytes: an enzyme characteristic of T cells. *Brit. J. Haemat.* 50, 241 (1982).



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## **Impaired Production of GM-CSA in Bone Marrow and Peripheral Blood Monocytes in Two Patients with Severe Congenital Neutropenia**

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The capacity of bone marrow and peripheral blood cells to stimulate colony formation by normal granulocyte-macrophage progenitor cells (CFU-GM) was investigated in two patients with severe congenital neutropenia using soft agar-gel culture techniques. In both patients, monocyte/macrophage-derived colony stimulating activity (GM-CSA) in conditioned medium was found to be significantly decreased compared to the control. Furthermore, the capacity of patients' unfractionated peripheral blood leukocytes to stimulate normal CFU-GM in overlays, was also found significantly decreased. In contrast, the number of bone marrow and peripheral blood CFU-GM was within the normal range in both patients. Patients' CFU-GM showed a normal pattern of *in vitro* differentiation when they were stimulated by exogenous GM-CSA. These data indicate that in some cases of severe congenital neutropenia, monocyte/macrophage-derived GM-CSA may be impaired. Whether this abnormality plays some role in the regulation of granulopoiesis in these patients is unclear.

**Keywords:** congenital neutropenia, granulocyte/macrophage colony-stimulating factor, monocytes

### **Introduction**

Congenital neutropenia represents a heterogeneous group of granulocytic disorders in childhood, characterized by persistent reduction of circulating neutrophils, usually associated with increased numbers of marrow and peripheral blood monocytes and eosinophils and pronounced maturation arrest in marrow granulocytic series. The clinical course may be benign or severe with repeated bouts of life-threatening infections. The condition is considered as a genetic disorder of granulopoiesis with sporadic [1], recessive [2], dominant [3] or sex-linked [4] appearance.

Granulopoiesis in patients with congenital neutropenia has been studied by several methods, including bone marrow cell cultures in soft agar-gel media. It has been shown that marrow granulocyte/macrophage colony forming units (CFU-GM) from these

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\* Correspondence to: Prof. N. Anagnostou

patients can proliferate in vitro and can lead to increased, normal or decreased numbers of colonies with normal or abnormal pattern of differentiation [1, 5–8]. However, regulatory factors of granulopoiesis have not been extensively studied yet in these patients. Some studies have shown sufficient production of granulocyte/macrophage colony-stimulating activity (GM-CSA) [9], while others have documented defective GM-CSA production in few cases [10].

In the present study, we investigated several parameters of the in vitro granulopoiesis in two cases of severe congenital neutropenia. Our findings document an impaired production of GM-CSA by the monocytes of these patients, which may be involved to some extent in the pathogenesis of granulocytopenia.

## Materials and Methods

### *Patients*

Two patients with severe congenital neutropenia, the so-called "infantile genetic agranulocytosis" or Kostmann's disease [2], were studied. The first patient (Case #1) was a 7 month old male admitted to the Aghia Sophia Children's Hospital of Athens for recurrent skin infections and a perianal abscess for which he was operated. The second patient (Case #2) was a 27 month old female who had been hospitalized many times in the Pediatric Clinic of the University of Thessaloniki School of Medicine because of recurrent skin, middle ear and pulmonary infections since the age of 10 months. In both cases, the diagnosis of severe congenital neutropenia was based on 1) the consistent absence or very low number of circulating neutrophils, 2) the concomitant monocytosis and eosinophilia, 3) the maturation arrest of marrow granulocytic series at the stage of promyelocyte/myelocyte, and 4) the presence of an increased number of monocytes and eosinophils in marrow smears. Familial neutropenia, isoimmune neutropenia, cyclic neutropenia and drug-induced neutropenia were excluded in these patients by appropriate tests and investigations of the medical history.

### *Preparation of cell suspensions*

Unfractionated peripheral blood leukocytes (UF PBL) were obtained by allowing heparinized peripheral blood to settle at room temperature for 1–2 hours. The separated leukocyte-rich plasma was then removed and the leukocytes were used either for preparation of stimulating soft-agar underlayers (see below) or as target cells for colony formation. Unfractionated bone marrow cell suspensions (UF BMC) were prepared similarly as above, except for the fact that the marrow cells were resuspended in supplemented McCoy's 5a medium containing 5% fetal calf serum (FCS).

Suspensions of light density non-adherent cells (LDNAC) were prepared from peripheral blood or bone marrow mononuclear cells isolated by centrifugation on Ficoll-Hypaque [11]. Several mononuclear cells were mixed in plastic Petri dishes (Falcon Plastics, Los Angeles, CA) with supplemented McCoy's 5a medium containing



5% FCS and were incubated for 2 hours at 37°C and 5% CO<sub>2</sub>. Afterwards, the non-adherent light density cells were collected by washing the dishes and centrifugation. The cells were then tested for viability and were further resuspended in medium. Previous studies in our laboratory have shown that approximately 95% of LDNAC obtained by this procedure were lymphocytes on May-Grunwald-Giemsa stained smears.

#### *Preparation of MoCM*

Monocyte-macrophage conditioned medium was prepared by adding 1 ml of supplemented McCoy's 5a medium containing 15% FCS [12] to each Petri dish immediately after the removal of LDNAC. The light density cells (LDAC) adherent to the bottom of the dishes were submitted to a further incubation for 5 days in a humidified incubator. The supernatant fluid, called monocyte-macrophage conditioned medium (MoCM) was collected, centrifuged, filtered, and stored at -20°C until use. The number of LDAC per plate was calculated by determining the difference between light density cells originally plated and LDNAC recovered after washing the plates. Approximately 97% of LDAC obtained by this procedure were monocytes-macrophages based on morphological criteria and staining for  $\alpha$ -naphthyl acetate esterase.

#### *Assays for colony formation*

All assays for colony formation were performed in triplicate as previously described [12]. 10<sup>6</sup> normal UF PBL in underlayers were used as a source of exogenous GM-CSA. Clusters of more than 50 cells were counted as colonies after 10–12 days of incubation. Cellular composition of picked-off colonies was determined after staining with 0.6% orcein in 60% acetic acid.

#### *Assays for GM-CSA*

Leukocyte-derived GM-CSA was assayed by incorporating 10<sup>6</sup> UF PBL in underlayers of 1 ml of 0.5% agar in supplemented McCoy's 5a medium containing 15% FCS. The underlayers were tested for GM-CSA capacity against 10<sup>6</sup> normal UF PBL suspended in 1 ml of 0.3% agar-medium.

Monocyte/macrophage-derived GM-CSA in conditioned medium was assayed by mixing 0.15 ml of MoCM with 1 ml of 0.3% agar-medium containing 5 x 10<sup>4</sup> normal human bone marrow LDNAC as target cells in 35x10 mm plastic Petri dishes.

#### *Statistical analysis*

All comparisons between patients and normal controls were based on the 95% confidence limit of the control values.



## Results

In both patients, bone marrow CFU-GM were found within normal limits when UF or LDNA marrow cells were stimulated in vitro by exogenous GM-CSA (Table 1). Autostimulation of patients' CFU-GM did not differ from the control. Furthermore, randomly selected colonies from stimulated cultures showed a normal pattern of CFU-GM differentiation to mature granulocytic forms (data not shown).

Table 1  
Bone marrow CFU-GM in the two patients

Cases	Colonies/ $10^5$	UF BMC*	Colonies/ $2.5 \times 10^4$	LDNAC*
	Without exogenous GM-CSA	With exogenous GM-CSA**	Without exogenous GM-CSA	With exogenous GM-CSA**
No.1	$4.3 \pm 0.5$	$42.6 \pm 8.7$	0	$50.3 \pm 8.1$
No.2	$2.7 \pm 2.3$	$32.7 \pm 3.3$	0	$43.3 \pm 9.0$
Controls	$4.7 \pm 4.1$ (n = 12)	$31.5 \pm 9.3$ (n = 11)	0 (n = 6)	$44.3 \pm 7.8$ (n = 6)

\* Values are expressed as means  $\pm$  1SD (UF BMC, unfractionated bone marrow cells. LDNAC, light density non-adherent cells).

\*\*  $10^6$  normal human unfractionated peripheral blood leukocytes derived from the same donor were used in underlayers as a source of exogenous GM-CSA.

Patients' peripheral blood CFU-GM are presented in Table 2. In both patients, CFU-GM numbers were within normal limits when UF or LDNA cells were assayed for colony formation in stimulated cultures with exogenous GM-CSA. In patient No. 1, autostimulation of blood CFU-GM was also within the normal range.

Table 2  
Peripheral blood CFU-GM in the two patients

Cases	Colonies/ $10^6$	UF PBL*	Colonies/ $2.5 \times 10^5$	LDNAC*
	Without exogenous GM-CSA	With exogenous GM-CSA**	Without exogenous GM-CSA	With exogenous GM-CSA**
No.1	$3.7 \pm 0.9$	$29.3 \pm 3.3$	0	$44.3 \pm 8.5$
No.2		$28.4 \pm 4.3$	0	$41.8 \pm 11.6$
Control	$4.3 \pm 0.5$ (n = 20)	$27.9 \pm 5.6$ (n = 47)	0 (n = 21)	$49.7 \pm 9.5$ (n = 21)

\* Values are expressed as means  $\pm$  1SD (UF PBL, unfractionated peripheral blood leukocytes. LDNAC, light density non-adherent cells).

\*\*  $10^6$  normal human unfractionated peripheral blood leukocytes derived from the same donor were used in underlayers as a source of exogenous GM-CSA.

Table 3  
GM-CSA production by the patients' unfractionated peripheral blood leukocytes (UF PBL)

Cases	UF PBL seeded in underlayers		Colonies in overlayers	
	Total number per dish	Corresponding monocytes*	Per dish	Per 10 <sup>5</sup> monocytes in underlayer
Case No.1	10 <sup>6</sup>	1.9 × 10 <sup>5</sup>	30.3 ± 3.8	15.9 ± 3.9
Case No.2	10 <sup>6</sup>	1.4 × 10 <sup>5</sup>	22.7 ± 4.3	16.2 ± 4.6
Controls** (n = 31)	10 <sup>6</sup>	0.5 × 10 <sup>5</sup>	28.4 ± 5.4	56.8 ± 9.8

\* Calculated from differential count.

\*\* In all cultures, 10<sup>6</sup> UF PBL taken from the same normal donor were used as target cells. The values are expressed as means + 1SD of the colony numbers obtained from 3 to 4 Petri dishes in each case.

Table 3 shows the capacity of patients' UF PBL to stimulate colony formation by normal human CFU-GM. In both cases, colony numbers were significantly decreased compared to control values ( $p < 0.001$ ) when the number of monocytes in the underlayers was adjusted. All three cellular types of colonies (granulocytic, monocytic and mixed) were observed, and their distribution did not differ statistically from the control.

The levels of monocyte/macrophage-derived GM-CSA in conditioned medium are presented in Table 4. Although the numbers of LDAC retained on the bottom of plates after the removal of LDNA cells were significantly higher than the corresponding control numbers ( $p < 0.01$ ), conditioned media derived from these cells were found to be less capable in stimulating colony formation by normal CFU-GM than media derived from control LDAC ( $p < 0.001$ ).

Table 4  
Monocyte/macrophage-derived GM-CSA in conditioned medium in the patients studied

Cases	LDC plated*		Mean number of  LDAC per plate  (×10 <sup>6</sup> )	Number of colonies	
	Origin	Number of cells  (×10 <sup>6</sup> )		Per 0.15 ml** MoCM	Per ml MoCM derived from  10 <sup>6</sup> monocytes
No.1	Blood	4	1.84	17.3 ± 1.9	62.7 ± 5.7
	Marrow	10	2.41	18.3 ± 4.3	50.6 ± 3.8
No.2	Marrow	10	1.58	15.7 ± 2.3	66.2 ± 5.5
Controls (n = 8)	Blood	5	1.25	26.3 ± 4.0	140.3 ± 9.3
	Marrow	10	1.02	24.7 ± 3.8	161.4 ± 13.8

\* LDC, Light density cells.

\*\* Values (means ± 1SEM) derived from 3 to 4 plates. In all assays, 5 × 10<sup>4</sup> normal human bone marrow LDNAC were used as target cells.



## Discussion

In patients with severe congenital neutropenia, as well as in the members of their families, the numbers of bone marrow and peripheral blood CFU-GM have been reported to be normal [1, 5, 7, 13–16], decreased [6, 8, 17] or increased [8, 17]. In some of these patients, CFU-GM showed a normal *in vitro* pattern of differentiation to mature granulocytes, suggesting that microenvironmental factors are probably implicated in the manifestation of granulocytopenia in this disorder [5, 6, 8, 9, 16]. The successful treatment of 5 patients with severe congenital neutropenia with recombinant granulocyte colony-stimulating factor (G-CSF) [18] supported this hypothesis. In our patients, the number of bone marrow and peripheral blood CFU-GM, as well as the *in vitro* differentiation pattern of these cells to mature granulocytes, were within the normal limits when they were stimulated by exogenous GM-CSA.

On the other hand, neutrophils derived from bone marrow samples or from *in vitro* cultures exhibited abnormalities in shape and granule formation under electron microscopy [1, 19, 20]. Furthermore, in some cases, the *in vitro* differentiation of marrow CFU-GM was entirely abnormal since only colonies composed of macrophages and occasionally of eosinophils were found [7, 14, 17]. These observations indicate that severe congenital neutropenia may be related to some intrinsic defect at the stem cell level. This suggestion was strengthened by the successful bone marrow transplantation in a child suffering from the disease [21].

*In vitro* GM-CSA production by monocytes/macrophages was found significantly decreased in our patient. Similar results have been reported in a few cases using different approaches [10, 17]. However, other investigators have found normal GM-CSA production by patients' monocytes/macrophages when UF PBL seeded in underlayers were tested for GM-CSA capacity against normal or patients' CFU-GM [1, 8, 9]. Defective GM-CSA production by monocytes/macrophages in conditioned media has also been reported in some neutropenic states of adults [22–24].

Another, although indirect evidence for the impaired GM-CSA production in our patients is the fact that autostimulation of patients' UF PBL and UF BMC was within the normal range, despite the significantly elevated numbers of monocytes in the seeded cells due to monocytosis (Table 3). In two other cases of congenital neutropenia, a high number of spontaneously formed colonies has been observed [17]. However, in both cases, bone marrow CFU-GM were found significantly increased, suggesting that in these patients, a different pathogenic mechanism is involved than in our patients.

Our data suggest that GM-CSA production is impaired in certain cases of severe congenital neutropenia. Although a patient whose macrophages are unable to produce sufficient amounts of GM-CSA may potentially become neutropenic, it appears difficult to assume that this type of defect is the sole event in the pathogenesis of congenital neutropenia. The involvement of bone marrow microenvironment in the pathogenetic mechanism of this disorder has been previously suggested, but not documented [6, 16]. Nishihara et al. [10] provided some evidence for impaired production of GM-CSA in congenital neutropenia. Furthermore, deficient hemopoiesis related to a dysfunction of the stromal cells, has already been well recognized in S1/S1d mice [25]. Recently, Coulombel et al. [15] observed that the reproduction *in vitro* of the clinical picture of the disease depends on the presence of an adherent layer support in long-term cultures.



They postulated that a defect in interaction of granulocytic cell population with cells of the microenvironment may be involved in some cases of severe congenital neutropenia. On the other hand, the possibility of a defect in GM-CSA receptors on granulocytic cells, permitting cell proliferation and differentiation only in the presence of high concentrations of GM-CSA, cannot be excluded.

All the above discussed data indicate the great heterogeneity of cellular and humoral mechanisms which are involved in the pathogenesis of severe congenital neutropenia. In some cases, GM-CSA production by bone marrow and peripheral blood monocytes may be impaired, as in the case of our patients. Whether this abnormality has a central role in the regulation of granulopoiesis in these patients remains as yet unknown.

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## **Expression of Chromosomal Protein HMG-1 in Transformed and Normal Human Cells**

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Western blot analysis showed differences in the expression of chromosomal high mobility group protein (HMG-1) in human leukemic cell lines and normal peripheral nonproliferating leukocytes. The amount of HMG-1 was increased in fast growing transformed cells. The data suggest that HMG-1 has an important function in cell proliferation.

**Keywords:** HMG-1 protein, antibodies, leukemia, proliferation

### **Introduction**

At present, the role of HMG proteins has been intensively studied in eukaryotic cells. It is clear that these proteins somehow influence DNA synthesis or transcription [1, 2], but the data on the roles of HMG-1 and HMG-2 *in vivo* are still controversial. The proteins can be posttranslationally modified and thus activated [3, 4, 5].

The relative abundance ( $10^5$ – $10^6$  molecules per nucleus) of HMG-1 suggests a general structural rather than a gene-specific role [6]. HMG-1 binds preferentially on single-stranded DNA [7, 8] and it participates in the destabilization of double-stranded DNA [9]. It is also supposed to work as a nucleosome assembly factor [10].

The expression of HMG-1 is not so worked out as that of the HMG-14 and HMG-17 [11, 12, 13], and the findings presented previously do not confirm the correlation between the expression of HMG-1 and proliferation activity of various cells [13, 14] (but see [12]). For the elucidation of this problem, we chose several human leukemic cell lines as a model of very fast growing cells, and compared them with normal blood leukocytes.

### **Materials and Methods**

#### *Cell lines and culture conditions*

In this study, the following human leukemic cell lines have been used: myeloblastic leukemia ML-1 was obtained from J. Minowada (Fujisaki Cell Center, Okayama, Japan). K 562 (erythroleukemia), U 937 (histiocytic lymphoma), Raji (Burkitt's lym-



phoma) were purchased from the American Type Culture Collection (Rockville, MD, USA). T lymphoblastic leukemia Jurkat was given by J. Kovár (Institute of Molecular Genetics, Prague, Czechoslovakia).

The cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum.

### *Isolation of leukocytes*

Peripheral blood diluted 1:2 with PBS was layered on equal volume of 15% Verografin (Spofa, Prague, Czechoslovakia). After centrifugation (1000 x g, 20 min) the ring of leukocytes was removed. Leukocytes from five healthy blood donors have been used.

### *Antibody production*

BALB/c mice were initially injected with pure bovine HMG-1 protein (25 µg) (kindly provided by M. Stros, Institute of Biophysics, Brno, Czechoslovakia), cross-linked to bovine albumin (25 µg). The cross-linked proteins were initially injected subcutaneously with complete Freund's adjuvant and thereafter, the mice were boosted at regular intervals with pure HMG-1 alone (15 µg per injection). The sera were produced ten days after the last booster.

### *Immunoblot analysis*

To determine the expression of HMG-1 in investigated cells, the pellet of cells (0.2 g) was mixed in 1 ml of lytic buffer (0.01 M Tris-HCl, pH 8.2 / 0.14 M NaCl / 2 mM EDTA / 2% Triton X-100 / 1 mM PMSF) for 1 hour at 4°C. After centrifugation (10,000 x g, 20 min) the supernatant was dialysed against 0.01 M Tris-HCl, pH 6.8. Extracted proteins were resolved by SDS-PAGE on 12.5% gels [15]. The proteins were then electro-transferred to a nitrocellulose membrane and reacted with anti-HMG-1 IgGs (1:50 dilution). The mouse HMG-1 antibody was localized on the membrane by reaction with peroxidase-conjugated pig anti-mouse IgGs.

## **Results and Discussion**

We have found the expression of protein HMG-1 in the following human leukemic cell lines: ML-1, K 562, Raji, U 937 and Jurkat by using mouse polyclonal antisera against HMG-1.

In each immunoblot strip, bands appeared at m.w. 28 kD (HMG-1). The appearance of other bands was caused by the polyclonality of antisera (Fig. 1). All tested leukemic lines were very fast growing cells with high turnover and they represented various kinds of leukemia.

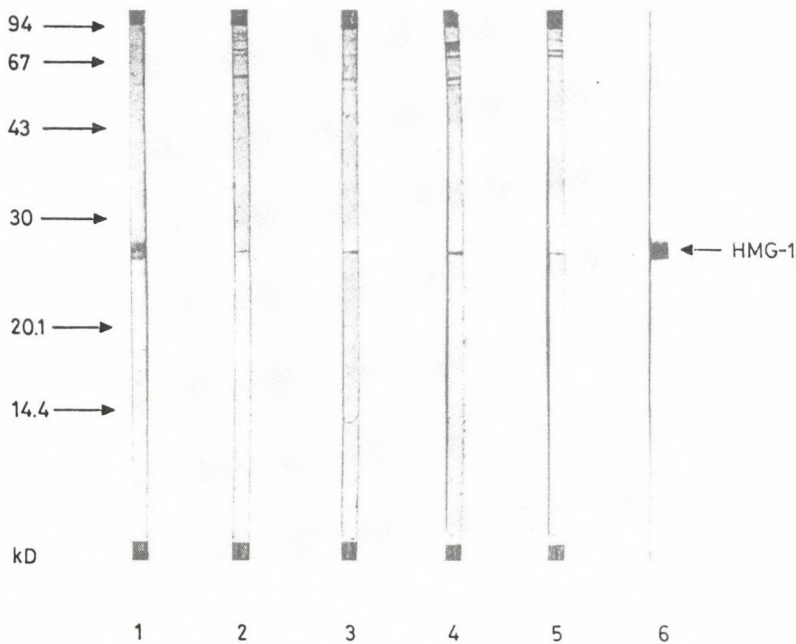


Fig. 1. Immunoblot of HMG-1 expression in different leukemic cell lines. SDS-page resolved protein extract of transformed cells was transferred to nitrocellulose membrane. HMG-1 proteins were immunodetected with the HMG-1 antisera; lane 1, ML-1; lane 2, K 562; lane 3, U 937; lane 4, Raji; lane 5, Jurkat; lane 6, pure HMG-1 protein

On the other hand, HMG-1 was not detected in normal leukocytes isolated from the blood of different healthy donors. The missing of 28 kD bands in these leukocytes can be explained by low HMG-1 concentration in the quiet cells and therefore being under the detection limit of this method (Fig. 2).

From previous studies, the general importance of HMG-1 in cell proliferation is not clear, yet. Mosevitsky et al. [14] investigated the distribution of HMG-1 in different tissues and they found that low amounts of HMG-1 were present in the nuclei of rat regenerating liver and hepatoma (active synthesis DNA) and in the adult liver and brain (transcription).

There are no differences between the quantitation of HMG-1 from chicken thymus (contains transcriptionally active and replicating lymphocytes) and chicken erythrocytes characterized by quiet cells, too [13].

However, the synthesis and stability of HMG-1 were decreased during the myogenesis of rat skeletal cell lines [12]. Our results confirm the suggestion that HMG-1 plays a role in cell proliferation. We can conclude that there are differences in the expression of HMG-1 in transformed cell lines of hemopoietic origin and normal blood cells.

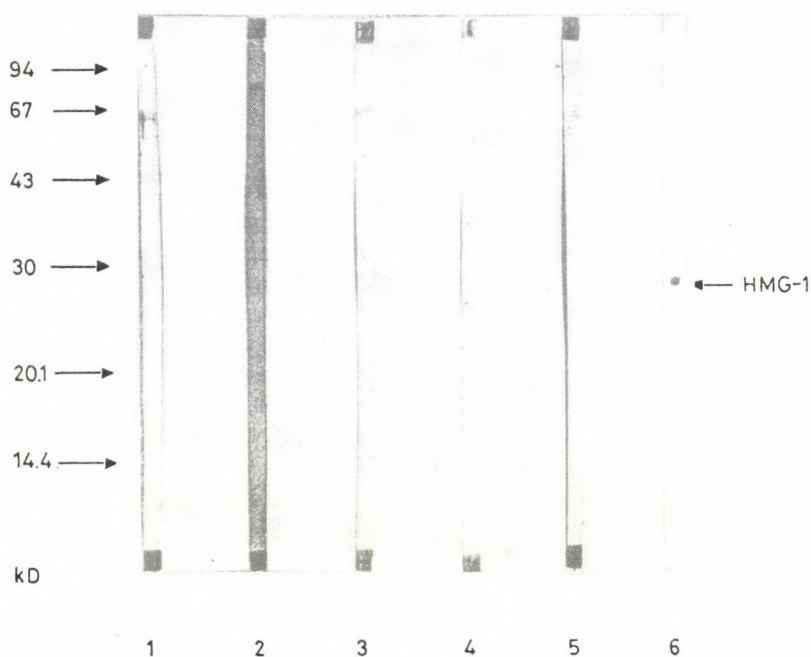


Fig 2. Immunoblot of HMG-1 expression in normal leukocytes. SDS-page resolved protein extract of normal cells was transferred to nitrocellulose membrane. HMG-1 proteins were immunodetected with the HMG-1 antisera; lanes 1–5, leukocytes from five donors; lane 6, pure HMG-1 protein

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## **Growth Kinetics and Blast-Colony Forming Cell Binding Capacity of Stromal Cells in Various Haematological Malignancies**

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Growth kinetics of bone marrow stromal layers from normal, AML, ALL and CML patients was studied. Significantly reduced time for confluency was observed in AML patients in complete remission, in CML patients in chronic phase, or CML patients after allogeneic bone marrow transplantation. The functional capacity of these stromal layers did not differ: they all bound similar amounts of blast colony forming cells (BL-CFC) from normal bone marrow. The stromal layers from bone marrow transplanted patients varied in their BL-CFC binding capacity: two CML patients (10.5 and 49 months after transplantation) showed normal values, while two ALL patients (1.5 and 3 months, respectively, after transplantation) as well as one patient transplanted for CML (19.5 months after transplantation) showed significantly reduced BL-CFC binding capacity.

**Keywords:** ALL, CML, bone marrow stromal layers, blast-colony forming cells, adhesive properties

### **Introduction**

Haemopoietic microenvironment plays a decisive role in haemopoiesis *in vivo* and in long term bone marrow cultures *in vitro* [1, 2]. Stromal cells, the extracellular matrix components (collagen, fibronectin, laminin, haemonection, proteoglycans) as well as growth factors secreted or bound by the stroma or the extracellular matrix regulate the normal haemopoiesis [3, 4, 5, 6, 7].

*In vitro* culture procedures, involving haemopoietic colony formation, proved to be essential to allow a quantitative approach to study stem cell attachment and differentiation.

By the two overall applied techniques (Dexter's long term bone marrow culture [8], and Gordon's co-culture system [9]) new data could be obtained for the better understanding of the role of haemopoietic microenvironment in pathologic situations.

Although the altered growth of clonally grown bone marrow stromal cells (CFU-F) has been reported in some haematological malignancies [10, 11, 12, 13, 14], its clinical significance remained unclear. This has induced us to study the growth kinetics and a special functional feature (the binding capacity of a primitive progenitor cell type, the so-called blast colony forming cell, BL-CFC) of stromal layers obtained from the bone marrow of patients with various haematological malignancies.



## Materials and Methods

### *Preparation of stromal layers*

Rib segments, obtained with informed consent from patients who had been operated for non-haematological diseases, were used as source of normal bone marrow cells. The cells were flushed with 5 ml  $\alpha$ -MEM medium (Gibco, Grand Island, N.Y), supplemented with 2–5% foetal calf serum (FCS, Flow Laboratories, Scotland). Bone marrow cells from patients with chronic myeloid leukaemia (CML), acute myeloid leukaemia (AML) and acute lymphoid leukaemia (ALL), respectively, were obtained from sternal puncture for diagnostic purposes.

Stromal layers were prepared according to Gordon's technique [9, 16]. Briefly, bone marrow mononuclear cells separated on  $1.077\text{g/cm}^3$  Percoll gradient (Pharmacia, Sweden) were diluted and plated at a final concentration of  $5 \times 10^5/\text{ml}$  in  $\alpha$ -MEM supplemented with 15% FCS and  $2 \times 10^{-6}$  M hydrocortisone hemisuccinate in 35 mm petri dishes.

Cultures were incubated at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  in air. Stromal layers were fed at weekly intervals by replacement complete media until a confluent monolayer of adherent stromal cells were formed on the surface of the petri dish and virtually all haemopoietic cells had disappeared.

### *Blast colony forming cell (BL-CFC) assay*

Plastic adherent cells were removed from nucleated bone marrow cells by incubating them in a  $25\text{ cm}^2$  polystyrene tissue culture flask for two hours at  $37^\circ\text{C}$ . Cells remaining in the suspension were referred to as plastic non adherent bone marrow mononuclear cells (PNABMC).

$5 \times 10^5$  PNABMC in 1 ml  $\alpha$ -MEM medium supplemented with 15% FCS were co-incubated with each confluent stromal layer at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  for two hours [9, 16]. Then the stromal layers were washed three times to remove non-attached cells and the remaining cells were covered with 1 ml  $\alpha$ -MEM medium with 15% FCS and 0.3% agar. The dishes were incubated for 5 days and aggregates of more than 20 cells were counted as colonies. In order to exclude the possibility of growth of blast colonies from haemopoietic cells surviving among stromal cells in confluent layers, in some samples stromal cells were covered with the above mentioned agar mixture with no added cells and colony formation was tested as stated above. In these plates no colony formation was observed.

### *Statistical analysis*

The significance of the differences between the growth of normal and leukaemic bone marrows was tested by Student's two paired t test.

## Results

### *Comparison of the growth of stromal layers derived from normal donors or from patients with AML, ALL and CML*

Table 1 summarizes the stromal cell growth of 32 normal bone marrows, of bone marrows from 17 patients with acute leukaemias (8 AML in complete remission, 3 untreated AML at the time of diagnosis, 6 patients with ALL) 5 patients with CML and 6 patients who were successfully transplanted for CML.

Table 1  
Comparison of the growth of stromal layers from patients with various haematological malignancies

Origin of stroma	N*	Time to confluence mean $\pm$ SD (days)	p**
normal b. m.	32	29.1 $\pm$ 2.6	
AML b. m. (CR)	8	20.4 $\pm$ 4.9	< 0.001
AML b. m. (untreated)	3	28.0 $\pm$ 12.0	N. S.
ALL b. m.	6	28.0 $\pm$ 4.4	N. S.
CML b. m.	5	22.4 $\pm$ 3.1	< 0.001
CML b. m. (after BMT)	6	22.0 $\pm$ 2.6	< 0.001

b. m.: bone marrow, plated cell count:  $5 \times 10^5$ /petri in the presence of hydrocortisone, AML: acute myeloid leukaemia, ALL: acute lymphoid leukaemia, CML: Chronic myeloid leukaemia, BMT: allogenic bone marrow transplantation, CR: complete remission, N.S.: not significant

\* number of observations

\*\* referred to normal

Bone marrow stromal cells derived from patients with AML in complete remission, ALL and CML, in chronic phase or after allogenic bone marrow transplantation (BMT), respectively, formed confluent layers significantly faster than stromal cells derived from normal bone marrow.

### *Comparison of blast colony forming cell binding capacity of normal and leukaemic stromal layers*

A standard dose of  $5 \times 10^5$  PNABMC from normal bone marrow resulted in  $98.3 \pm 34.6$  blast cell colonies when co-cultured with normal bone marrow derived stromal layers. Neither the 5 AML derived nor the ALL or CML derived stromal layers differed significantly in binding BL-CFCs from normal bone marrow (Table 2).

If, however, BL-CFC binding capacity of patients after successful allogenic bone marrow transplantation was studied, only the stromal layers derived from the bone marrows of two patients, transplanted for CML 10.5 and 49 months, respectively,

Table 2  
Capacity of normal, AML, ALL and CML stroma to bind blast colony forming cells from normal bone marrow

Origin of stroma (initials of patients)	N*	Colonies/5 × 10 <sup>5</sup> PNABMC** (mean ± SD)
normal b. m.	5	98.3 ± 34.6
AML b. m. (CR)	5	103.0 ± 23.0
ALL b. m. (T.A.)		87.0 ± 19.3
ALL b. m. (A.L.)		145.0 ± 44.4
ALL b. m. (N.Z.)		105.0 ± 12.3
ALL b. m. (V.A.)		173.0 ± 24.7
CML b. m. (G.I.)		148.3 ± 22.9
CML b. m. (Sz.I.)		121.0 ± 25.5
CML b. m. (B.I.)***		114.1 ± 9.8

\* number of observations (see ref. 27)

\*\* Plastic non adherent bone marrow cells

\*\*\* in blastic phase

previously, showed normal binding capacity. Stromal layers from 2 ALL patients 1.5 and 3 months, respectively, and one CML patient 19.5 months after transplantation showed significantly reduced BL-CFC binding capacity (Table 3).

Table 3  
Capacity of stroma from CML or ALL patients after allogenic bone marrow transplantation to bind blast colony forming cells from normal bone marrow

Origin of stroma (initials of patients)	Time after BMT (months)	Colonies/5 × 10 <sup>5</sup> PNABMC* mean ± SD
CML b. m. (Cz.M.)	10.5	164.4 ± 26.3
CML b. m. (CS.B.)	19.5	43.8 ± 15.1
CML b. m. (G.L.)	49.0	105.0 ± 11.2
ALL b. m. (S.A.)	1.5	21.3 ± 7.0
ALL b. m. (A.L.)**	3.0	31.1 ± 14.1
Normal b. m.***		98.3 ± 34.6

\* Plastic non adherent bone marrow cells

\*\* After autologous bone marrow transplantation

\*\*\* Mean of 5 experiments, see Table 2

## Discussion

Bone marrow stromal cells from AML patients in complete remission, from CML patients in chronic phase or after successful bone marrow transplantation showed significantly faster growth than normal bone marrow stromal cells.



Since these altered growth kinetics could not be observed either in the few bone marrows obtained from untreated AML patients or from ALL patients, we do not postulate it to be a specific leukaemia-associated functional feature. The fact that except for the freshly diagnosed, untreated AML patients all bone marrows were obtained from patients after one or more cytostatic courses suggests the possible role of regenerative processes in the phenomenon observed.

Some previous data have already reported reduced fibroblastic colony (CFU-F) formation in ALL but not in AML patients [11], while others suggested decreased CFU-F level in patients with acute leukaemias [15], being recovered at complete remission. In myeloproliferative disorders (chronic phase CML, polycythaemia vera, essential thrombocythaemia), however, CFU-F level was similar to the normal [10, 12], supporting the idea that CFU-F does not measure complex stromal function.

Co-culture system represents an ideal system to study growth kinetics and haemopoiesis supporting activity (BL-CFC binding) of the bone marrow stroma [9]. Applying this assay, all leukaemic stromas studied had normal haemopoiesis supporting activity, independently of type of leukaemia or treatment of the patients. In three out of five patients after bone marrow transplantation, however, reduced BL-CFC binding capacity was observed in spite of the normal or faster growth of stromal layers. In two ALL patients (S.A. and AL) where only 1.5 or 3 months elapsed after bone marrow transplantation, reduced function of stroma supports the generally accepted idea of damaged stromal function after cytostatics or irradiation [17, 18]. In one patient (A.L.) this assumption was supported by the fact that before autologous transplantation (Table 2), supranormal binding of BL-CFC was observed, while 3 months after autologous bone marrow transplantation this was reduced to 50% of normal (Table 3).

The reduced BL-CFC binding in one CML patient nearly 20 months after BMT, however, could not be explained by a short term radiation injury. Long term bone marrow damage after irradiation has been reported in animal experiments, bone marrow cultures and humans [reviewed by Knospe, 19]. Although one finding definitely does not allow us to draw further conclusions, it seems to support our earlier observation that patients transplanted for CML show a significantly delayed recovery of granulocyte-macrophage progenitors compared to that of patients transplanted for AML and ALL [20].

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## **The Pattern of Activation Antigen Expression on T-Lymphocyte Subpopulation in Infectious Mononucleosis**

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The peripheral blood mononuclear cells of patients with infectious mononucleosis (IM) have been characterized by the determination of activation antigens using a panel of 11 monoclonal antibodies (MoAbs) belonging to 9 clusters. The activation of CD8<sup>+</sup> peripheral blood mononuclear cells of IM patients was found to be determined by HLA-DR and CD45RO MoAbs. The antibodies of the CD30, CD40 and CD70 termed antigens also showed an increased expression as compared to the controls. In contrast, the CD25, CD69, CD71, and CD45 antigens were expressed at a low rate on the surface of the CD8<sup>+</sup> cells. We found, on the other hand, a very low percentage of B lymphocytes in the peripheral blood, which reflects on the virus caused antigen modulation and on the effectivity of activated CD8 cells with the characteristic expression of the above outlined markers in IM.

In six asymptomatic patients, the percentage of CD8+HLA-DR+, as well as of CD8+CD45RO+ cells proved to be lower than that in the active phase of the disease. The number of B cells showed normal value in the clinically asymptomatic cases.

**Keywords:** infectious mononucleosis, T-cell, activation antigens

### **Introduction**

Infectious mononucleosis (IM) caused by the Epstein-Barr virus (EBV) is characterized by the presence of atypical mononuclear cells.

Immunological studies have demonstrated that the majority of expanded lymphocyte populations are not EBV positive B lymphocytes [1, 2, 3]. Peripheral blood mononuclear (PBM) cells from patients with IM during the acute phase of the disease include an increased number of HLA-DR<sup>+</sup> T-lymphocytes [4, 5, 6], suggesting that these cells are activated *in vivo*. However, the mechanism of their activation and proliferation is still unclear.

In this study, we investigated the expression of differentiation and activation antigens on PBM cells of 10 IM patients in an active phase of the disease. Using double marker analysis with 11 monoclonal antibodies (MoAbs), we demonstrated the expression of some activation antigens on CD8 cells, giving more evidence on the existence of the activation of T-cells in IM.



## Materials and Methods

### Patients

Ten IM patients, 8 males and 2 females, were studied. Age: 1.5–19 years (mean: 13.95). The diagnosis was based on the usual clinical symptoms of acute IM, atypical mononuclear cells, the positive serological test for heterophile antibody and the presence of IgM antibody to EBV capsid antigen. Peripheral blood from 5 age- and sex-matched healthy donors served as control samples.

### Methods

Peripheral blood mononuclear (PBM) cells were isolated from heparinized blood using Ficoll-Hypaque gradient centrifugation. Binding of the various monoclonal antibodies (MoAbs) was assessed with indirect immunofluorescence using FITC conjugated rabbit anti-mouse Ig, DAKO as second antibody.

The B cell associated Abs used were: CD19 (B4), CD20(B1), CD21(B2) from Coulter Corporation. The immune monitoring Kit of Becton-Dickinson (CD3/DR, CD4/CD8, CD14/CD14, CD8/CD57) and CD8 FITC and MoAbs against activation antigens and phycoerythrin conjugated rat anti-mouse Ig (Becton-Dickinson) as second antibody were used for the double marker analysis. MoAbs from the panel of the IV. International Workshop on Human Differentiation Antigens (Vienna, 1989) were used to detect the activation antigens on CD8<sup>+</sup> cells (Table 1).

Table 1  
Activation monoclonal antibody panel used in the study

MoAbs CD	Mol. weight	Characteristics
CD25	55kD	activated T and B cells, B-CLL*, HCL**, B-cell lines, IL-2 chain
CD30	116kD	activated T and B cells, T and B cell lines, M. Hodgkin, anaplastic large cell lymphoma (Ki-1)
CD40	50kD	activated B, EBV-CLL*
CD69	28/34kD	activated T and B cells, early activation antigen activation inducer molecule
CD70	75/50kD	activated T and B cells, M. Hodgkin, anaplastic large cell lymphoma (Ki-24)
CD39	80kD	B-cell, intracell, Ca <sup>++</sup> regulation
CD71	90kD	activated T and B, B-CLL*, B-NHL***, anaplastic large cell lymphoma transferrin receptor
CD45	220kD	pan-leukocyte marker
CD45RO	180kD	T-subset

CLL\* = chronic lymphocytic leukaemia

NHL\*\*\* = non-Hodgkin lymphoma

HCL\*\* = hairy cell leukaemia

Analysis of all samples was done using a FACStar flow cytometer (Becton-Dickinson). The relative fluorescence intensity of 10.000 cells per sample was determined.

## Results

The proportion of CD3<sup>+</sup>, CD8<sup>+</sup>, and HLA-DR<sup>+</sup> cells was significantly higher in the IM patients than that of the controls (Table 2). The proportion of CD4<sup>+</sup> cells was lower than in the controls, while the ratio of CD56<sup>+</sup> cells remained normal. The B-cells showed extremely low values when investigated with CD19, CD20, and CD21 MoAbs. Using double marker analysis, the CD3<sup>+</sup> as well as the CD8<sup>+</sup> cells proved to be simultaneously HLA-DR positive, showing the presence of activated CD8<sup>+</sup> cells in the PBM cells of patients (Table 3).

Sample : PE 0111 MONU CD8 1 008

Date: 16-Jan-90 11:45

Cytometer: FACSTAR

File : PECD31025

FL1 : CD8

FL2:UCHL1(CD45RO)

FL3 :

FSC 75.245

SSC 11.215

FL1 0.255

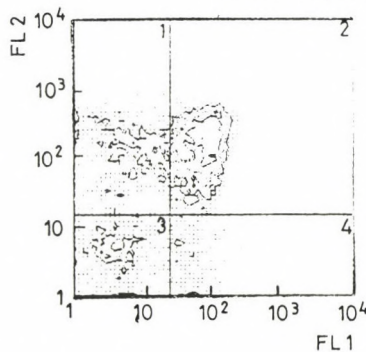
FL2 0.255

1 min

3 —

7 .....

15 —



Sample : PE 0111 MONU CD8 1 008

Date: 16-Jan-90 11:15

Cytometer: FACSTAR

File : PECD31025

FL1 :

FL2: UCHL1

FL3 :

Parameters: FL1 FL2		Contour statistics				Total events: 5000		
		Gated events: 4562						
#	X & Y Lower	X & Y Upper	Events	% Gated	% Tot	X & Y Mean	X & Y Mode	Peak
1	1.00	21.54	952	20.87	19.04	8.20	1.00	10
	16.08	10000				167.51	346.48	
2	24.94	10000	2786	61.07	55.72	75.53	92.95	36
	16.08	10000				185.69	223.46	
3	1.00	21.54	597	13.09	11.94	5.22	4.31	8
	1.00	13.89				5.13	4.31	
4	24.94	10000	227	4.98	4.54	59.13	51.79	9
	1.00	13.89				4.76	1.00	

Fig. 1. Two parameter dot-plot of CD8-FITC(x-axis) and CD45RO(UCHL1)-PE (y-axis) fluorescence showed high number of double marker positive cells

Table 2  
The distribution of the differentiation antigens on PBM cells in patients with IM (a  $\pm$  SE)

Cases	Abs. ly. count/mm <sup>3</sup>	CD3% (T3)	CD4% (T4)	CD8% (T8)	HLA-DR% (single+)	CD57% (Leu-7)	CD19% (B4)	CD20% (B1)	CD21% (B2)	CD14% (Mo2)
IM patients n = 10	1891.09 $\pm$ 884.71	85.24 $\pm$ 6.08	13.91 $\pm$ 2.03	66.21 $\pm$ 4.46	6.57 $\pm$ 1.21	6.64 $\pm$ 0.92	0.42 $\pm$ 0.14	1.14 $\pm$ 0.75	1.24 $\pm$ 0.56	0.25 $\pm$ 0.07
Controls n = 5	2880.40 $\pm$ 641.5	53.18 $\pm$ 5.70	36.73 $\pm$ 4.59	23.09 $\pm$ 1.80	8.65 $\pm$ 1.16	8.36 $\pm$ 2.19	9.6 $\pm$ 1.6	12.1 $\pm$ 2.4	16.8 $\pm$ 3.0	3.01 $\pm$ 1.01
p <	NS	0.01	0.001	0.001	NS	NS	0.001	0.001	0.001	0.001



Table 3  
The expression of the activation antigens on CD8<sup>+</sup> T-cells in patients with IM (a  $\pm$  SE)  
Double marker positive cells

Cases	CD3/HLA-DR %	CD8/HLA-DR %	CD8/CD25 %	CD8/CD30 %	CD8/CD69 %	CD8/CD70 %	CD8/CD71 %	CD8/CD40 %	CD8/CD45RO %	CD8/CD45 %
IM patients	63.17	51.22	2.60	10.43	1.87	13.82	5.30	24.37	51.75	12.23
n = 10	$\pm 6.08$	$\pm 11.73$	$\pm 1.06$	$\pm 4.13$	$\pm 0.84$	$\pm 5.64$	$\pm 2.31$	$\pm 6.98$	$\pm 7.15$	$\pm 5.64$
controls	5.1	0.50	0.28	0.11	0.12	0.04	0.13	0.13	2.11	9.95
n = 5	$\pm 2.0$	$\pm 0.35$	$\pm 0.15$	$\pm 0.04$	$\pm 0.08$	$\pm 0.01$	$\pm 0.06$	$\pm 0.04$	$\pm 0.43$	$\pm 2.31$
p <	0.001	0.001	NS	0.01	NS	0.01	NS	0.001	0.001	NS

High levels of the CD45RO molecule, similar to that of the HLA-DR Ag on the CD8<sup>+</sup> cells, were found (Fig. 1). On the other hand, only moderate increase of the expression of CD30, CD70 and CD40 antigens were observed on CD8<sup>+</sup> cells in double marker studies ( $p < 0.01$ ). The MoAbs of the CD25, CD69, CD71 and CD45 clusters did not react with the CD8<sup>+</sup> lymphocytes of patients.

In six cases, the proportion of activated CD8<sup>+</sup> cells was determined after the EBV infection (4–6 months). The percentage of CD8+HLA-DR+ cells was significantly lower in the asymptomatic patients than that of the active phase of the disease ( $19.26 \pm 6.82\%$  versus  $51.22 \pm 11.73\%$ ), but this result proved to be higher than that of the controls ( $0.5 \pm 0.35\%$ ).

The proportion of CD8+CD45RO+ cells was also significantly lower in the six asymptomatic cases ( $1.33 \pm 0.61\%$  versus  $51.75 \pm 7.15\%$ ) than that found in the active phase.

## Discussion

Earlier studies demonstrated that the lymphocytosis manifested in acute IM is characterized by the expansion of T-lymphocytes, the majority of which are of the CD8<sup>+</sup> cytotoxic/suppressor type [1, 2, 3]. The expansion is thought to result from the activation and proliferation of T-lymphocytes in vivo induced by EBV<sup>+</sup> B lymphocytes [7, 8]. The occurrence of the HLA-DR antigen bearing T-lymphocytes in IM is the major evidence of in vivo cellular activation [3, 4, 6]. It was surprising that Tac antigen (p55) expression of these cells was not detectable by flow cytometric immunofluorescence [9, 10]. The results of an affinity cross linking study seemed to indicate that the IL-2 receptor expressed on IM lymphocytes is p70, the second chain of the IL-2 receptor distinct from p55 [11].

In the present study, the PBM cells from 10 acute IM patients were found to possess simultaneously the CD3, CD8 and HLA-DR antigens supporting the presence of activated CD8<sup>+</sup> cells (as well as CD3<sup>+</sup>) in the blood. In addition, the CD8<sup>+</sup> cells expressed a high level of CD45RO molecule, which is involved in the process of T-cell activation [12].

Antigens of the leucocyte common antigen (LCA) family are expressed on virtually all leucocytes. Earlier data demonstrated that LCA MoAbs generally fall into two major clusters. CD45 MoAbs detect epitopes characteristic of all members of the LCA family, whereas CD45RO recognise restricted epitopes which are observed on some but not all LCA members. The increased expression of UCHL-1 (CD45RO) was observed in high-grade B and T-cell lymphomas [12] as well as in our IM cases, suggesting that CD45RO has an important functional role in lymphocyte activation.

The CD30 antigen is present on some lymphoid cell lines, e.g. on all cell lines derived from Hodgkin's disease, HTLV-1<sup>+</sup> lines as well as the EBV lymphoblastoid B-cell lines. CD30 did not occur during inflammatory events, with the exception of IM [12]. Similarly to this earlier result, the MoAb of the CD30 cluster gave a positive reaction with the CD8<sup>+</sup> cells in our investigated cases, proving the activated state of these cells in IM.



Among permanent cell lines, the CD70 activation molecule is apparently highly expressed only in those cell lines which are related to activated T and B-cells. Kinetic studies showed that the expression of the CD70 antigen reaches its highest level on day 4 [7]. The expression of the CD70 antigen was elevated on CD8<sup>+</sup> cells in IM patients, too. Another activation molecule, the CD40, is a putative receptor for a soluble product or cellular ligand supporting proliferation of B-cells, showing homology to the growth factor receptors [12]. The expression of the CD40 antigen was also found to be elevated on activated CD8<sup>+</sup> cells in IM.

Similarly to the earlier reported data [9, 10, 11], the CD25 antigen (Tac, p55) was not elevated on PBM cells of our IM patients, suggesting another mechanism of cell activation in the defence against viral infection of the immune system.

In conclusion, the activation of CD8 cells in acute IM patients was characterized by the high expression of HLA-DR and CD45RO antigens. CD45RO identifies a subpopulation of T cells which provides maximum help for PWM stimulated immunoglobulin synthesis [13]. Nevertheless, the possibility that the priming of suppressor and MHC-specific cytotoxic T lymphocytes generated by MHC class I antigens may also contribute to the increase of the CD45RO<sup>+</sup> cells should also be considered [14].

The increased CD8+CD45RO<sup>+</sup> T-cells in the active phase of IM might play a role in the pathogenesis of IM.

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## **Characterization of T Lymphocyte Subsets in Hairy Cell Leukaemia: Influence of Splenectomy and Correlations with the Clinical Stage of the Disease**

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Peripheral blood mononuclear cell surface markers were studied in a series of 26 hairy cell leukaemia patients 19 of whom were splenectomized previously. Patients with non-symptomatic and stable disease were distinguished from those with symptomatic and/or progressive disease (also termed "active" clinical stages). In all HCL patients as a group, the absolute number of CD4+ MN cells did not differ statistically from that of the controls, while the number of CD8+ MN cells was significantly increased. The reduction of the CD4/CD8 ratio in the peripheral blood of HCL patients as compared to the controls was explained by the reduction of this ratio in patients with "active disease", while the CD4/CD8 ratio of patients with non-symptomatic and stable disease did not differ statistically from that of the controls. The CD4/CD8 ratio was found to be influenced mainly by the clinical stage of the disease, and not by the effect of splenectomy.

**Keywords:** clinical stage, hairy cell leukaemia, splenectomy, T-lymphocyte subsets

### **Introduction**

Both cell surface marker [1] and DNA rearrangement studies [2] indicate that in the overwhelming majority of the cases, the neoplastic cells of HCL are of B cell origin. Though pancytopenia is characteristic of HCL, the leukaemic form of the disease is not very rare, leading to considerable variations in the number of circulating B cells. Monocytopenia has been described to occur in the majority of untreated as well as splenectomized HCL patients [3].

Studies regarding the phenotypic and functional characteristics of T cells gave heterogeneous results. The CD4/CD8 ratio was found to be normal in untreated patients with HCL, while in splenectomized HCL patients this ratio decreased due to the increase in the number of CD8+ peripheral blood mononuclear (PBMN) cells [4]. In the removed spleen, the majority of the T cells were CD8 positive [4]. Others have found a correlation between the CD4/CD8 ratio and disease activity: in patients with active disease, the CD4/CD8 ratio was significantly decreased when compared to both the patients in clinical remission and to healthy controls [5, 6]. The decrease in the percentage of CD4 and the increase in the percentage of the CD8+ MN cells resulted in a decrease in the CD4/CD8 ratio. During the interferon-alpha therapy, the CD4/CD8 ratio returned to normal [7,8]. Some T cell functions, e.g. the T cell response to different antigens and mitogens were found to be impaired [9]; while the T-helper capacity and the T cell

cytotoxicity were normal in both untreated and in splenectomized HCL patients [6, 10]. Because of the importance of the T cell subsets in host defence and in immunoregulation, we studied the PBMN cells of HCL patients with monoclonal antibodies directed against T cell antigens.

## Materials and Methods

### *Patients*

Twenty six HCL patients (12 males and 14 females) were studied: their age at the time of diagnosis was 22–79 years (mean: 54.3 years). The diagnosis of HCL was based on the usual cytologic, cytochemical, histopathologic and immunologic criteria of the disease: in the 19 splenectomized patients (cases 1–19), it was also confirmed by spleen histology. The disease was found to be of B cell origin in all these cases by cell surface marker and/or immunoglobulin gene rearrangement studies as described previously [13]. Splenectomy was performed 5 to 123 months (mean: 53 months) prior to our study. Patient 18 was atypical having a negative tartarate-resistant acid phosphatase (TRAP) test in peripheral blood smears, however, all other criteria of HCL were fulfilled. Patients were untreated for at least three weeks at the time of the study.

The number of healthy donors whose peripheral blood samples served as controls varied from 18 to 28.

### *Determination of clinical stage*

The clinical staging criteria of Porzsolt et al. [12] were used. According to this, we distinguish patients with non-symptomatic and stable disease from those with symptomatic and/or progressive disease. The latter will also be mentioned as patients in "active" clinical stages of the disease. Furthermore, we evaluated patients according to the presence or absence of the spleen separately.

### *Isolation of peripheral blood mononuclear cells*

Mononuclear cells were separated from peripheral blood on a Ficoll-Uromiro gradient. Interphase cells were resuspended in RPMI 1640 containing 10% fetal calf serum (FCS, Gibco) and antibiotics. Monocytes were not removed because hairy cells are capable of adherence and clumping [13], and thus potentially selective cell losses could have occurred. The viability of the final cell suspension was greater than 95% as tested by the exclusion of trypan blue.



### *Cell surface markers*

Binding of the monoclonal antibodies (MoAbs) was assessed with indirect immunofluorescence using FITC conjugated rabbit anti-mouse immunoglobulin, DAKO as second antibody. T cell associated antibodies were CD3 (T3), CD2 (T11), CD4 (T4), CD8 (T8) from Ortho Diagnostic Systems. Cells prepared the same way, but without addition of the first layer antibody served as negative controls. Results of studies using B cell associated as well as activation associated MoAbs have been reported separately [13].

### *Statistics*

Statistical analysis was performed using Student's t-test. When comparing more groups, analysis of variance has been performed. The absolute number of lymphocytes has a skewed distribution, thus the geometrical mean and 95% confidence interval values were counted.

## **Results**

The number of PBMN cells was found to be increased in all the clinical stages of the disease. Though the number of PBMN cells does not belong to the functional criteria that decide the clinical stage, all the seven patients who were studied in the leukaemic phase of the disease (PBMN cells < 10 G/l) belonged to the active clinical stages. In one of these patients, the disease was non-symptomatic but progressive, while five further patients had symptomatic and progressive disease.

The results of the cumulated evaluation of all the HCL patients are shown in Table 1. The percentage of PBMN cells carrying pan-T markers as well as that of the CD4+ PBMN cells was found to be decreased, the percentage of CD8+ PBMN cells did not differ statistically from that of the controls. On the other hand, the absolute number of CD3+ PBMN cells was normal, while that of the CD2+ MN cells was even found to be increased as compared to the healthy controls. The absolute number of CD4+ MN cells did not differ statistically from that of the controls, while the number of CD8+ MN cells was significantly increased. As a result of these changes, the CD4/CD8 ratio was greatly reduced in the group of HCL patients.

To evaluate the effect of splenectomy and that of clinical stage, we had to divide the HCL patients into subgroups. It was not possible to separate the effect of these factors completely, as all our HCL patients with non-symptomatic stable disease were splenectomized earlier, while the data of patients in the symptomatic and/or progressive clinical stages of the disease could be evaluated separately according to the absence or presence of the spleen.

In the non-symptomatic stable clinical stage of the disease, the percentage and absolute number of MN cells carrying pan-T markers was normal (Table 2). Though the percentage of CD4+ MN cells was found to be decreased, but due to the elevated number

Table 1

Reactivity of PBMN cells from HCL patients and controls with MoAbs against T cells (mean and 95% confidence intervals)

Percentages	CD3+ %	CD2+ %	CD4+ %	CD8+ %	CD4/CD8
HCL	38.6 (28.1–49.1)	42.9 (31.4–54.5)	21.1 (15.6–26.6)	21.0 (15.9–26.9)	1.1 (0.9–1.3)
(n)	(22)	(23)	(22)	(22)	(22)
Controls	60.6 (55.8–65.4)	57.3 (51.4–63.2)	40.5 (36.5–44.5)	24.4 (20.8–28.0)	1.89 (1.5–2.3)
(n)	(28)	(18)	(25)	(27)	(25)
P<	0.001	0.05	0.001	NS	0.0001
Absolute numbers*	CD3+ G/l	CD2+ G/l	CD4+ G/l	CD8+ G/l	PBMN cells G/l
HCL	1.7 (1.2–2.5)	2.1 (1.5–2.9)	1.1 (0.8–1.5)	1.1 (0.8–1.6)	6.1 (4.0–9.4)
Controls	1.3 (1.1–1.4)	1.1 (1.0–1.4)	0.8 (0.7–1.0)	0.5 (0.4–0.6)	2.1 (1.9–2.3)
P<	NS	0.05	NS	0.001	0.001

\* geometrical mean

of PBMN cells, the absolute number of the CD4+ MN cells did not differ statistically from that of the controls. On the other hand, the percentage of CD8+ MN cells was normal, while their absolute number was found to be increased.

In the group of splenectomized HCL patients with symptomatic and/or progressive disease, the percentage of MN cells carrying pan-T markers was significantly decreased, while their absolute number was greatly increased (Table 3). The absolute numbers of the CD4+ as well as of the CD8+ PBMN cells were found to be normal and increased, respectively.

In those HCL patients who have not been splenectomized and were studied in the symptomatic and/or progressive clinical stages of the disease, the percentage of MN cells carrying pan-T markers was decreased, while the absolute number of CD3+ MN cells was normal and that of the CD2+ MN cells increased (Table 4). The ratio of CD4+ MN

Table 2

Reactivity of PBMN cells from splenectomized HCL patients studied in the non-symptomatic stable clinical stage of the disease with MoAbs against T cells

Patient	PBMN cells (G/l)	CD3+ % (G/l)	CD2+ % (G/l)	CD4+ % (G/l)	CD8+ % (G/l)
1	3.408	56.9 (1.940)	85.5 (2.913)	19.0 (0.647)	42.6 (1.450)
2	4.080	—	80.5 (3.284)	31.3 (1.277)	31.1 (1.268)
3	4.896	62.5 (3.060)	63.5 (3.109)	36.0 (1.762)	22.0 (1.077)
4	2.516	36.8 (0.926)	57.8 (1.454)	43.9 (1.105)	26.8 (0.674)
5	4.656	89.3 (4.157)	77.2 (3.594)	36.0 (1.676)	25.6 (1.191)
6	1.440	53.5 (0.770)	51.8 (0.746)	34.6 (0.498)	17.3 (0.249)
7	2.074	41.6 (0.862)	50.1 (1.039)	21.8 (0.452)	27.7 (0.574)
8	5.060	63.0 (3.188)	61.0 (3.086)	41.3 (2.089)	24.6 (1.244)
9	3.055	40.0 (1.222)	43.5 (1.329)	25.7 (0.785)	16.4 (0.501)
10	2.280	18.9 (0.430)	21.9 (0.499)	12.0 (0.274)	9.7 (0.221)

*Percentages (mean; 95% confidence intervals)*

HCL	51.4 (36.2–66.5)	59.4 (45.8–73.0)	30.1 (23.0–37.2)	24.4 (18.2–30.6)
Controls	60.6 (55.8–65.4)	57.3 (51.4–63.2)	40.5 (36.5–44.5)	24.4 (20.8–28.0)
(n)	(28)	(18)	(25)	(27)
P<	NS	NS	0.01	NS

*Absolute numbers (G/l; mean; 95% confidence intervals)*

HCL	3.1 (2.3–4.2)	1.4 (0.8–2.6)	1.7 (1.0–2.8)	0.9 (0.5–1.4)	0.7 (0.4–1.1)
Controls	2.1 (1.9–2.3)	1.3 (1.1–1.4)	1.1 (1.0–1.4)	0.8 (0.7–1.0)	0.5 (0.4–0.6)
(n)	(31)	(28)	(18)	(25)	(27)
P<	0.01	NS	NS	NS	0.05



Table 3

Reactivity of PBMN cells from splenectomized HCL patients studied in the symptomatic and /or progressive clinical stages of the disease with MoAbs against T cells

Patient	PBMN cells sejt (G/l)	CD3+ % (G/l)	CD2+ % (G/l)	CD4+ % (G/l)	CD8+ % (G/l)
11	7.680	—	—	—	—
12	5.616	52.5 (2.948)	81.1 (4.554)	30.7 (1.724)	39.4 (2.213)
13	11.060	—	33 (3.649)	4.8 (0.531)	14.2 (1.571)
14	61.085	11.4 (6.964)	16.4 (10.002)	7.9 (4.826)	8.7 (5.314)
15	2.772	21.7 (0.601)	34.0 (0.942)	14.9 (0.413)	24.6 (0.681)
16	4.452	45.3 (2.017)	55.1 (2.453)	18.3 (0.815)	45.3 (2.017)
17	18.500	—	4.1 (0.759)	—	—
18	84.150	5.8 (4.881)	6.4 (5.386)	4.5 (3.787)	5.6 (4.712)
19	2.2	65 (1.3)	—	—	—
<i>Percentages (mean; 95% confidence intervals)</i>					
HCL		33.6 (9.6–57.6)	32.8 (8.0–57.6)	13.5 (3.5–23.5)	23.0 (6.7–39.3)
Controls		60.6 (55.8–65.4)	57.3 (51.4–63.2)	40.5 (36.5–44.5)	24.4 (20.8–28.0)
(n)		(28)	(18)	(25)	(27)
P<		0.001	0.01	0.001	NS
<i>Absolute numbers (G/l, mean; 95% confidence intervals)</i>					
HCL	10.2 (3.9–27.0)	2.9 (1.2–7.0)	2.9 (1.3–6.6)	1.3 (0.5–3.7)	2.2 (1.0–4.7)
Controls	2.1 (1.9–2.3)	1.3 (1.1–1.4)	1.1 (1.0–1.4)	0.8 (0.7–1.0)	0.5 (0.4–0.6)
(n)	(31)	(28)	(18)	(25)	(27)
P<	0.001	0.001	0.001	NS(0.052)	0.001

Table 4

Reactivity of PBMN cells from non-splenectomized HCL patients studied in the symptomatic and/or progressive clinical stages of the disease with MoAbs against T cells

Patient	PBMN (G/l)	CD3+ % (G/l)	CD2+ % (G/l)	CD4+ % (G/l)	CD8+ % (G/l)
20	3.564	63.0 (2.245)	66.4 (2.366)	—	—
21	19.152	22.7 (4.348)	26.7 (5.114)	8.4 (1.608)	12.8 (2.451)
22	25.220	10.7 (2.699)	8.7 (2.194)	8.9 (2.245)	8.0 (2.017)
23	5.478	18.5 (1.013)	27.0 (1.479)	15.4 (0.843)	23.5 (1.287)
24	1.914	49.6 (0.949)	—	23.9 (0.457)	13.1 (0.251)
25	8.100	14.0 (1.260)	30.0 (2.430)	21.0 (1.701)	18.0 (1.458)
26	22.680	2.9 (0.657)	4.0 (0.907)	3.9 (0.885)	5.5 (1.247)
<i>Percentages (mean, 95% confidence intervals)</i>					
HCL		26.2 (6.4–46.0)	27.0 (5.2–48.7)	13.7 (6.1–21.2)	13.5 (6.9–20.1)
Controls		60.6 (55.8–65.4) (28)	57.3 (51.4–63.2) (18)	40.5 (36.5–44.5) (25)	24.4 (20.8–28.0) (27)
P<		0.001	0.001	0.001	0.05
<i>Absolute numbers (G/l; mean; 95% confidence intervals)</i>					
HCL	8.5 (3.9–18.5)	1.6 (0.8–2.8)	2.1 (1.3–3.6)	1.1 (0.6–2.0)	1.2 (0.5–2.7)
Controls	2.1 (1.9–2.3)	1.3 (1.1–1.4)	1.1 (1.0–1.4)	0.8 (0.7–1.0)	0.5 (0.4–0.6)
(n)	(31)	(28)	(18)	(25)	(27)
P<	0.001	NS	0.05	NS	0.001

cells was decreased, while their absolute number did not differ statistically from that of the controls. The absolute number of CD8+ MN cells was found to be increased also in this group of HCL patients.

## Discussion

Though HCL is a rare disease and the number of cases in the single studies are rather limited, it is very important to divide these patients into subgroups to be able to study clinical effects separately [14]. Studies aimed at describing the immunophenotype of untreated HCL focus on the cell surface markers of the neoplastic cells [15]. Nevertheless, we believe that factors like the absolute number of MN cells or the clinical stage of the disease are of inevitable importance and should be considered when evaluating results of cell surface marker studies.

While pancytopenia is characteristic for untreated HCL, some patients have the leukaemic form of the disease. Thus, the percentage of a given MN cell subset itself is not very informative. The wide range of variation in the absolute number of MN cells in our patients explains that results of evaluations considering percentages or absolute numbers of certain subpopulations differ greatly. Though the percentage of some T cell subsets might be low [15], we found no deficiency in the absolute number of T cells in our HCL patients considering either subgroup with either of the T cell markers studied.

We found that the decreased CD4/CD8 ratio in the peripheral blood of all HCL patients as a group is in accordance with earlier studies [4, 6]. Nevertheless, in patients with non-symptomatic stable clinical disease this ratio did not differ statistically from that of the controls. The CD4/CD8 ratio was found to be decreased only in the symptomatic and/or progressive clinical stages of the disease, thus confirming the impact of disease activity on the CD4/CD8 ratio [5]. According to our data, the clinical stage is of greater importance in influencing the CD4/CD8 ratio than the presence or absence of the spleen. Furthermore, we have found the CD4/CD8 ratio of PBMN cells of otherwise healthy patients after posttraumatic splenectomy to be normal [16]. In contrast to the data of Cawley et al. [4], these results argue further against the role of splenectomy in the decrease of the CD4/CD8 ratio.

In conclusion, we have found the absolute number of CD2+, CD3+, CD4+ and CD8+ PBMN cells in HCL to be not deficient. The decrease in the CD4/CD8 ratio in HCL is explained by the increase in the number of CD8 MN cells and seems to be influenced mainly by the clinical stage of the disease and not by the effect of splenectomy.

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MAGYAR  
TUDOMÁNYOS AKADÉMIA  
KÖNYVTÁRA

## **Recurrent Attacks of Hemolytic Uremic Syndrome**

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In 1955 Gasser and his co-workers [1] were the first to describe the so-called hemolytic uremic syndrome (HUS); since then, the number of reports has steadily increased. Some authors consider HUS as a unique syndrome [2], while others suggest that HUS is both heterogenic and heterogeneous [3]. It is generally emphasized that HUS never recurs [4, 5]. However, this view should be reconsidered due to the numerous reports on a recurrent form of HUS [6-9], which is beginning to be recognized as an important subset or variant of this syndrome [10]. This report describes a case, where three similar recurrent episodes of hemolytic anemia, thrombocytopenia and uremia had occurred during the past eight years.

**Keywords:** hemolytic uremic syndrome, thrombocytopenia, hemolytic anemia

### **Case Report**

A 13 year old girl was admitted for jaundice of 48 hours duration. Four days previously, she had had headaches, backpains and fever of 39°C. She received erythromycin and aspirin and the fever fell within 48 hours; at that time, she noticed hyperpigmentation of the urine associated with abdominal pain and diarrhea. Her past history involved the following:

a) at the age of five, she was hospitalized for fever, anemia, uremia and hematuria. She was discharged with the diagnosis of acute glomerulonephritis. Since then, she has been receiving 1.200.000 IU of benzathin penicillin i.m. every 20 days.

b) at the age of seven, she was hospitalized due to hematuria, jaundice, uremia and skin eruption, following a febrile episode. She was discharged with the diagnosis of 'paroxysmal hemoglobinuria'.

c) at the age of ten, she was admitted to another Children's Hospital because of high fever, jaundice and hematuria. She was discharged with the diagnosis of hemolytic uremic syndrome. The laboratory data at that time were: Hb 8.6 g/dl, reticulocytes 2.4%, platelets  $50 \times 10^9/l$ , BUN 100 mg/dl, serum bilirubin 4.5 mg/dl (unconjugated 3.7 mg/dl), urine hemoglobin 3+, many erythrocytes in the sediment. Erythrocyte G-6-PD was normal and L.E. cells were negative.



### Present findings and course

On admission, physical examination disclosed extreme pallor of the skin, jaundiced sclerae and several small petechiae at the epigastrium. Liver and spleen were not palpable. Temperature and blood pressure were normal.

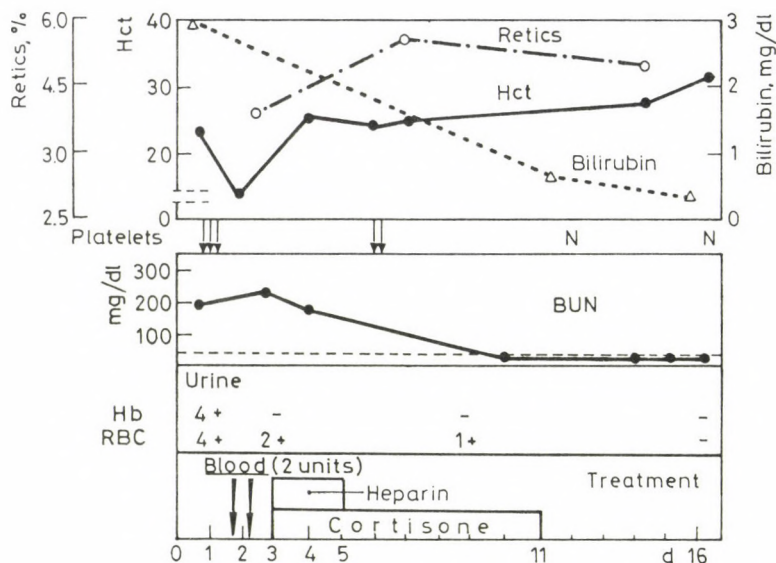


Fig. 1. Main laboratory features and treatment during the fourth episode (the number of arrows in platelets denotes severity of decrease, N=normal number)

Rapid fall of the hematocrit from 22% to 13% was observed on the second day, therefore 2 units of blood were urgently transfused (Fig. 1). As microangiopathic hemolytic anemia (schistocytic forms of erythrocytes in the peripheral blood smear) was suspected, 20,000 IU of heparin/24 h was given i.v., but it was stopped on the third day of treatment, as the results of the coagulation tests did not confirm the diagnosis of consumptive coagulopathy. The possibility of thrombotic thrombocytopenic purpura was considered and cortisone was given for about a week. From the fourth day, a stabilization and then gradual increase of hematocrit, reduction of jaundice and return of blood urea nitrogen to normal was observed. The patient was discharged in good clinical condition (Ht 32%, BUN 20 mg/dl and total serum bilirubin 0.93 mg/dl). The data of her follow-up 42 days after her discharge were all normal.

### Discussion

We classified the present case as HUS, based on the clinical features and on the characteristic triad of hemolytic anemia, thrombocytopenia and uremia. The erythrocyte fragmentation corroborated the diagnosis of microangiopathic hemolytic anemia.

Thrombotic thrombocytopenic purpura is the only other diagnosis that can be considered seriously; classical TTP seems unlikely because of the age of the patient, the absence of severe neurological manifestations and the rapid recovery. No direct indications of intravascular coagulation were found in our patient and the twice repeated coagulation tests were all negative. This, however, does not exclude intravascular coagulation, since normal or increased levels of fibrinogen and Factors VIII, IX and XI (usually done a few days after the onset of the syndrome), represent an expression of a 'rebound' phenomenon [5].

Other conditions which may mimic HUS at least partially, such as autoimmune hemolytic anemia of different etiology, deficiency of erythrocytic G-6-PD, paroxysmal nocturnal hemoglobinuria and Evans' syndrome could be excluded because of the absence of their pathognomonic features or tests.

The main interest of our case is the recurrence of such successive episodes, which were all serious enough to require hospitalization. All three episodes seem to be identical to the one we observed: the main clinical and laboratory findings appear to be similar.

We were unable to find one single factor which could have triggered each attack; therefore, we cannot be certain whether it is always the same or each time a different provoking agent, exogenous or intrinsic. The same difficulty is faced by the authors of similar cases with recurrent attacks [7].

Gianantonio, who has probably the widest experience regarding this syndrome, based on a long follow-up of over 1.000 children in Buenos Aires, considers the non-relapse as the crucial criterion for the diagnosis of HUS [4]. This view is contradicted to some extent by recent reports of two or more recurrences of HUS [6, 7, 9]. Furthermore, Kaplan [10] analyzed 30 patients (27 of them children) in whom one to fourteen recurrent episodes of HUS occurred over periods ranging from three months to 20 years. In this review, a recurrence was defined as more than one discrete episode of HUS, so patients with relapses rather than recurrences were excluded. However, the prognosis did not appear to be related to the number of recurrences. The mortality rate is 30%. Although these 30 published cases of recurrent HUS have a common feature that differentiates them from the other 'typical' cases of HUS, their analysis failed to show that recurrent HUS consists of a discrete clinicopathologic entity. So, for the time being, there are no safe prognostic features or criteria to predict which patient will manifest in the future a recurrent episode of HUS.

To explain the uniqueness of an attack, non-exposure to an offending agent cannot be seriously considered, since this hypothetical agent remains unknown and therefore cannot be avoided. It is possible that different etiologic factors may prevail in various parts of the world; if the agent is infectious [5, 10] and probably viral, then perhaps lasting immunity is obtained more easily in the population at risk of Argentina than in other populations.

Convincing epidemiologic evidence exists for an association between hemolytic uremic syndrome occurring in childhood and infections with verotoxin-producing strains of *E. coli* and the Shiga toxin of *Shigella* [11]. There have been no reports associating childhood HUS with drugs except for a possible association with Metronizadole [12]. In contrast, infections have rarely been implicated in the pathogenesis of adult HUS but this syndrome can be induced by certain drugs. Thus women taking oral contraceptives [13] or conjugated estrogens [14] and patients receiving chemotherapeutic agents,



especially mitomycin [15], have increased susceptibility to developing HUS which can be recurrent in women on oral contraceptives [13]. Moreover, recurrent hemolytic uremic syndrome induced by Quinine has recently been reported in adults and was found to be associated with the presence of Quinine-dependent IgG and IgM antibodies reactive with the platelet glycoproteins 1b/1x and 11b/111 [16].

As far as therapy is concerned in our case, it is doubtful whether the administration of heparin and/or cortisone [1, 2] had actually affected the course of the disease, since the previous episodes regressed spontaneously and completely, without any therapeutic interventions. It is also worth pointing out, that even after the fourth episode, no residual renal damage could be demonstrated.

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## **Congenital Factor XIII Deficiency. A family report.**

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Four sisters born to consanguineous muslim parents with a bleeding disorder since birth are presented. They also had prolonged umbilical cord bleeding and history of delayed wound healing. Since childhood, they have been developing spontaneous ecchymotic spots. Three out of four sisters had Factor XIII deficiency. Their mother's sisters, who have been developing ecchymotic spots were found to have normal clot stability and Factor XIII levels. Family study indicates autosomal recessive mode of inheritance of the congenital Factor XIII deficiency.

**Keywords:** congenital, Factor XIII deficiency

### **Introduction**

Factor XIII deficiency, though a rare disorder, has been documented in all parts of the world. Presently, about 100 patients have been reported. The clinical manifestations and abnormal laboratory tests seen in Factor XIII deficiency have been established [6]. The mode of inheritance of the disease is suggested to be either sex linked or to have an autosomal pattern. A final view is not yet available. The present communication is to contribute to the observations that support an autosomal recessive inheritance of the disease. A consanguineous family has been described. Three of their 6 siblings tested for Factor XIII deficiency were suspected of having this disease.

### **Family Report**

The muslim parents had a history of first degree consanguinity, they had 6 children (5 daughters and 1 son). The details of their clinical profile are given below.

A 13 year old girl (T) with history of umbilical cord bleeding at birth which required 2 stitches, and a history of a tendency to bruise easily and ecchymotic spots and prolonged wound healing. There is no history of menorrhagia.

The propositus, a 9 year old girl (R), had a history of umbilical cord bleeding which required two stitches and blood transfusion to control umbilical cord bleeding. An increased post-traumatic bleeding and also prolonged wound healing were included in her history. Her examination revealed ecchymotic spots on legs, swelling of left thigh with overlying tense skin.

A 5 year old girl (F) also had a history of a tendency to bruise easily, epistaxis and prolonged bleeding following trauma. Her history also included prolonged wound healing. On examination, ecchymotic spots were present.



A 3 year old girl (N) also had a history of umbilical stump bleeding at 12 hours of age which required a few stitches and a unit of blood transfusion. Her history also included prolonged wound healing. On examination, ecchymotic spots were present on the left lower knee.

A 2 year old girl died of prolonged bleeding and inadequate management.

A 3 year old brother died of prolonged bleeding after head injury and intracranial haemorrhage. He had prolonged bleeding from umbilical stump at birth and from circumcision wound.

The 30 year old mother (N) had blue spots and had bruised easily since birth. None of the episodes were so severe as to require hospitalization or blood transfusion. There was no history of menorrhagia or postpartum haemorrhage. Family history (Fig. 1) revealed that her mother and three aunts had a similar history of bluish discolouration and a tendency to bruise easily. On examination, her data were essentially normal.

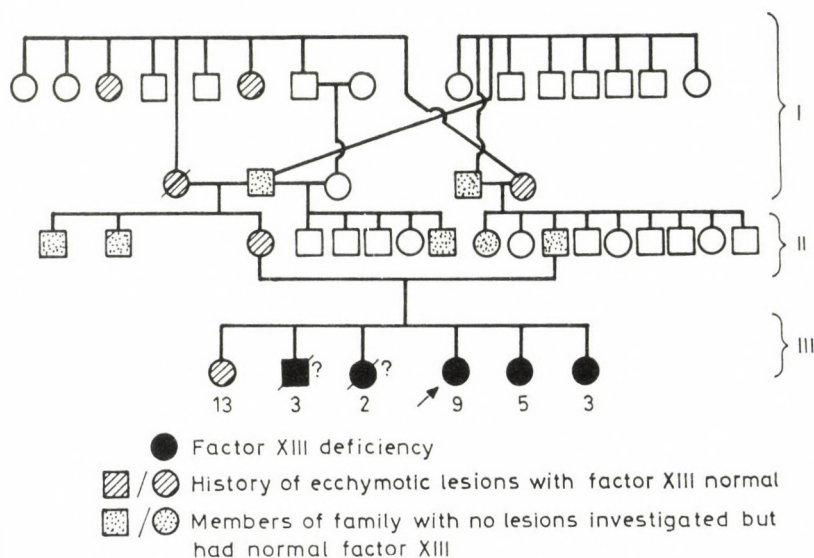


Fig. 1. Kindred of Factor XIII. deficient persons. Roman numerals indicate the 3 generations studied. The first degree consanguinity is marked by a thick line

The father, a 36 year old man (I.A.), had no history of any bluish discolouration or tendency to bruise. The family history suggested that his mother had had a history of tendency to bruise easily.

The performed investigations are summarized in Table 1. The whole family was investigated for bleeding time, platelet count, whole blood coagulation time, and one stage plasma prothrombin time (PPT). Prothrombin consumption index, clot retraction, activated partial thromboplastin time (APTT), and clot solubility in 5M urea were performed by standard methods [2]. All the family members had normal haemoglobin levels, platelet counts, red cell and platelet morphology, screening coagulation tests were

Table 1  
Screening tests in the family with factor XIII deficiency

Tests	T 13 yr.	R 9 yr.	F 5 yr.	N 3 yrs.	Mother N 30	Father I.A 36	Normal control
Bleeding time	4 min.	5 min.	5 min.	4 min.	4 min.	2 min.	2.5 min.
Whole Blood Clotting time	9 min.	9 min.	10 min.	9 min.	9 min.	8 min.	7-12 min.
Plasma pro- thrombin time	13 sec	13 sec	12 sec	12 sec	12 sec	12 sec	12 sec
Prothrombin Consumption	—	66.6%	64.3%	64.3%	—	—	25.7%
Activated Part- ial Thrombo- plastin time	45 sec	45.5 sec	43 sec	44 sec	45 sec	47 sec	41 sec
Clot solubility 5M / urea (in 24 hrs.)	Insoluble	Soluble	Soluble	Soluble	Insoluble	Insoluble	Insoluble
Haemoglobin g/dl	12.0	12.0	10.7	10.4	12.0	14.0	—
Platelet count	2.0 lacs	2.0 lacs	3.4 lacs	2.2 lacs	2.5 lacs	2.1 lacs	1.25-4.5
Total PF3	—	10 sec	10 sec	9 sec	—	—	8 sec
PF3 release							
ADP o	—	37 sec	35 sec	33.5 sec	—	—	26.5 sec
—20	—	26.5 sec	21 sec	20.5 sec	—	—	17 sec

normal. Urea clot stability showed clot solubility within one hour. Both parents, one daughter, grandparents from both sides and maternal grandmother's sisters were normal. One aunt from both sides and 3 maternal uncles tested were also normal.

### Discussion

Urea clot stability test can be abnormal in the presence of heparin, fibrin degradation products (FDPS) and in dysfibrinogenemia [6]. The investigations in this family did not suggest any dysfibrinogenemia and raised FDP levels as evident by normal coagulation studies (Table 1).

Bleeding manifestations in Factor XIII deficiency occurs only in homozygous state when the Factor XIII levels are very low and its activity is less than 1% of the normal [9]. However, in the heterozygous state, the laboratory parameters are essentially normal and the individuals are clinically symptomatic or may develop minor symptoms due to stress or trauma.

Nearly all patients with Factor XIII deficiency have bleeding from umbilical cord during the first few days of their life [3, 6, 9]. All the children in this family had similar umbilical cord bleeding requiring stitches and blood transfusion in the first few hours



after birth. All of them had ecchymoses, haematomas and prolonged bleeding following trauma. All the sibs had a history of poor wound healing and wounds lasting for 10–14 days. Poor wound healing has been attributed to Factor XIII deficiency [1, 3, 5]. There is a higher incidence of intracranial haemorrhage in Factor XIII deficiency [1, 5, 6] than in other inherited bleeding disorders. In the present family, one boy died of intracranial haemorrhage following an injury.

Haemarthrosis is reported in the literature [1, 3]. Other bleeding manifestations include spontaneous abortions in female patients [7].

The disorder appears to be inherited as an autosomal recessive trait [3, 6, 9, 10, 11], but sex linked inheritance has also been postulated [13]. Quantitative assay of Factor XIII has indicated that the inheritance is autosomal recessive [10]. The present family study also supports the autosomal recessive inheritance of Factor XIII deficiency (Fig. 1). The parents, one sister, grandparents, maternal grandmother's sisters may be heterozygotes as qualitatively the Factor XIII levels were normal. They also had minimal clinical symptoms, whereas the other 3 daughters are homozygotes with severe clinical pictures requiring blood transfusion, and their urea clot solubility test was found to be positive. The earlier cases reported from this laboratory did not show any inheritance pattern to suggest genetic disorder [12].

Factor XIII consists of two A sub-units joined as a dimer and two B sub-units. Factor XIII transglutaminase activity has been described in A sub-units [8]. In homozygous state, the transglutaminase activity may be virtually absent. In heterozygotes, transglutaminase activity may be reduced to intermediate levels [2, 10]. Based on the qualitative tests, patient T seems to be a heterozygote. Her clinical picture, however, indicates a congenital Factor XIII deficiency. If the Factor XIII activity were measured by the semiquantitative or quantitative methods, she may actually turn out to be a homozygote.

The molecular and genetic heterogeneity of Factor XIII and its disorders still remain to be explored.

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## **Dysmegakaryocytopoiesis and Thrombocytosis in a Patient with Acute Myelomonocytic Leukemia and Long Evolution**

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A patient with acute myelomonocytic leukemia (M<sub>4</sub>), dysmegakaryocytopoiesis and thrombocytosis is presented. Immunophenotyping and blast colony assay showed the presence of blasts with IaDr, CD 33 and CD 14 antigens. Cytogenetic analysis and level of thrombopoietin were normal. This atypical case represents yet another example of the transitional zone between atypical myeloproliferative disease and acute leukemia, and the apparent absence of any cytogenetic abnormality is noteworthy.

**Keywords:** AMMoL, dysmegakaryocytopoiesis, long evolution, thrombocytosis

### **Introduction**

In 1976, Rowley and Potter [1] reported a patient with acute myelomonocytic leukemia and thrombocytosis in whom an insertional translocation between the homologues of chromosome 3 was detected. Subsequently, other similar cases were described [2,3,4]. It has also been reported, that acute myelomonocytic leukemia can be accompanied by dysmegakaryocytopoiesis, and that this type of leukemia may be associated with thrombocytosis and translocation of chromosome 3 [5]. To the best of our knowledge, however, no case has yet been reported of FAB type M<sub>4</sub> leukemia with thrombocytosis, dysmegakaryocytopoiesis, and normal karyotype.

### **Case report**

A 62 year old man developed general malaise and furunculosis in October, 1988. Five months later, the WBC was found to be elevated with many immature cells. We saw the patient for the first time in March, 1989. Except for moderate splenomegaly, the results of the physical examination were normal. Laboratory findings: hemoglobin 106 g/L, RBC  $3.1 \times 10^{12}/L$ , WBC  $17.6 \times 10^9/L$ , and platelet count  $1400 \times 10^9/L$ . The differential white cell count consisted of 31% myeloblasts, 1% myelocytes, 6% polymorphonuclear cells, 14% lymphocytes and 47% monocytes. A sternal bone marrow aspirate showed hypercellularity with an abundance of megakaryocytes, some of which were micromegakaryocytes and megakaryocytes with one to three nuclear lobes. Dysmegakaryocytopoiesis was characterized as type III according to Jinnai et al. [5]. Large



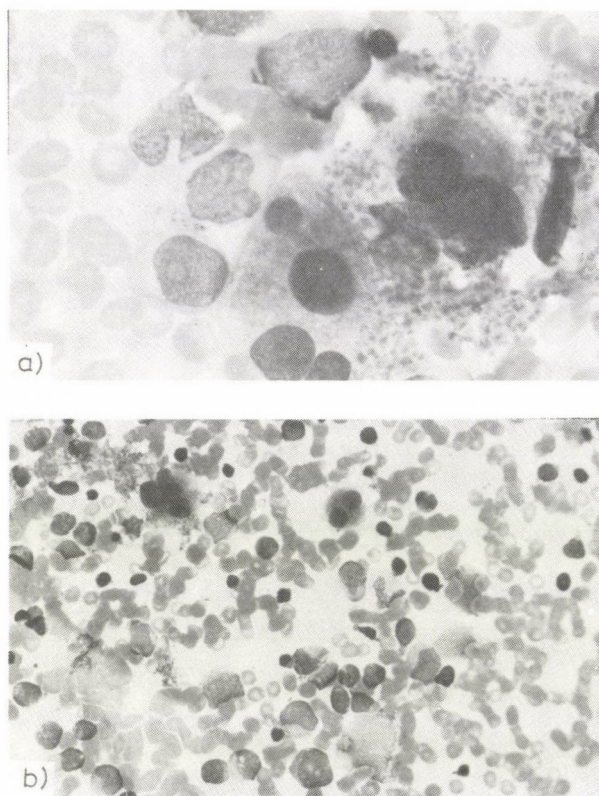


Fig. 1. Bone marrow aspirate morphology (May-Grunwald Giemsa staining) showing abnormal megakaryocytes, platelet clumping and blasts.

a) magnification X 400

b) magnification X 1000

clumps of platelets were noted (Fig. 1 a,b). Sixty percent of the cells were myelomonoblasts. All blasts were Sudan black B positive and 10% of the blasts were weakly PAS positive. Bone marrow trephine biopsy confirmed the diagnosis of an acute myelomonocytic leukemia (FAB M4) with abundance of abnormal megakaryocytes. Thrombopoietin (TSF) serum analysis revealed slightly but not significantly lower percentage of  $^{35}\text{S}$  incorporation in this patient (done by Dr. Ted McDonald) when compared to normal human serum [6]. Il-6 in plasma samples and bone marrow cells conditioned medium assayed by the method of Aarden et al. [7] showed elevated levels of biologically active Il-6. Immunophenotyping with monoclonal antibodies revealed the presence of a clone of blasts with phenotype: HLA-DR $^{+}$ , CD 33 $^{+}$ , CD 13 $^{+}$ , CD 14 $^{+}$ , GP IIb/IIIa - which is consistent with the diagnosis of acute myelomonocytic leukemia (FAB M4 type). Chromosome analysis with banding studies gave a normal karyotype. Leukemic progenitor assay (CFU-L) showed that blast colony formation was PHA-LCM dependent. Morphologic and immunophenotyping studies of blast colony cells showed myelomonocytes with CD 33 $^{+}$  antigen positivity and GP IIb/IIIa antigen negativity. Seventeen

months after the first symptoms in April, 1990, the patient started losing his appetite and weight. His temperature increased to 39°C and his general condition deteriorated (Karnofsky index 30%). He was readmitted to the hospital. WBC was  $87 \times 10^9/L$  with 79% myelomonoblasts. The platelet count fell to  $300 \times 10^9/L$ . The bone marrow showed hypercellularity with 90% of blasts with many clumps of platelets, and rare megakaryocytes with signs of dysmegakaryocytopoiesis. Immunophenotyping revealed a presence of blasts which showed HLA-Dr and CD 33 positivity and CD 14 negativity. Cytogenetic analysis again showed a normal karyotype. Chemotherapy with cytosine arabinoside and rubidazone was initiated and the patient received three courses of the same therapy without achieving a remission. One month after the last course of chemotherapy, the patient died with signs of myocardial infarction.

### Discussion

In the majority of patients with acute leukemia, the platelet number is usually low [5]. However, a few cases of acute myeloid leukemia with a high number of platelets and megakaryocytes with dysplastic features have been described [3, 4]. Almost all these patients had chromosomal abnormalities, i.e. insertional chromosome translocation between chromosome 3 homologous or paracentric inversion of 3q [3, 4]. So far, no cases of acute myeloid leukemia associated with dysplastic megakaryocytes and thrombocytosis, but with normal cytogenetic findings have been reported.

The prognosis of these patients is poor and the majority die within a few months. Treatment did not improve the prognosis; untreated patients did even better than the treated ones [3, 4]. Our patient's duration of survival, who was not treated during the first 17 months, was the longest of any other reported cases [1, 3, 4]. After 17 months without treatment, our patient deteriorated with high temperature, elevation of WBC and percentage of blasts followed by a decrease in the number of platelets (from 1000 to  $300 \times 10^9/L$ ). At this stage, treatment with cytosine arabinoside and rubidazone was introduced and the patient died after the third course of chemotherapy.

According to Bernstein et al. [3], AML patients who have only karyotypically abnormal cells have short survival whereas patients with both chromosomally normal and abnormal cells have longer survival. The presence of only karyotypically normal cells could explain the long survival in our case. Long survival could also be explained by the peculiar haematopoiesis characterized by relatively well preserved thrombocytopoiesis, platelet and neutrophil counts in spite of non-remission state, with few infections and no bleeding complications.

This atypical case represents another example of the transitional zone between atypical myeloproliferative disease and acute leukemia.

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## **Portal Vein Thrombosis in a Patient with Severe Haemophilia A and Post-Hepatitis Liver Cirrhosis**

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The occurrence of a portal vein thrombosis in a haemophilia A patient is reported. The patient, a 53 year old male, had been followed by us for the past 20 years in our out-patient Clinic. He was hospitalized recently for a suspected hepatic cirrhosis. Severe ascites, hepato-splenomegaly together with weight loss and mild fever were present. During the hospitalization, an ultrasound and CT scan of the liver confirmed the cirrhotic pattern and showed the presence of a portal vein thrombosis. There were no changes in the underlying coagulation defect, in fact, the patient had recurrent haemarthrosis. Furthermore, with the ultrasound examination, some focal hepatic lesions — probably due to a hepatocellular carcinoma — were also observed. The patient died because of massive haematemesis due to rupture of oesophageal varices.

**Keywords:** haemophilia A, vein thrombosis, liver cirrhosis

### **Introduction**

Thromboembolic disease is rare in patients with congenital coagulation disorders [1]. Spontaneous deep venous thrombosis with pulmonary embolism was described in patients with Factor V deficiency [2]. Superficial phlebitis was seen by us in a patient with haemophilia A [3] and in one with Factor VII deficiency [4]. Post-operative deep vein thrombophlebitis and pulmonary embolism were described in patients with Factor VII deficiency [5]. Massive femoral artery thrombosis, requiring amputation, was observed in a patient with hypofibrinogenaemia [6]. Other clotting defects such as dysfibrinogenemia and Hageman trait have also been associated with thrombosis [7, 8]. Myocardial infarction and atherosclerotic disease were described in Hageman trait, haemophilia A and B patients [9, 10, 11, 12]. In the latter two coagulation disorders, cerebral thrombosis was also observed [13, 14]. The occurrence of deep vein thrombophlebitis in association with rectal carcinoma was described in a patient with Hageman trait [1]. To the best of our knowledge, no paraneoplastic thrombosis has ever been described in haemophilia A or B patients.

The aim of this case report is to describe the occurrence of a portal vein thrombosis in a haemophilia A patient with hepatocellular carcinoma.

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\* To whom correspondence should be addressed

## Case Report

The patient, a 53 year old male affected with severe haemophilia A, had been followed by us for the past 20 years as an outpatient. In the first 10 years he used antihaemophilic concentrates for post-traumatic haemarthrosis in moderate doses. In 1981, he was admitted to the hospital a number of times for dental extractions and was transfused with over 10.000 U of Factor VIII concentrates. After two years, a post-transfusion chronic hepatitis was diagnosed with hepatomegaly, hypertransaminasaemia and HBs Ab in the serum. HBs Ag and HDV Ab were never detected. Abnormal serum transaminases persisted for five years without any symptoms, and HBs Ab remained unchanged. Screening for HIV antibodies was always negative. On February 9, 1988, the patient was hospitalized for mild fever, anorexia, diffuse abdominal pain and weight loss. Severe ascites was present. Oesophageal varices were seen by oesophagoscopy. Ultrasound and CT scans of the liver confirmed the diagnosis of hepatic cirrhosis and showed also some small focal lesions near the falciform legament. A tentative diagnosis of evolution in hepatocellular carcinoma was made for the gradual and severe increase of alpha-fetoprotein (171  $\mu\text{g/L}$  with normal values between 0–10  $\mu\text{g/L}$ ). Furthermore, ultrasound examination showed the presence of portal vein thrombosis, with dilated portal and hepatic veins and splenomegaly. No changes in the underlying coagulation defect were present (PTT 100 sec.). Moreover, a prolonged Prothrombin Time (51%) and a low platelet count ( $62 \times 10^9/\text{L}$ ) were observed according to liver cirrhosis, whereas the antithrombin III levels were within the normal range. In fact, the patient had recurrent haemarthrosis in the right knee, and incidental epistaxis for which he was treated with Factor VIII concentrates. During the patient's stay in the hospital, with appropriate diuretic therapy, the ascites diminished slightly. After a month (on March 14, 1988), the patient was discharged in fairly good condition, but he was soon readmitted due to the reappearance of a severe ascites and anaemia. Diuretic therapy became useless and two paracentesis were made. On May, 1988, the patient died from a shock syndrome after a severe haematemesis. At the family's specific request, no autopsy was performed.

## Discussion

The propositus had post-hepatitis liver cirrhosis. A diagnosis of hepatocellular carcinoma could also be surmised on the basis of ultrasound scan pattern of the liver and a gradual, significant increase of alpha-fetoprotein in the serum. In agreement with this interpretation is the also known fact of an increased incidence of liver carcinomas in post-hepatitis B patients. However, this increased incidence of hepatocarcinoma invalidates persons with a persistence of HBs antigen. No biopsy of the liver was made due to the risk of bleeding: in fact, at this time, the patient was also thrombocytopenic. The presence of portal vein thrombosis is in agreement with this diagnosis and was probably caused by the persistence of portal hypertension. This thrombotic event is a frequent sequelae of liver cirrhosis and of hepatocellular carcinoma, but to our knowledge, it has never been described in a haemophiliac patient. It is important to remark that no changes in the coagulation defect were present, and the patient was haemorrhagic at the time of the onset of the thrombosis. Furthermore, he was not transfused with antihaemophilic



concentrates in the pre-diagnostic period. The coagulation abnormalities underlying malignant diseases are undoubtedly of considerable interest [15, 16, 17]; various tumors have been shown to produce coagulation activators [18]. A possible mechanism of thrombosis could be the tumor production of tissue factor procoagulants (tissue thromboplastin) or direct activators of Factor X. Some blood coagulation factors (prothrombin/thrombin, Factor VII and X) were also identified in situ in certain types of tumors [19].

In conclusion, it would seem that the Factor VIII defect is not able to protect against thrombosis when prolonged and severe stasis is present in the portal system due to liver cirrhosis and/or liver carcinoma.

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## OBITUARY

Professor Dr. Stefan Berceanu

Professor Dr. Stefan Berceanu, Director of the Department of Haematology, Bucarest (Roumania), passed away on November 9, 1990, still fully active, after a severe illness. He was an outstanding haematologist, the founder of modern haematology and clinical immunology in Roumania, a dedicated scientist and great teacher. Practically all Roumanian haematologists and many pathologists trained under his guidance in the in-patient departments and laboratories, and by attending his lectures and undergraduate and postgraduate courses. Stefan Berceanu was a highly cultured humanist.

Professor Berceanu was a member of a number of international scientific societies. In 1980, the National Academy Dei Lincei awarded Professor Berceanu the Prize of the Di Guglielmo Foundation (Rome). He was the President of the Roumanian Council for the Transplantation of Organs, and the President of the Committee of Experts for Haematological Studies of the Roumanian Academy.

The research activities of Professor Berceanu covered a broad spectrum of haematology and immunology. He contributed valuable data to the diagnosis and pathogenesis of myelosclerosis and leukemia, as well as to the protean signs of the paraneoplastic syndrome. He was among the first to suggest that immunodeficiency predisposes for autoimmune diseases. It is of interest that Professor Berceanu diagnosed the first case of AIDS in Roumania in 1985 and the first two HTLV-I lymphomas in 1989.

Besides his scientific work, Professor Berceanu was also a playwright and a writer of philosophical essays on classic and scientific humanism, the biological condition of human beings, the role of art and science. Two of his essays (Interferences; Philosophy and Medicine) as well as a monograph (Essays and Meditations) were published by the Eminescu publishing house. Professor Berceanu was the author of five plays (e.g. The Keys of the Breda Town; The Strangers; White Souls, Black Souls) which deal primarily with the human condition and man's ever present aspiration for creation and realisation. One play was presented at the Bucharest National Theater, and another was broadcast on radio. Professor Berceanu also gave lectures on the philosophy of culture and science.

Professor Stefan Berceanu was a fine man, an exceptional character gifted with a "magic charm" who was always ready to help his colleagues and students, happy to share his knowledge. He was deeply committed not only to his research work — ever interested in new fields of research, new ideas — but also to his patients. His books "Clinical Haematology", "Treatise of Blood Diseases", "The Biology and Pathology of Immunity", "Immunology and Immunopathology" are just a few of the examples of his work, of his striving to pass on his knowledge to others. In recognition of his merits, Professor Stefan Berceanu was posthumously awarded membership of the Roumanian Academy.

Professor Berceanu's demise is a great loss for his colleagues, pupils, for Roumanian scientific life. His presence and guidance, his humaneness and devotion to his profession, his country and his family will always be remembered.





## Abstracts

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*Phosphorylation and subcellular distribution of calpastatin in human hematopoietic system cells.* Y. Adachi, A. Ishida-Takahashi, Ch. Takahashi, E. Takano, T. Murachi and M. Hatanaka (Institute for Virus Research, Kyoto University, Kyoto 606, Japan). *J. Biol. Chem.* 266, 3968 (1991).

Calpastatin is an endogenous inhibitor protein acting specifically on calpain. The phosphorylation of calpastatin was investigated in human hematopoietic system cell lines. Microheterogeneity of calpastatin was observed, in which 118- and 116-kDa forms were named calpastatin a and b, respectively. The phosphorylation of both calpastatins was identified in all cell lines examined and occurred mainly at serine residues with trace amounts of phosphothreonine *in vivo*. The incubation of cells with 12-O-tetradecanoylphorbol-13-acetate increased the incorporation of  $^{32}\text{P}$ -orthophosphate into calpastatin a. Two-dimensional maps of  $^{32}\text{P}$ -labeled phosphopeptide from both calpastatins were identical except for additional minor spots for calpastatin a. [ $^{35}\text{S}$ ]methionine-labeled calpastatins a and b were localized mainly in the cytosol, and only 6% of cellular calpastatins were detected in the membrane fraction. By contrast, more than 30% of the  $^{32}\text{P}$ -labeled calpastatins a and b were distributed in the membrane fraction. Thus, the phosphorylation of calpastatin may be in-

involved in regulating the calpain-calpastatin-protein kinase system by its subcellular distribution.

G. Gárdos

*Identification and characterization of a ouabain-like compound from human plasma.* J.M. Hamlyn, M.P. Blaustein, S. Bova, D.W. DuCharme, D.W. Harris, F. Mandel, W.R. Mathews and J.H. Ludens (Department of Physiology and Hypertension Center, University of Maryland School of Medicine, Baltimore, MD, USA). *Proc. Natl. Acad. Sci. USA* 88, 6259 (1991).

The plasma membrane sodium-potassium pumps that regulate intracellular sodium in most animal cells have specific, high-affinity receptors for the digitalis glycosides and their aglycones. This has fostered speculation that there is an endogenous ligand. An endogenous substance from human plasma that binds with high affinity to this receptor and that is indistinguishable from the cardenolide ouabain was purified and structurally identified by mass spectroscopy. This human ouabain-like compound (OLC) displaces [ $^3\text{H}$ ]ouabain from its receptor, inhibits Na,K-ATPase and ouabain-sensitive  $^{86}\text{Rb}^+$  uptake, and has cardiotonic actions quantitatively similar to commercial ouabain. Immunoreactive OLC was detected in the plasma of many mammals, and high

concentrations were found in the adrenals. The circulating OLC may modulate intracellular  $\text{Na}^+$  and affect numerous  $\text{Na}^+$  gradient-dependent processes including intracellular  $\text{Ca}^{2+}$  and pH homeostasis in many tissues. Furthermore, altered circulating levels of OLC may be associated with the pathogenesis of certain forms of hypertension.

G. Gárdos

*Isolation and identification of hemin as an endogenous  $\text{Na}^+/\text{K}^+$ -ATPase inhibitor from porcine blood cells.* T. Yasuhara, M. Mori, K. Wakamatsu and K. Kubo (Tsukuba Research Laboratories, Takeda Chemical Industries, Ltd., Wadai 7, Tsukuba 300-42, Japan). *Biochem. Biophys. Res. Comm.* 178, 95 (1991).

A substance which is a potent inhibitor of  $\text{Na}^+/\text{K}^+$ -ATPase activity and competitively displaces [ $^3\text{H}$ ]ouabain binding to this enzyme was isolated from porcine blood cells. From its chemical and physicochemical properties, this activity was identified as hemin (chloroproteohemin IX). Hemin showed a dose dependent curve for  $\text{Na}^+/\text{K}^+$ -ATPase inhibitory activity similar to that of ouabain and displaced [ $^3\text{H}$ ]ouabain binding as potent as 1/100 of ouabain itself.

G. Gárdos

*Potentiation of hyperthermia-induced haemolysis of human erythrocytes by photodynamic treatment. Evidence for the involvement of the anion transporter in this synergistic interaction.* C. Prinsze, K. Tijssen, T.M.A.R. Dubbelman and J. van Steveninck (Sylvius Laboratories, Department of Medical Biochemistry, Leiden, The Netherlands). *Biochem. J.* 277, 183 (1991).

Heat treatment of human erythrocytes led to increased passive cation permeability, followed by haemolysis.  $\text{K}^+$  leakage was linear up to a loss of about 80% in the temperature

range 46–54°C. Kinetic analysis of the results revealed an activation energy of 246 kJ/mol, implicating a transition in the membrane as critical step. Pretreatment of erythrocytes with 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate, chymotrypsin or chlorpromazine caused a potentiation of subsequent heat-induced  $\text{K}^+$  leakage. Photodynamic treatment of erythrocytes with Photofrin II, eosin isothiocyanate or a porphyrin- $\text{Cu}^{2+}$  complex as sensitizer also induced an increase in passive cation permeability, ultimately resulting in colloid osmotic haemolysis. The combination of photodynamic treatment immediately followed by hyperthermia had a synergistic effect on  $\text{K}^+$  leakage. Analysis of the results by the Arrhenius equation revealed that both the activation energy and the frequency factor of heat-induced  $\text{K}^+$  leakage were decreased significantly by preceding photodynamic treatment, suggesting that hyperthermia and photodynamic treatment have a common target for the induction of  $\text{K}^+$  leakage. Several lines of reasoning indicate that this common target is band 3. A model is thus proposed for the observed potentiation of hyperthermally induced  $\text{K}^+$  leakage by photodynamic treatment, in which photo-oxidation of band 3 results in increased sensitivity to subsequent thermal denaturation. These phenomena may be of more general significance, as photodynamic treatment and hyperthermia interacted synergistically with respect to  $\text{K}^+$  leakage with L929 fibroblasts also.

G. Gárdos

*Kinetics of hemolysis of normal and abnormal red blood cells in glycerol-containing media.* A. Sauer, T. Kurzion, D. Meyerstein and N. Meyerstein (Department of Chemistry and Dr. J. Kaufmann Laboratory, Unit of Physiology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel). *Biochim. Biophys. Acta* 1063, 203 (1991).



The kinetics of hemolysis of erythrocytes in glycerol-containing media was studied spectrophotometrically. The hemolytic process starts by a rapid process, obeying a first order rate law, which is followed by a slow change in absorbance. The kinetics of hemolysis may be described by (a) the maximum absorption,  $E_{\max}$ , due to cellular expansion, (b) the rate constant,  $k$ , of the fast process and (c) the final absorption at its end,  $E_{\inf}$  and the ratio  $E_{\inf}/E_{\max}$ . At pH 6.85 in normal human cells,  $k=0.72 \text{ min}^{-1}$  while in hereditary spherocytosis cells,  $k=1.06 \text{ min}^{-1}$ , iron deficiency  $k=0.52$  and  $\beta$ -thalassemia minor  $k=0.36 \text{ min}^{-1}$ . The percentages of  $E_{\inf}/E_{\max}$  were 35.3 in control cells, while they were 9.8, 50.0 and 88.3 in spherocytosis, iron deficiency and thalassemia, respectively. Thus these kinetic parameters may help to distinguish and understand the above mentioned erythrocyte disorders. At physiological pH (7.4–7.2), no hemolysis was detected in the medium used. When the pH decreased, hemolysis occurred, its rate increasing gradually until pH 6.3. On further acidosis, the hemolytic rate slowed down again. Addition of DIDS to the whole blood prior to the test inhibits hemolysis. Similar effect of DIDS was noted in washed cells; this effect was partially reversed by albumin. These results suggest that a process involving band 3 affects the rate and degree of glycerol-induced hemolysis of normal red blood cells.

G. Gárdos

*Uncoupling of the spectrin-based skeleton from the lipid bilayer in sickled red cells.* S.C. Liu, L.H. Derick, S. Zhai, and J. Palek (Division of Hematology-Oncology and Departments of Biomedical Research and Medicine, St. Elizabeth's Hospital, Tufts University School of Medicine, Boston, MA, USA). *Science* 252, 574 (1991).

The distribution of spectrin and band 3 in deoxygenated reversibly sickled cells was visualized by immunofluorescence and im-

munoelectron microscopy. Antibodies against band 3, the major lipid-associated transmembrane protein, labeled the entire cell body, including the entire length of the long protruding spicule, whereas antibodies against spectrin labeled only the cell body and the base region of the spicules. The results suggest that the formation of long spicules during sickling is associated with a continuous polymerization of hemoglobin S polymers, presumably through gaps in the spectrin-actin meshwork, and a subsequent uncoupling of the lipid bilayer from the submembrane skeleton.

G. Gárdos

*Specific binding of tritium-labeled inositol 1,4,5-trisphosphate to human platelet membranes: ionic and GTP regulation.* S.B. Hwang (Merck Sharp & Dohme Research Laboratories, Department of Biochemical Regulation, Rahway, NJ, USA). *Biochim. Biophys. Acta* 1064, 351 (1991).

Specific, saturable and reversible binding of tritium-labeled inositol 1,4,5-trisphosphate ( $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ ) to human platelet membranes is demonstrated. The  $\text{Ins}(1,4,5)\text{P}_3$ -binding sites are abundant and display high selectivity for  $\text{Ins}(1,4,5)\text{P}_3$ . Other inositol phosphates exhibit much lower affinity for this site. The specific  $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  binding was found to be modulated by pH, monovalent and divalent cations, and GTP. A sharp increase in binding occurs at slightly alkaline pH. The monovalent cations,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Li}^+$  almost double the binding at 30 mM.  $\text{Mg}^{2+}$  inhibits the specific  $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  binding. At low concentrations of  $\text{Ca}^{2+}$ , the binding is inhibited, but at concentrations higher than 5 mM the binding is potentiated and increases by almost 5-fold at 100 mM. Similar pattern of the effects is also observed for  $\text{Mn}^{2+}$  and  $\text{Sr}^{2+}$ . The specific  $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$  binding is specifically inhibited by GTP. Other nucleotides also inhibit the binding but at higher concentrations. From saturation binding stu-



dies,  $\text{Ca}^{2+}$  potentiation seems to be due to the conversion of the receptor from the low-affinity state to the high-affinity one. In the absence of  $\text{Ca}^{2+}$ , the Scatchard plot is nonlinear and concave, and statistically can be fitted best with two equilibrium dissociation constants ( $K_d$  values),  $0.19 \pm 0.11$  and  $13.2 \pm 18.1$  nM, respectively, for high- and low-affinity binding sites. However, in the presence of 100 mM  $\text{CaCl}_2$ , the Scatchard plot reveals only the high-affinity binding sites with a  $K_d$  value of  $0.32 \pm 0.15$  nM. The specific  $\text{Ins}(1,4,5)\text{P}_3$  receptor in human platelets could therefore exist in multiple conformational states to regulate the intracellular  $\text{Ca}^{2+}$  concentration.

Ágnes Enyedi

*Low molecular weight kininogen binds to platelets to modulate thrombin-induced platelet activation.* F.J. Meloni and A.H. Schmaier (Thrombosis Research Center and Department of Medicine, Temple University School of Medicine, Philadelphia, Pennsylvania, USA). *J. Biol. Chem.* 266, 6786 (1991).

The kininogens, high molecular weight kininogen (HK) and low molecular weight kininogen (LK), are multifunctional, single-gene products that contain bradykinin and identical amino-terminal heavy chains. Studies were performed to determine if LK would bind directly to platelets.  $^{125}\text{I}$ -LK specifically bound to gel-filtered platelets in the presence of  $50 \mu\text{M Zn}^{2+}$ . HK effectively competed with  $^{125}\text{I}$ -LK for the same binding site ( $K_i = 27 \pm 9$  nM,  $n=5$ ). Similarly, the  $K_i$  for LK inhibition of  $^{125}\text{I}$ -LK binding was  $12 \pm 1$  nM ( $n=3$ ). Albumin, fibrinogen, factor XIII, and kallikrein did not inhibit  $^{125}\text{I}$ -LK binding to unstimulated platelets.  $^{125}\text{I}$ -LK (66 kDa) was not cleaved upon binding to platelets. The binding of  $^{125}\text{I}$ -LK to unstimulated platelets was found to be fully reversible by the addition of a 50 molar excess of unlabeled LK at both 10 and 20 min. LK binding to platelets was saturable with an apparent  $K_d$  of  $27 \pm 2$  nM (mean  $\pm$  S.E.,  $n=9$ )

and  $647 \pm 147$  binding sites/platelet. Both LK and HK at plasma concentrations inhibited thrombin-induced platelet aggregation. LK and HK at about 5% of plasma concentration also inhibited thrombin-induced secretion of both stirred and unstirred platelets. Both kininogens were found to be noncompetitive inhibitors of proteolytically active thrombin binding to platelets. The kininogens did not inhibit D-phenylalanyl-prolyl-arginine chloromethyl ketone-treated thrombin from binding to platelets. These studies indicated that both kininogens have a region on their heavy chain which allows them to bind to platelets. Further, kininogen binding by its heavy chain modulates thrombin activation of platelets since it prevents proteolytically active thrombin from binding to its receptor.

Ágnes Enyedi

*Proteasome and its novel endogenous activator in human platelets.* M. Yukawa, M. Sakon, J. Kambayashi, E. Shiba, T. Kawasaki, H. Ariyoshi and T. Mori (Hematology Research Unit, Department of Surgery II, Osaka University Medical School, 1-1-50, Fukushima, Fukushima-ku, Osaka 553, Japan). *Biochem. Biophys. Res. Comm.* 178, 256 (1991).

Proteasome, a high molecular weight multicatalytic protease, was purified from the cytosolic fraction of human platelets for the first time. The biochemical properties of the enzyme including substrate specificity, optimal pH and effects of various inhibitors were almost identical with those of other cells. During the purification with a Heparin-Sepharose chromatography, a novel endogenous activator of the protease was identified and was partially purified. The activator enhanced both chymotrypsin or trypsin like activities of the proteasome in a dose related manner and was inactivated by heating at  $56^\circ\text{C}$  for 30 min. This newly identified activator may serve as an important regulator or cofactor of intracellular activities of the proteasome.

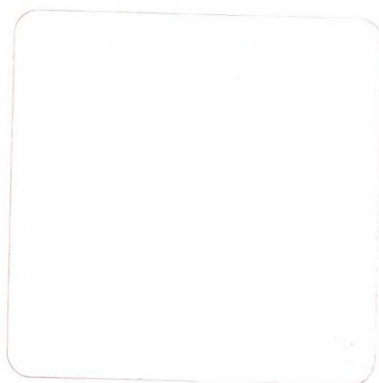
Ágnes Enyedi

*Rat platelets are deficient in internal  $\text{Ca}^{2+}$  release and require influx of extracellular  $\text{Ca}^{2+}$  for activation.* J.W.M. Heemskerk, M.A.H. Feijge, E. Rietman and G. Hornstra (Departments of Biochemistry and Human Biology, Biomedical Center, University of Limburg, Maastricht, The Netherlands). *FEBS Letters* 284, 223 (1991).

Calcium fluxes were studied in fura-2-labeled rat platelets. Thrombin, ADP and ionomycin induced rapid mobilization of internally stored  $\text{Ca}^{2+}$ , which resulted in only a moderate increase of cytosolic  $[\text{Ca}^{2+}]_i$ . Throm-

bin and ADP stimulated influx of extracellular  $\text{Ca}^{2+}$ , which was monitored as uptake of  $^{45}\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$ . With either agonist, the influx of  $\text{Ca}^{2+}$  magnified the initial increase of  $[\text{Ca}^{2+}]_i$ . Since responses of rat platelets were dependent on external  $[\text{Ca}^{2+}]$ , it was concluded that  $\text{Ca}^{2+}$  influx complements the mobilization of internal stores to reach sufficiently high  $[\text{Ca}^{2+}]_i$  for full activation. A regulatory effect of protein kinase C modulators was observed on both agonist-induced elevation of  $[\text{Ca}^{2+}]_i$  and receptor-mediated  $\text{Ca}^{2+}$  entry.

*Agnes Enyedi*



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## **Serum Erythropoietin in Regularly Transfused Thalassemic Patients\***

M. Kalmanti<sup>1</sup>, Th. Kalmantis<sup>2</sup>, Th. Liacopoulou<sup>3</sup>, K. Tsoumakas<sup>3</sup>, V. Ladis<sup>3</sup>,  
C. Kattamis<sup>3</sup>

<sup>1</sup>Department of Ped. Hematology-Oncology, Univ. of Crete Medical School, University Hospital of Iraklio, Iraklio 1352, Crete, Greece; <sup>2</sup>2nd Department of Internal Medicine, Hematology Unit, Univ. of Athens, Medical School; <sup>3</sup>1st Department of Pediatrics, University of Athens, Medical School, Athens, Greece.

(Received: 7 March, 1991; accepted: 23 April, 1991)

Serum erythropoietin levels were measured in 67 regularly transfused thalassemic patients with pre-transfusion hematocrit ranging from 25–32% and in 40 normal individuals. In patients, mean erythropoietin levels were slightly increased (mean 91.5 miu/ml) as compared to normal individuals (mean 42 miu/ml). The distribution of erythropoietin (Ep) was wide in thalassemic patients. 40% had normal or decreased and 60% increased Ep levels. A reverse relation between pretransfusion Hct and erythropoietin activity was observed only among patients with normal erythropoietin levels and splenectomized patients with high erythropoietin titers suggesting that a normal feedback between tissue hypoxia and erythropoietin activity occurs in these groups. The effect of regular blood transfusions in reversing tissue hypoxia resulting from anemia in the majority of regularly transfused thalassemic patients seems to be satisfactory, as it is assessed by serum erythropoietin levels.

**Keywords:** erythropoietin, tissue hypoxia, regular transfusions, thalassemia, splenectomy

### **Introduction**

Clinical and experimental observations demonstrate the inverse relationship between the erythropoietin (Ep) activity and the degree of tissue hypoxia [1]. When renal function is intact, a direct correlation has been demonstrated between elevated erythropoietin titers and the degree of tissue hypoxia [2, 3]. Thalassemic patients as well as patients with other chronic hemolytic anemias are expected to have high erythropoietin levels since tissue oxygenation is impaired [4, 5, 6] in relation to the severity of the anemia. The present policy of the treatment of thalassemics consists of frequent transfusions to preserve pretransfusional Hb above 10 g/dl, in an attempt to prevent bone marrow expansion and to minimize tissue hypoxia [7].

\* Presented in part at the 2nd International Conference on Thalassemia and Hemoglobinopathies (Iraklio, Crete, Greece 1987).

As erythropoietin activity seems to be a good index of tissue oxygenation, it was thought worthwhile to determine erythropoietin activity in thalassemic patients in order to investigate the effectiveness of frequent transfusion treatment on tissue oxygenation.

## Patients and Methods

### Patients

The present study included 67 patients (41 males, 26 females) aged 3 to 25 years, who are regularly transfused amongst whom twenty-nine patients had been splenectomized. Pretransfusion hematocrit (Ht) levels ranged between 25–32%. The interval between transfusion was 14–25 days. All patients had the clinical phenotype of either severe or mild thalassemia major [8] and belonged to the most common hematological phenotypes, namely 46 were homozygous of high  $A^2$  thalassemia, 6 double heterozygous of  $\beta$  high  $A^2$  and  $\beta^0$  high  $A^2$  and F, 4  $\beta$  high  $A^2$  and  $\delta\beta$  (high F) thalassemia, 1  $\beta$  high  $A^2$  and  $\delta\beta$  lepore, while 3 were not characterized.

None of the patients had impaired renal function, while 20/67 had increased levels of transaminases without any other liver abnormality. Serum erythropoietin levels and hematocrit values correspond to samples obtained prior to transfusion.

For multiple assays of erythropoietin measurement, serum was collected under sterile conditions prior to transfusion from 5 ml of peripheral blood and was kept at  $-70^\circ\text{C}$ . Hematocrits were obtained concomitantly with the serum. Serum samples obtained from 40 normal controls (20 males, 20 females) aged 3 to 25 years, were run simultaneously with the patients' sera. Erythropoietin was measured with a sensitive *in vitro* enzyme immunoassay method (JCL Clinical Research Corp. [2, 9, 10]).

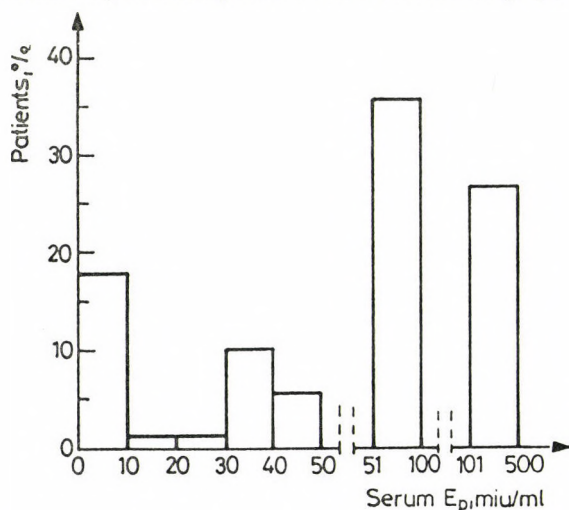


Fig. 1. Histogram of Ep levels in patients studied

### Statistical methods

The unpaired *t*-test was used for the comparison of the mean values of Ep and Ht. The Pearson correlation coefficient was used for possible correlations between Ht and Ep [11].

### Results

The histogram of the percentage of patients' population and of the serum erythropoietin titers is depicted in Fig. 1. Normal controls had serum Ep levels ranging from 25–58 mIU/ml with a mean value of 42 mIU/ml  $\pm$  8.6. In contrast, in thalassemic patients the Ep level had a wide range between 1/505 mIU/ml, with a mean value of 91.50 ( $t = 0.39$ ,  $p > 0.1$ ). 60% of the patients were found to have increased Ep levels while 20% had very low ones and 20% had normal values (Table 1).

Table 1  
Serum Ep levels (mIU/ml)

	Range	Mean	<i>t</i> -test
Normal controls	25–58	42	0.39
Patients	1–505	91.5	

No correlation was found between Ep and Ht in any of the patients studied (Table 2, Fig. 2), while a weak correlation was observed in the 23 splenectomized patients with the highest Ep levels and in the 13 patients who had normal Ep values (Table 2, Fig. 3).

Table 2  
Serum Ep levels and correlation between Ht and Ep in the normal controls and in the group of patients studied

	No.	%	Mean Ep (mIU/ml)	<i>r</i> (Ht-Ep)
Normal controls	40	100	42	
All patients	67	100	91.5	0.16
Patients with normal Ep	13	19.4%	41.4	-0.22
Patients with low Ep	13	19.4%	4.2	-0.05
Patients with high Ep	41	61.2%	135.2	0.14
Splenectomized patients	29	43%	101.6	-0.05
Splenectomized patients with high Ep	23		119.0	-0.27



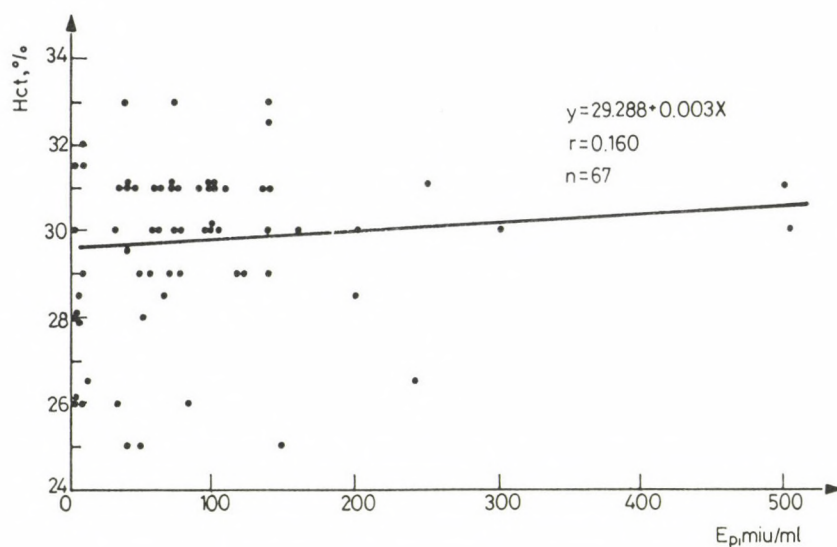


Fig. 2. Correlation between Hct and Ep in all thalassemic patients

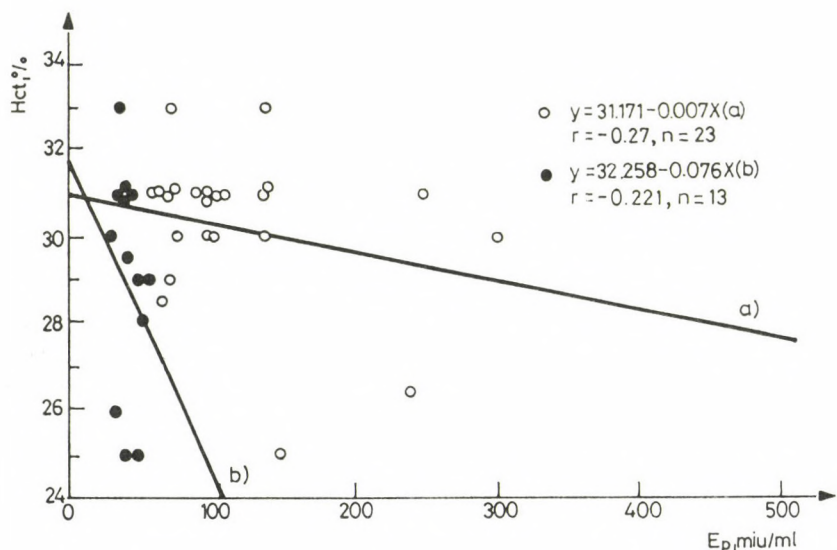


Fig. 3. Correlation between Hct and Ep in 23 splenectomized patients (o) and 13 patients with normal Ep levels (●)

## Discussion

During the past years, the regular transfusion programs with maintenance of hemoglobin in adequate levels have improved the quality of the life of thalassemic patients, due to the decrease of side effects resulting from tissue hypoxia [4, 12, 13].

The normal hemoglobin function involves two naturally occurring regulation mechanisms [7]. A short-term mechanism is associated with the rapid increase in 2-3-DPG concentration either in response to a decrease in arterial oxygen concentration or to an increase in the oxygen affinity of hemoglobin [5, 6, 14].

On the other hand, a long term effect corresponds to the increased synthesis of erythropoietin in prolonged hypoxia for a final net elevation of the hemoglobin concentration [13, 14]. Thalassemia belongs to this group of anemias where erythropoietin production is increased proportionally to the degree of the anemia [15]. Serum erythropoietin levels in thalassemic patients have not been studied extensively. The few existing reports refer to an indirect evaluation of erythropoiesis using  $\text{Fe}^{59}$  incorporation into the red blood cells of experimental animals by sera obtained from heterozygotes [15,16], or with evaluation of erythroid committed populations in the peripheral blood of thalassemic patients [17]. In our study, all the 67 patients were under a regular transfusion program every 14–25 days and in all of them, the lowest hematocrit was 25%. Although the range of erythropoietin levels found was wide, the mean value was not as high as would be expected in other hemolytic anemias [1, 12], and not statistically significant when it was compared to normal controls. Forty percent of the patients studied were found to have normal or low erythropoietin levels. This finding suggests that these patients who are regularly transfused do not suffer from severe hypoxia which stimulates erythropoietin production [18]. Similar results by evaluation of erythropoietic activity with in vitro culture assays and with thymidine incorporation into red cells were found in 30% of thalassemic patients studied by Manor [7]. Sixty percent of our patients exhibited high levels of hormone, although their range was not as high as that detected in other anemias [4, 14, 19]. This finding might imply that even in this group of patients, tissue hypoxia is adequately controlled by regular blood transfusions. Similar findings have been reported in a small group of patients with sickle cell anemia [18].

Splenectomized patients had erythropoietin levels which did not differ from that of the non-splenectomized ones with high titers. Patients in this group who exhibited the highest erythropoietin levels were found to have a correlation between erythropoietin activity and the degree of anemia. This observation suggests that in the majority of the splenectomized patients, the feed-back mechanism of low tissue oxygen supply and increased production of erythropoietin is normalized. Our findings in this study suggest that in regularly transfused thalassemic patients, tissue hypoxia is controlled as reflected by serum erythropoietin levels, even in patients with the lowest acceptable hematocrit levels.

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## **Effects of Oral Contraceptive on Fibrinogen Levels in African Women**

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Fibrinogen levels were determined in 100 women on oral contraceptive pills (OCP), aged 17-46 years, and 100 apparently healthy women with regular menstruation and no previous history of hormonal therapy, aged 20-40 years, who served as controls. The women on OCP had significantly higher plasma fibrinogen concentration than the control ( $P < 0.001$ ). There was a cumulative increase in plasma fibrinogen level from three months of usage. We conclude that prolonged use of OCP may result in the development of circulatory and vascular disorders related to hyper-fibrinogenaemia and other clotting factors.

**Keywords:** fibrinogen, oral contraceptive pill, low dose oestrogen, vascular disorders

### **Introduction**

The possibility that oral contraceptives may increase the risk of venous thromboembolism has been raised previously [1]. Since then, several epidemiological studies have indicated that women on oral contraceptives have a higher risk of developing thrombosis [2, 3]. The possibility of a connection between the use of hormonal contraceptives and the risk of thrombosis has been suggested [4]. Enhanced activity of several coagulation factors, particularly factor VII [5, 6], and factors VIII, X and fibrinogen [7, 8] have been observed in association with pregnancies as well as the use of contraceptives, but the findings were not without contradictions. Some authors [7, 9] attribute these contradictions to the variation in the components and the differences in dosage in the various contraceptive pills.

Most of the published epidemiological studies and papers on haemostasis deal with Caucasian subjects. In view of the fast increase of Nigerian women receiving oral contraceptives as a means of birth control, and considering the possible risk of thrombosis, this study was designed to investigate the changes in plasma fibrinogen levels in Nigerian women on oral contraceptives.

## Materials and Method

### Patients

Two hundred women, aged 17–46 years, were grouped as follows:

Group A: 100 apparently healthy women with regular menstruation and no history of hormonal therapy for the 6 months preceding the investigation served as controls. The mean age was  $30.00 \pm 6.27$  years.

Group B: 100 women who had been receiving oral contraceptives (combined, biphasic) for at least 3 months prior to the study, with a mean age of  $23.03 \pm 3.69$  years.

The spectrum of the social and economical status of the subjects participating in the study was typical for an urban, Nigerian city.

### Method

Venous blood was drawn from the women in Group A and B from a vein in the arm, as near as possible to their mid-cycle, with a minimum of stasis, into a clean, disposable plastic syringe. 10 ml of blood was collected from each subject. 9 ml of blood was mixed with 1 ml of 31.3 g/l sodium citrate solution. After mixing, the blood was spun at  $2,500 \times g$  for 10 minutes in a bench centrifuge to separate the plasma. The plasma was then decanted and kept at room temperature ( $25\text{--}26^\circ\text{C}$ ) until the tests were done.

The plasma fibrinogen level was subsequently measured by the clot-weight procedure of Ingram [10]. Measurements were made on the same day the samples were collected. The packed-cell volume (PCV) used for correction of the weighed fibrinogen clot was determined on both heparinised and citrated blood by a standard micro-haematocrit method.

The significant difference between the means was determined by Student's *t* test.

Table 1  
Mean (+ SD) of Plasma Fibrinogen Values for Women on Oral Contraceptives and Controls

Subjects	N	Fibrinogen Levels (g/l)
Control (C)	100	$2.9 \pm 1.02$
Oral Contraceptive (O/C)	100	$3.20 \pm 0.95^*$

\*  $P < 0.001$



## Results

Table 1 shows the mean ( $\pm$  SD) of plasma fibrinogen levels for the women on oral contraceptives and for the control group. The women receiving oral contraceptives had higher plasma fibrinogen concentration than the control ( $P < 0.001$ )

The analysis of the effect of prolonged usage of the oral contraceptive pill on plasma fibrinogen levels (Table 2) showed cumulative increase from  $3.09 \pm 0.9 \mu\text{g/l}$  for those on OCP for less than 6 months to  $3.37 \pm 1.47 \text{ g/l}$ , for those on OCP for more than 1.5 years.

Table 2  
Plasma Fibrinogen Levels Relationship With Duration Of Oral Contraceptive Usage

Duration (Months/Year)	N	Fibrinogen Levels (g/l)
1.5 Years – 4 Years	69	$3.37 \pm 1.47 \text{ g/l}$
7 Months – 1 Year	21	$3.33 \pm 0.91 \text{ g/l}$
3 Months – 6 Months	11	$3.09 \pm 0.94 \text{ g/l}$

## Discussion

Our results suggest that oral contraceptives do increase circulating fibrinogen levels. Such increases were cumulative and directly dependent on the duration of usage.

The raised fibrinogen levels in Nigerian women on OCP could not but seem to be induced by OCP and thus represent an important factor for the development of thrombo-embolic complications. Alternatively, the increased fibrinogen could be a result of decreased fibrinolysis in the presence of adequate fibrinogen synthesis by the liver. The latter assumption is supported by the finding of an enhanced spontaneous fibrinolytic activity in Nigerian males compared to females [11, 12], a factor these authors have attributed to a level of protection from atheroma and atherosclerosis in African males.

Interestingly, the majority of the women in this study (95%) who were on low dose oestrogen pills (0.03 mg) had lower plasma fibrinogen levels ( $3.00 \pm 2.50 \text{ g/l}$ ) than those (5%) who were receiving high dose oestrogen pills (0.05 mg); ( $3.50 \pm 0.60 \text{ g/l}$ ). However, these observed raised fibrinogen levels are within the normal range, although the increase may constitute a risk factor for vascular disease in African women on OCP.

Our study further indicates a cumulative and progressive increase in plasma fibrinogen concentration after prolonged usage of OCP. It is not possible, however, to infer a conclusion based on the small number of women (11) who had received the treatment in the early period of three to six months. We, however, believe that Nigerian women on OCP could be predisposed to develop circulatory and vascular disease as related to hyper-fibrinogenaemia and other clotting factors as the duration of usage is continued.

In order to assess the significance of abnormal coagulation fibrinolytic changes in our women on OCP, a more comprehensive prospective study is planned.



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## **Peripheral Blood Leukocyte Subpopulations a Long Time After Posttraumatic Splenectomy**

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Peripheral blood leukocyte subpopulations have been determined in 50 patients a long time (2 to 20 years) after posttraumatic splenectomy. These otherwise healthy individuals had significant lymphocytosis and monocytosis, while the absolute number of granulocytes did not differ statistically from that of the controls. The absolute number of CD2+, CD3+ as well as CD4+ and CD8+ peripheral blood mononuclear cells was found to be elevated, while the number of CD21+, CD20+ and HLA-DR+ PBMN cells was significantly decreased. The absolute number of sIgM+ as well as CD16+ MN cells did not differ statistically from that of the controls. Two further patients were found to have developed B-chronic lymphocytic leukaemia 5 and 31 years following posttraumatic splenectomy, respectively.

**Keywords:** posttraumatic splenectomy, lymphocyte subsets, chronic lymphocytic leukaemia

### **Introduction**

Splenectomized individuals are at an increased risk of overwhelming postsplenectomy infection (OPSI). This clinical syndrome, caused in the majority of cases by *Streptococcus pneumoniae*, is rapidly fatal in the majority of affected patients [1]. The risk of OPSI depends on the underlying illness for which splenectomy has been performed, the time elapsed since splenectomy and the age of the patient. The risk is the lowest in otherwise healthy adults splenectomized because of splenic injury [2, 3]. Several alterations of the humoral and cellular immune response have been described in splenectomized individuals. These include decreased serum IgM [4, 5] and tuftsin [6] levels, and impaired antibody response to antigenic stimuli [7]. Data on antibody response to pneumococcal vaccination are controversial, as both impaired [8] and normal antibody responses [9] have been described to depend also on the indication of splenectomy [10]. Lymphocytosis and monocytosis are well-known sequelae of splenectomy, moreover various changes of lymphocyte subpopulations and their function have been recognized [4, 11–13].

Because of the importance of different lymphocyte subpopulations in host defense, we performed cell surface marker determinations in the peripheral blood of otherwise healthy asplenic individuals. Lymphocytosis, monocytosis and T-lymphocytosis were observed while the number of B cells was found to be reduced. These alterations were not in correlation with either the age of the patients or with the time elapsed since splenectomy.

Table 1  
Peripheral blood mononuclear cell surface markers in healthy splenectomized patients (TSP)  
and in controls (mean, 95% confidence intervals)

		TSP	Controls	P<
Neutrophil	%	48.7 (45.8–51.6)	61.5 (59.5–63.5)	0.001
granulocytes	G/I*	4.22 (3.72–4.79)	4.20 (3.89–4.52)	NS
	(n)	(49)	(59)	
Lymphocytes	%	38.1 (36.7–39.4)	32.3 (30.7–33.9)	0.05
	G/I	3.11 (2.87–3.38)	2.11 (1.92–2.32)	0.001
	(n)	(50)	(31)	
Monocytes	%	7.3 (6.3–8.3)	4.8 (3.8–5.8)	0.001
	G/I	0.53 (0.46–0.61)	0.27 (0.20–0.36)	0.001
		(49)	(31)	
CD20+(B1+)	%	3.7 (3.2–4.2)	9.5 (6.4–14.0)	0.001
	G/I	0.12 (0.10–0.14)	0.20 (0.13–0.31)	0.001
		(42)	(15)	
CD21+(B2+)	%	4.8 (3.9–6.0)	12.3 (7.3–20.6)	0.001
	G/I	0.16 (0.12–0.20)	0.26 (0.15–0.45)	0.05
		(42)	(15)	
HLA-DR+	%	8.3 (7.3–9.5)	16.3 (12.1–21.9)	0.001
	G/I	0.26 (0.22–0.31)	0.35 (0.26–0.47)	0.05
		(42)	(21)	
slgM+	%	10.0 (8.5–11.7)	16.7 (13.0–21.4)	0.001
	G/I	0.28 (0.23–0.36)	0.44 (0.31–0.64)	NS
		(41)	(25)	
CD3+(OKT3+)	%	67.3 (63.9–70.7)	60.6 (55.8–65.4)	0.05
	G/I	2.07 (1.86–2.29)	1.26 (1.11–1.42)	0.001
		(42)	(28)	
CD2+(OKT11+)	%	70.6 (66.7–74.5)	57.3 (51.4–63.2)	0.001
	G/I	2.16 (1.93–2.42)	1.15 (0.96–1.37)	0.001
		(42)	(18)	
CD4+(OKT4+)	%	43.9 (40.3–47.5)	40.5 (36.5–44.5)	NS
	G/I	1.32 (1.17–1.48)	0.82 (0.70–0.95)	0.001
		(42)	(25)	
CD8+(OKT8+)	%	28.9 (26.4–31.4)	24.4 (20.8–28.0)	0.05
	G/I	0.86 (0.75–1.0)	0.49 (0.42–0.57)	0.001
		(42)	(27)	
CD4/CD8		1.7 (1.4–1.9)	1.9 (1.5–2.3)	NS
		(42)	(25)	
CD16+(Leu-11+)	%	12.8 (10.9–14.7)	15.8 (12.0–19.6)	NS
	G/I	0.34 (0.27–0.43)	0.30 (0.25–0.36)	NS
		(42)	(16)	

\* in the case of the absolute cell numbers the geometrical mean is shown



## Materials and Methods

Fifty-two posttraumatic splenectomy patients have been studied immunohaematologically, 41 men and 11 women. Based on detailed medical examination, 50 of these splenectomized patients were found to be healthy. Two further patients were found to have developed chronic lymphocytic leukaemia CLL [14]. The age of the 50 otherwise healthy posttraumatic splenectomy patients ranged from 21 to 57 years (mean: 35.1 years, SD: 8.7 years). The time elapsed since splenectomy ranged from 2 to 20 years (mean 7.8 years, SD: 4.5 years). The number of healthy volunteers who served as controls is shown in Table 1 beneath the respective marker. All studies were performed prior to vaccination with Pneumo-23 (Mérieux, Lyon).

### *Peripheral blood mononuclear cell counts*

Leukocytes were counted from fingertip peripheral blood taken between 8 a.m. and 10 a.m. Differential counts were obtained by evaluating 200 cells in thin, well-spread May-Grünwald-Giemsa stained smears.

### *Isolation of peripheral blood mononuclear cells*

Mononuclear cells were separated from peripheral blood on a Ficoll-Uromiro gradient. Interphase cells were resuspended in RPMI 1640 containing 10% fetal calf serum (FCS, Gibco) and antibiotics. Adherent cells were removed by incubation in plastic Petri dishes at 37°C in 5% CO<sub>2</sub> humidified air for 30 min. The viability of the final cell suspension was greater than 95% as tested by the exclusion of trypan blue. The concentration of nonadherent cells was adjusted to 10 million/ml.

### *Cell surface markers*

Binding of the monoclonal antibodies (MoAbs) was assessed with indirect immunofluorescence using FITC conjugated rabbit anti-mouse immunoglobulin, DA-KO as second antibody. T-cell associated antibodies were CD3 (T3), CD2 (T11), CD4 (T4), CD8 (T8) from Ortho Diagnostic Systems Inc., Raritan, N.J.; CD21 (B1) and CD20 (B2) from Coulter Corporation, Florida; IgM from Heintel, Vienna, CD16 (Leu-11) from Becton-Dickinson, Mountain View, California. Cells prepared the same way, but without the addition of the first layer of antibody served as negative controls. The relative fluorescence intensity of 10,000 cells was determined by a FACSTAR flow cytometer (Becton-Dickinson, Mountain View, California).

### *Statistics*

The results were evaluated statistically with the Student's *t*-test for unpaired samples.

### **Results**

The posttraumatic splenectomy patients had significant lymphocytosis and monocytosis while the absolute number of granulocytes did not differ statistically from that of the controls as shown in Table 1. The proportion and absolute number of large granular lymphocytes was found to be significantly elevated as reported previously [15]. Cell surface marker determinations were carried out in 42 posttraumatic splenectomy cases (35 men and 8 women). Results of cell surface marker analysis (percentages as well as absolute numbers) are shown in Table 1.

The absolute number of CD2+, CD3+ as well as CD4+ and CD8+ PBMN cells was found to be significantly elevated. Thus, the CD4/CD8 ratio did not differ statistically from that of the controls. The absolute number of CD21+, CD20+ and sIgM+ as well as HLA-DR+ PBMN cells was significantly decreased as compared to the controls. The percentage and absolute number of CD16+ MN cells did not differ from that of the controls.

### **Discussion**

Immunity and infection susceptibility of splenectomized patients differs from that of the healthy individuals in several respects [1, 3, 11]. The *in vivo* cellular immunoreactivity of posttraumatic splenectomy patients was also found to be impaired [16]. The determination of the lymphocyte subpopulations in the peripheral blood is not enough to estimate the risk of OPSI in splenectomized individuals, nevertheless, it is necessary for the characterization of the defect in cellular immunity. The ratio and the absolute number of the T cell subpopulations is of basic importance also because of their immunoregulatory role. Having found the CD4/CD8 ratio to be normal is in agreement with the data of Chelazzi et al. [12] while others have found this ratio to be decreased [11, 17]. The low number of B lymphocytes in our study is in contrast with earlier data on increased values [4, 17] and is an unexpected result. Furthermore, we have found the serum level of the immunoglobulins and of the natural antibacterial antibodies in the same patient group to be normal or increased [19]. As the CD5+ B lymphocytes are committed to the production of the polyreactive autoantibodies [18], in future studies it seems to be advisable to determine the number of these cells to study the cellular background of autoimmunity in splenectomized individuals [20].

The number of NK cells was found to be elevated following posttraumatic splenectomy both when the morphological counterpart, large granular lymphocytes, were counted [21–23] or when the absolute number of CD16+ MN cells were taken into account [11, 24]. Our data confirm the elevation of LGLs, while we have found the number of CD16+ MN cells and NK cell activity to be normal [15]. The number of



patients in our study greatly exceeds those of earlier studies, furthermore, the indication of splenectomy — splenic trauma — was the same in all cases. Care was taken that patient age and time from splenectomy should allow a good comparability of results. These factors might explain the fact that we were able to establish the increase in the number of T cells and the decrease in the number of CD20+, CD21+ and HLA-DR+ B cells on largely homogeneous samples showing a small scattering of values.

In the course of this study, we found two cases who had a rapidly fulminant clinical course and in whom the retrospective diagnosis of OPSI seems to be justified. These two cases could not be studied immunologically, but are known not to have received anti-pneumococcal vaccination. Results of immunohaematological investigations in two further patients who were found to have developed CLL a long time after posttraumatic splenectomy have been reported separately [14]. Periodic haematologic investigation of posttraumatic splenectomy patients may provide important insight into the mechanisms and possible sequelae of the ensuing immunodeficiency.

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## Quantitative Assay of Terminal Deoxynucleotidyl Transferase (TdT) Activity Using Monoclonal Antibodies

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Terminal deoxynucleotidyl transferase (TdT) is an important marker for the diagnosis and the therapy of leukemia and lymphoma patients. In this study, we developed the quantitative method for the assay of TdT antigen in leukemic cells using a combination of monoclonal and polyclonal antibodies prepared for this enzyme. High correlation was obtained between the values measured by biochemical assay and enzyme-linked immunosorbent assay (ELISA). This method (ELISA), using antibodies to TdT, permits the rapid and quantitative estimation of TdT antigen in leukemic cells.

**Keywords:** acute lymphoblastic leukemia (ALL), biochemical assay, chronic myelogenous leukemia (CML) in blast crisis, enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF)

### Introduction

Terminal deoxynucleotidyl transferase (TdT) is an intricate enzyme which is restricted in cortical thymocytes and lymphoid precursors of bone marrow [1]. Elevated enzyme activities have been reported in most of non-T, non-B acute lymphoblastic leukemia (ALL), T-ALL patients and in patients with chronic myelogenous leukemia (CML) in lymphoid crisis [2,3]. Therefore, TdT is an important marker for the diagnosis and the therapy of leukemia and lymphoma cases [1, 4]. Thus far, two assay methods (biochemical and immunocytochemical assay) have been applied for the detection of TdT in blood cells. The biochemical assay of TdT is, however, time-consuming, it requires a large number of cells, radioisotopes and some equipment. The recent availability of specific antibodies to TdT allowed the detection of this enzyme in single cells by immunocytochemical technique [5]. Therefore, the immunocytochemical assay has been widely used for the analysis of TdT in blood cells. Recently, we purified TdT from calf thymus and prepared monoclonal antibodies to the purified TdT antigen [6]. In this study, we developed the quantitative method for TdT assay in leukemic cells using these prepared antibodies.

## Materials and Methods

### *Reagents and clinical samples*

NALM-18, KM-3, REH, BV-173 (TdT-positive human non-T, non-B lymphoid leukemia cell lines) and HL-60, U-937, SPI-801, SPI-802 (TdT-negative human myeloid or monocytic leukemia cell lines) were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS) at 37 °C in 5% CO<sub>2</sub> and 95% air. Bone marrow aspirates or blood were obtained from patients with hematological disorders with their informed consent and mononuclear cells were isolated by Ficoll-Hypaque centrifugation. Subjects included 4 AML, 4 ALL, 3 non-Hodgkin's lymphoma cases and three patients including CLL, MDS and myelofibrosis, two patients with CML in the blastic phase and three in the chronic phase. The diagnosis of leukemia and lymphoma was based on conventional studies. The blastic phase of CML was defined as 30% or more blasts in the bone marrow, the blood or both. Granulocytes or lymphocytes as the reference were obtained from the blood of five normal volunteers.

### *Cytochemistry and surface marker analysis*

The cell smears were prepared with 0.1 M PBS (pH 7.2) + 10% FCS by using a cytocentrifuge (Shandon Southern Products Ltd., Cheshire, England) and cytochemical staining of the slides was done routinely, as shown previously [4]. Surface marker analysis was done as described previously [7] (Table 1).

### *Immunofluorescent analysis of TdT*

Mononuclear cells isolated or leukemic cell lines were used for this study. Cells were layered onto slides by cytocentrifuge, fixed in methanol at 4 °C for 10 min, then processed for indirect immunofluorescence as described previously [6, 8]. For the reference, polyclonal antibodies prepared by Bollum et al. were also used for this study [9].

### *Biochemical assay of TdT activity*

TdT activity was assayed under the optimal conditions established previously [10]. Mononuclear cells isolated from the bone marrow or blood of patients, or leukemic cell lines were disrupted in 250 mM phosphate buffer (pH 7.5) and used for the enzyme assay. 0.1 units of oligo(dA)<sub>12-18</sub> was used as a primer in combination with 1 mM <sup>3</sup>H-dGTP as a substrate. In biochemical assay, the activity of less than 2.5 units/10<sup>8</sup> cells was regarded as negative, which corresponded to the negative results in immunofluorescent analysis.



### *Enzyme-linked immunosorbent assay (ELISA)*

The cultured cells from leukemia cell lines or the mononuclear cells obtained from patients were suspended in PBS buffer (pH 7.2) containing 0.025% Tween-20 and 10% FCS at a concentration of  $5 \times 10^7$  cells/ml, and were then disrupted. Cell extracts obtained after the centrifugation at  $12,000 \times g$  for 15 min were used for this assay. Normal lymphocytes or granulocytes were obtained from the blood of five volunteers.

Myeloma cell lines producing monoclonal antibodies against terminal deoxynucleotidyl transferase (TdT) were prepared as described previously [6, 8]. These cell lines were maintained in medium containing inactivated 10% FCS. The cultured hybridoma cells were administered into balb/c mice intraperitoneally. After 10 to 14 days, ascitic fluid was obtained from the injected mice, and was then fractionated using ammonium sulfate precipitation. After overnight dialysis, IgG fraction of the ascitis was purified using protein A Sepharose column. The cell extracts were obtained from the cells suspended at a concentration of  $5 \times 10^7$  cells/ml in PBS containing 0.025% Tween-20 and 10% FCS. The immunoplates were coated with purified TdT-monoclonal antibodies (71-5; 10 µg/ml). The cell extracts (100 µl) were then layered on monoclonal antibody-coated plate, incubated for 2 hours at room temperature, and washed with PBS containing 0.05% Tween-20 three times. Rabbit anti-TdT (PL-Biochem.,  $\times 100$  diluted from the original antisera) was layered on the plate, incubated for 2 hours at room temperature, then the plate was washed with PBS three times. Following this, the peroxidase-goat antirabbit IgG ( $\times 100$  dil) was layered on the plate and incubated for 2 hours at room temperature. After washing the plate with PBS three times, the substrate, 0.04% ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] + 0.015%  $H_2O_2$  in 2% citrate buffer (pH 4.0) was added, and then the plate was incubated for 15 min at room temperature. Finally, 100 µl of 0.001% sodium azide was added, following which the peroxidase product was measured by an Immunoreader (Japan Intermed Ltd., Japan) at 414 nm. Normal mouse IgG and IgM fractions were used as controls.

Using the extracts of a NALM-18 cell line, biochemical assay and ELISA of TdT were done concurrently. A standard curve was then prepared as shown in Fig. 2. In this cell line, high correlation ( $r = 0.96$ ) was observed between the values obtained by biochemical assay and ELISA. The samples were diluted appropriately, then the assay was carried out within the linear range of the standard curve. In ELISA, the level of TdT in the samples was assessed from OD values of the cell extracts by using a standard curve obtained from a NALM-18 cell line. If the activity of ELISA calculated from a standard curve corresponded to that of no less than 2.5 biochemical units/ $10^8$  cells, the activity was regarded as negative. In our previous study, blasts of leukemia cases or leukemic cell lines with less than 2.5 biochemical units/ $10^8$  cells was also negative on immunofluorescent analysis [10].

### **Results and Discussion**

Table 1 shows marker profiles of TdT-positive human lymphoid leukemia cell lines used for this study. All these cell lines had characteristics of immature precursor B cells. As shown in Fig. 1, two monoclonal antibodies (62b-5 and 71-5) prepared for this

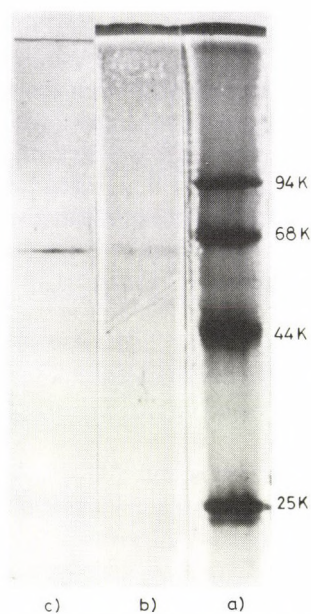


Fig. 1. Immunoblotting analysis of the fractions (crude human TdT) eluted from an immunoabsorbent column. (a) protein standards of known molecular weights [phosphorylase b ( $M_r = 94,000$ ), bovine albumin ( $M_r = 68,000$ ), ovalbumin ( $M_r = 44,000$ ) and chymotrypsinogen A ( $M_r = 25,000$ )]. (b) a lane treated with an anti-TdT monoclonal antibody, 62b-5. (c) a lane treated with an anti-TdT monoclonal antibody, 71-5

Table 1  
Marker Profiles of TdT-Positive Human Leukemia Cell Lines

	KM-3	REH	BV-173	NALM-18
E	negative	negative	negative	negative
S <sub>m</sub> I <sub>g</sub>	negative	negative	negative	negative
B <sub>1</sub>	0.38%	12.64%	11.19%	1.52%
B <sub>4</sub>	98.94%	99.77%	99.90%	96.45%
I <sub>a</sub>	96.08%	98.88%	84.90%	37.22%
J <sub>5</sub>	95.91%	82.94%	60.43%	52.20%
PNA	62.83%	65.43%	24.56%	99.47%
TdT	115 U/10 <sup>8</sup>	68.0 U/10 <sup>8</sup>	7.20 U/10 <sup>8</sup>	265 U/10 <sup>8</sup>
Origin	ALL	ALL	CML, BC	ALL



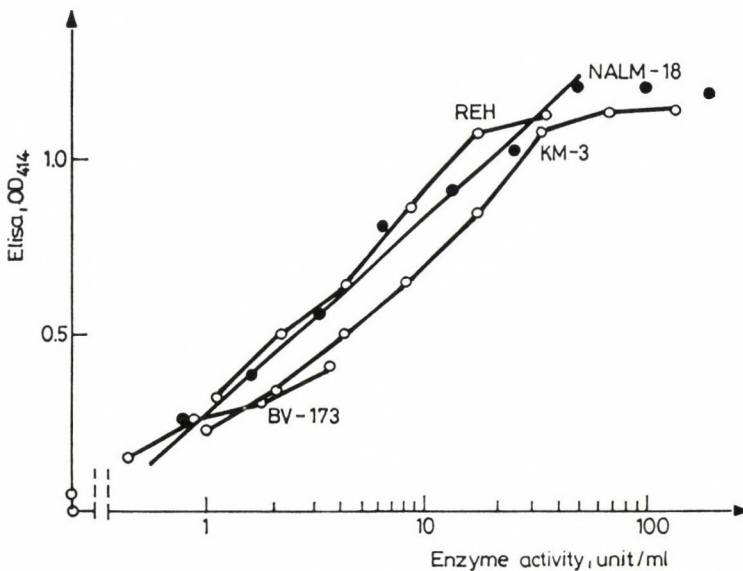


Fig. 2. The correlation between the enzyme activity obtained from biochemical assay and the values obtained from ELISA at OD<sub>414</sub> in human lymphoid leukemia cell lines (NALM-18, KM-3, REH and BV-173). The cells were disrupted in 250 mM phosphate buffer (pH 7.5), then centrifuged at 12,000  $\times$  g, for 15 min. The extracts were diluted with the same buffer serially, then TdT activity was assayed using ELISA and the biochemical method. The biochemical activity was represented as unit/ml. Although both monoclonal antibodies worked well for this ELISA, one (71-5) of two antibodies was used for this study as a primary antibody

study recognized 62 K dalton TdT for the extracts from a human lymphoid leukemia cell line, NALM-18. Figure 2 represents the correlation of TdT ELISA with TdT biochemical assay in four human lymphoid leukemia cell lines. In the NALM-18 cell line, a high correlation ( $r = 0.96$ ) was observed within the linear range between the values measured by biochemical methods and by the ELISA method. In this assay system (ELISA), cell extracts from biochemically TdT-negative leukemia cases or from biochemically TdT-negative leukemia cell lines (HL-60, U-937, SPI-801 and SPI-802) had negligible activity similar to that in lymphocytes or granulocytes. In addition, the values assessed from the cell extracts of TdT-positive leukemia cases or of TdT-positive leukemia cell lines by using ELISA method were significantly high and corresponded well to those obtained by using biochemical assay (Table 2). In our previous study [10], blasts of leukemia cases with less than 2.5 biochemical units/ $10^8$  cells was also negative on immunofluorescent analysis. Therefore, in this study, the activity of ELISA was regarded as negative, if the activity calculated from a standard curve corresponded to that of less than 2.5 biochemical units/ $10^8$  cells.

So far, Coleman et al. [11, 12] have reported the solid-phase immunoassay of TdT using polyclonal antibody-coated polystyrene beads. However, there is no report on the quantitative ELISA (enzyme-linked immunosorbent assay) of clinical samples by a



Table 2  
Biochemical Assay, ELISA and Immunofluorescent Analysis on TdT of Leukemic Cells

Sample	Biochemical Assay (unit/10 <sup>6</sup> cells)	ELISA (unit/10 <sup>6</sup> cells)	FTTC Staining
Clinical Case			
ALL	12.0	30.2	+
NHL	15.8	10.3	+
CML, BC	14.3	30.2	+
MDS	n. d.	0	-
ALL	56.9	37.0	+
AML	0	0	-
NHL	0	0	-
AML	0	0	-
AML	0	0	-
+CML	0	0	-
+CML	0	0	-
ALL	3.40	5.20	+
ALL	112	131	+
CML, BC	10.1	10.2	+
+CML	0	0	-
NHL	0	0	-
CLL	0	0	-
AML	0	0	-
Myelofibrosis	0	0	-
*lymphocytes	0	0	-
*granulocytes	0	0	-
Cell Line			
NALM-18	265	355	+
KM-3	115	160	+
REH	68.0	88.0	+
BV-173	7.20	3.10	±
U-937	0	0	-
HL-60	0	0	-
SPI-801	0	0	n.d.
SPI-802	0	0	n.d.

\*These lymphocytes and granulocytes were obtained from the blood of five normal volunteers. Zero in ELISA means that the level of TdT calculated from a standard curve was less than 2.5 units/10<sup>8</sup> cells. +CML: CML in chronic phase. CML, BC: CML in blastic phase. n. d.: not done.

combination of monoclonal and polyclonal antibodies to TdT. In this study, we used the monoclonal antibody (71-5) as a primary antibody on which TdT antigens are adsorbed specifically.

A polyclonal antibody was used for strengthening the reaction as a secondary antibody. Although the immunofluorescent analysis is useful as the qualitative assay examining the presence or the absence of TdT antigen in leukemic cells, quantitative correlation between antigen content as expressed by percent positive cells or by the intensity of positive cells and biochemical activity is not good [11, 13]. Accordingly, it is rather difficult to assess the content of this enzyme on the basis of the immunofluorescent analysis.

This quantitative assay (ELISA) using a combination of monoclonal and polyclonal antibodies to TdT allows the reliable estimation of the enzyme activities and the rapid assay of many samples without using radioisotopes.

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## **Abnormal Haemoglobins, Thalassaemia and G-6-PD Enzyme Deficiency in Rajasthan (Western-India)**

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Venous blood samples of 2922 apparently healthy individuals (both male and female) belonging to four scheduled tribes (408 Bhil, 340 Damor, 190 Garasia, and 186 Mina), five scheduled castes (108 Meghwal, 93 Labana, 86 Mochi, 76 Garg, and 58 Yadaw) and eleven general castes (215 Baniya, 156 Brahmin, 153 Muslim, 130 Patel, 128 Bohra-Muslim, 122 Suthar, 115 Patidar, 112 Rajput, 92 Bhoi, 92 Sompura, and 62 Panchal) of the Dungarpur district of Rajasthan state were investigated by standard techniques for evidence of erythrocyte genetic disorders, abnormal haemoglobins, thalassaemia syndromes, and G-6-PD enzyme deficiency ( $G^d$ ). Abnormal haemoglobins were encountered in 122 (4.17%) subjects. Of these, 93 (3.18%) were Hb-AS, 14 (0.47%) Hb-SS, 12 (0.41%) Hb-AD, 2 (0.06%) Hb-AE, and 1 (0.03%) Hb-SC. A higher incidence of these mutant genes was found in scheduled tribes (6.85%) as compared to scheduled castes (3.08%) and general castes (2.32%).  $\beta$ -thalassaemia syndromes were encountered in 111 (3.79%) subjects; 96 (3.28%)  $\beta$ -thalassaemia traits, 13 (0.44%) HbS-thalassaemia, and 2 (0.06%) HbD-thalassaemia.  $G^d$  was found in 329 (11.25%) subjects. Of these, 203 (18.06%) belonged to scheduled tribes, 39 (9.26%) to scheduled castes, and 87 (6.31%) to general castes. In our present communication, the incidence and interaction of these mutant genes in different ethnic groups of scheduled tribes (S.T.), scheduled castes (S.C.), and general castes (G.C.) are discussed. Furthermore, their distribution in various ethnic groups residing in different districts of Rajasthan state (Western-India) is also reviewed.

**Keywords:** abnormal haemoglobins, thalassaemia syndromes, G-6-PD enzyme deficiency, genes

### **Introduction**

Charles Darwin [1] acknowledged "natural selection" as the principal driving force for evolution of living organisms. His evolutionary theory was largely based on the understanding of selection and its consequences for "origin of species". The concept of "fitness" was a central theme of the theory of evolution. The Darwinian concept of selection is now understood in population genetics as the operational force that determines the relative fitness of a genotype in the population, thus affecting the gene concerned [2]. It is also recognized that selection has been playing an important role on the one hand, in the evolution of living organisms in achieving change in gene frequency by differential survival and reproduction of genotype, and on the other hand, playing a conservative role by producing equilibrium between mutation and selection [3]. One of the best examples for balanced polymorphism or natural selection is sickle cell haemoglobin (Hb-S) discovered by Herrick [4]. The heterozygote (Hb-AS) is protected against malaria and does not suffer from sickle cell disease (Hb-SS), whereas the normal

homozygote (Hb-AA) is vulnerable to malaria [5, 6]. Furthermore, it was also confirmed that the malaria parasite, *Plasmodium* is unable to penetrate or infect the plasma membrane of red blood cells containing Hb-S [7]. Other examples of natural selection, G-6-PD enzyme deficiency (sex-linked gene inherited from females), and thalassaemia syndromes which are also found to be protective factors against the malaria parasite. The relationship between malaria and these mutant genes has been investigated and it is now generally accepted that malaria exerts a selective pressure for the maintenance of higher frequencies of sickle cell and G<sup>d</sup> genes. In Africa, high frequencies of these mutant genes are found in areas where malaria endemicity is high and vice versa. This has been repeated and confirmed by several workers [8–12]. The relationship between other abnormal haemoglobins such as Hb-E, C, D, etc., and malaria is still controversial. However, these are, perhaps, due to different environmental or biogeographical conditions. Besides producing a varying degree of anaemias, abnormal haemoglobins, thalassaemia syndromes, and G<sup>d</sup> genes in human beings, these are also genetic markers. Therefore, many workers are screening the various populations residing in different parts of the world for evidence and are still engaged in getting to understand the mysteries of these erythrocyte mutant genes.

From India, many workers reported the prevalence of erythrocyte mutant genes, abnormal haemoglobins, thalassaemia syndromes and G<sup>d</sup> basically in the tribal population of different states. However, studies based on these genes in other ethnic groups are still scanty. From Rajasthan state, these mutant genes are also reported in S.T., S.C., and G.C. of certain districts. But previously, no such study was performed in the different ethnic groups residing in hyperendemic areas of malaria. Therefore, the present study was undertaken and conducted in a tribal district, Dungarpur where over 65% of the tribal populations live in hyperendemic malarious areas. Simultaneously, abnormal haemoglobins, G<sup>d</sup>, and thalassaemic genes reported from different districts of Rajasthan have also been reviewed.

### Material and Methods

Both male and female adult subjects from four endogamous scheduled tribes (S.T.), five scheduled castes (S.C.) and eleven general castes (G.C.) were studied (Table 1). The characteristics of the tribes are: dark skin, coarse black hair, flat nose, and medium-size stout body. They live basically in a hilly region (Arawali Hills) in near isolation where hypermalaria endemicity still exists. Other communities, S.C. and G.C. live in towns and villages where malaria endemicity is low. Inter-caste marriages generally do not occur.

Random intravenous blood samples were collected in EDTA vials from 2922 apparently healthy and unrelated subjects belonging to twenty ethnic groups of S.T., S.C., and G.C. (Table 1). For minor surgical operations, the subjects were admitted either to the General Hospital Dungarpur or to camps organized at various places in the Dungarpur district (1987–1989). Samples were investigated within 48 h for evidence of mutant haemoglobins, thalassaemia syndrome, and G<sup>d</sup>. Horizontal paper and cellulose acetate-electrophoresis were performed for the identification and quantitative evaluation of abnormal haemoglobins and Hb-A<sub>2</sub>. Other electrophoretic techniques, as described



Table 1

Distribution and incidence (%) of abnormal Hbs,  $\beta$ -thalassaemia, and G<sup>d</sup> genes in scheduled tribes, scheduled castes, and general cast groups of Dungarpur district (Rajasthan)

Ethnic groups	Number tested	Haemoglobin phenotypes					Total	Types of $\beta$ -Thalassaemia			Total	G-6-PD deficiency (G <sup>d</sup> )
		Hb-AS	Hb-SS	Hb-AD	Hb-AE	Hb-SC		$\beta$ -Thal. trait	HbD -Thal.	HbS -Thal.		
A. Scheduled tribes												
1. Bhil	408	26(6.37)	6(1.47)	—	—	—	32(7.84)	20(4.90)	—	8(1.96)	28(6.86)	60(14.70)
2. Mina	186	12(6.45)	—	3(1.61)	—	—	15(8.06)	11(5.91)	—	—	11(5.91)	36(19.35)
3. Damor	340	17(5.00)	2(0.58)	1(0.29)	—	—	20(5.88)	29(8.52)	—	1(0.29)	30(8.82)	67(19.75)
4. Garasia	190	9(4.73)	1(0.52)	—	—	—	10(5.26)	11(5.78)	—	2(1.05)	13(6.84)	40(21.05)
	1124	64(5.69)	9(0.80)	4(0.35)			77(6.85)	71(6.31)		11(0.97)	82(7.29)	203(18.06)
B. Scheduled castes												
1. Meghwal	108	5(4.62)	1(0.92)	—	—	—	6(5.55)	4(3.70)	—	—	4(3.70)	18(16.66)
2. Labana	93	3(3.22)	—	1(1.07)	—	—	4(4.30)	1(1.07)	—	—	1(1.07)	12(12.90)
3. Mochi	86	1(1.16)	1(1.16)	1(1.16)	—	—	3(3.48)	1(1.16)	—	—	1(1.16)	8( 9.30)
4. Garg	76	—	—	—	—	—	—	—	—	—	—	1( 1.31)
5. Yadav	58	—	—	—	—	—	—	—	—	—	—	—
	421	9(2.13)	2(0.47)	2(0.47)			13(3.08)	6(1.42)			6(1.42)	39( 9.26)
C. General caste group												
1. Brahmin	156	—	—	1(0.64)	—	—	1(0.64)	—	—	—	—	1( 0.64)
2. Baniya	215	5(2.32)	—	2(0.93)	1(0.46)	—	8(3.72)	2(0.93)	1(0.46)	—	3(1.39)	32(14.88)
3. Rajput	112	—	—	1(0.89)	—	—	1(0.89)	—	—	—	—	3( 2.67)
4. Patel	130	3(2.30)	—	—	1(0.76)	—	4(3.07)	4(3.07)	—	1(0.76)	5(3.84)	8( 6.15)
5. Patidar	115	3(2.60))	—	1(0.86)	—	—	4(3.47)	4(3.47)	—	—	4(3.47)	5( 4.34)
6. Suthar	122	4(3.27)	2(1.63)	—	—	—	6(4.91)	2(1.63)	—	—	2(1.63)	5( 4.09)
7. Sompura	92	—	—	1(1.08)	—	—	1(1.08)	1(1.08)	—	—	1(1.08)	1( 1.08)
8. Bhoi	92	1(1.08)	—	—	—	—	1(1.08)	—	—	—	—	—
9. Panchal	62	—	—	—	—	—	—	—	—	—	—	—
10. Muslim	153	2(1.30)	1(0.65)	—	—	—	3(1.96)	3(1.96)	1(0.65)	—	4(2.61)	12(7.84)
11. Bohra-Muslim	128	2(1.56)	—	—	—	1(0.78)	3(2.34)	3(2.34)	—	1(0.78)	4(3.12)	20(15.62)
	1377	20(1.45)	3(0.21)	6(0.43)	2(0.43)	1(0.03)	32(2.32)	19(1.37)	2(0.14)	2(0.14)	23(1.67)	87( 6.31)
Total	2922	93(3.18)	14(0.47)	12(0.41)	2(0.06)	1(0.03)	122(4.17)	96(3.23)	2(0.06)	13(0.44)	111(3.79)	329(11.25)



electrophoretic patterns and double heterozygous abnormal haemoglobins. Other standard methods; estimation of Hb-F, sickling test, solubility test, osmotic fragility test, staining of blood smears and BCB dye decolourization test were also performed as described earlier [14, 15] for the confirmation of mutant haemoglobin and G<sup>d</sup>.  $\beta$ -thalassaemia trait was diagnosed if Hb-A<sub>2</sub> was over 3.5% together with increased resistance of erythrocytes to haemolysis and abnormal erythrocyte morphology. HbS-thalassaemia and HbD-thalassaemia were diagnosed if more than 10% Hb-F occurred together with Hb-S and Hb-D, respectively. In the present investigation, family studies were not done and the data presented here are based only on haematological analysis.

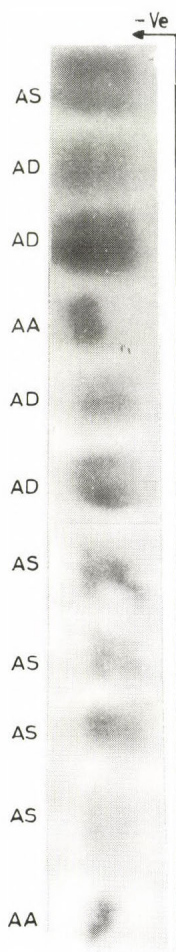


Fig. 1. Paper electrophoresis at pH 8.6 showing Hb-AD, and AS pattern

## Results

Electrophoretic examination of 2922 haemolysates revealed abnormal haemoglobins in 112 subjects (4.17%); 77 subjects (6.85%) belonged to scheduled tribes, 13 subjects (3.08%) to scheduled castes, and 32 subjects (2.32%) to general castes (Table 1). Heterozygous abnormal haemoglobins (Hb-AS, Hb-AD, and Hb-AE) were encountered in 93 (3.18%), 12 (0.41%), and 2 (0.06%) subjects, respectively. In the present series, one subject (0.03%) had a double heterozygous haemoglobin (Hb-SC) and in fourteen subjects (0.47%), homozygous mutant haemoglobin (Hb-SS) was observed. The frequency (%) of abnormal haemoglobins varied from 5.26 to 8.06 in S.T., 3.48 to 5.55 in S.C., and 0.64 to 4.91 in G.C. communities. Relative distribution and incidence of mutant haemoglobins in various ethnic groups have been depicted in Table 1 and their relative electrophoretic migration patterns are shown in Figs 1, 2 and 3.

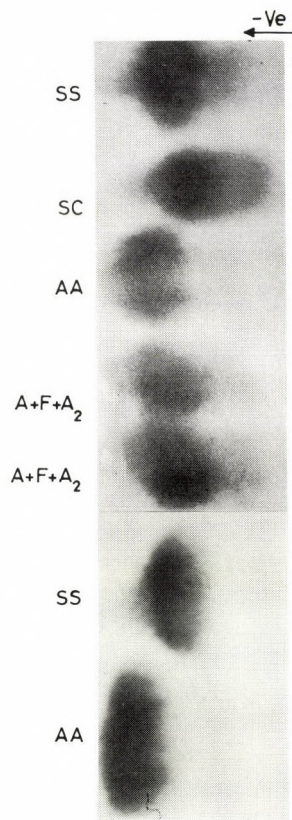


Fig. 2. Paper electrophoresis at pH 8.6 showing Hb-SS, SC, and  $\beta$ -thalassaemia trait (HbA + F + A<sub>2</sub>) pattern

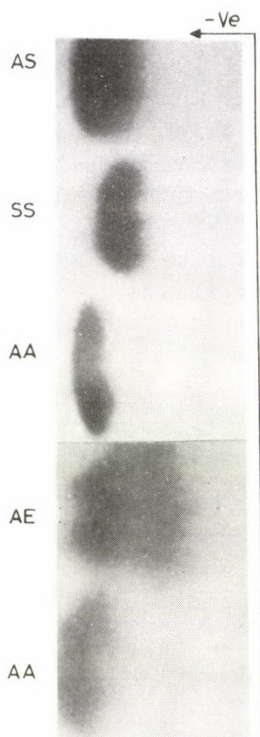


Fig. 3. Paper electrophoresis at pH 8.6 showing Hb-AS, SS and AE pattern

Of the 2922 unrelated subjects, 96 (3.28%) showed an increased level of Hb-A<sub>2</sub> (mean 6.9%), a reduced osmotic fragility and abnormal erythrocyte morphology. Of these 17 also had a raised level of Hb-F (mean 6.6%). All these were considered or diagnosed as  $\beta$ -thalassaemia cases. A high incidence (%) of this gene was observed in S.T. (6.31%), followed by S.C. (1.42%) and G.C. (1.67%) (Table 1). Interaction between  $\beta$ -thalassaemia and mutant haemoglobin genes was also observed. These are cases of HbD-thalassaemia and were found in two subjects (0.14%) of G.C., and eleven (0.97%) of S.T., and two (0.14%) of G.C. communities, respectively (Table 1). On electrophoresis, Hb-F+D and Hb-F+S patterns without Hb-A were found. As regards the overall incidence (%) of  $\beta$ -thalassaemia syndromes, 3.79 was observed in the present study and its frequency varied from 5.91 to 8.82% in S.T., 1.08 to 3.84% in G.C., and 1.07 to 3.70% in S.C. communities (Table 1).

BCB dye decolourization test was performed for rapid qualitative screening of 2922 unrelated subjects for the evidence of G<sup>d</sup> gene. Of these, 329 (11.25%) had G<sup>d</sup>. The highest incidence 18.06% of G<sup>d</sup> was observed in S.T. followed by 9.26% in S.C. and



Table 2

Relative distribution and incidence (%) of abnormal Hbs., thalassaemia and G<sup>d</sup> genes in various ethnic groups of different districts of Rajasthan State (India)

Place of district	Ethnic/ caste groups	Hb phenotypes			Types of Thalassaemia (%) :	G <sup>d</sup>	Reference No.
		Hb-AS	Hb-SS	Hb-AD			
A. Scheduled tribes							
Udaipur	Garasia	31.14	—	—	—	—	(17)
	Meena	—	—	—	—	—	
	Bhil	4.28	—	—	—	—	
	Gamit	14.67	—	—	—	—	
	Bhil	4.2	—	—	β-Thal. trait (2.9)	16.3	(18)
Kotra Tehsil (Udaipur)	Bhil	—	—	—	—	16.8	(26)
Kherwara Tehsil (Udaipur)	Mina	4.29	—	—	—	—	(19)
Udaipur region	Bhill	5.7	—	—	β-Thal. trait (3.0)	13.5	(20, 27)
	Mina	4.4	0.5	—	β-Thal. trait (4.8)	21.2	
	Garasia	1.1	—	—	β-Thal. trait (5.0)	17.2	
	Damor	—	—	—	β-Thal. trait (7.9)	17.3	
	Gameti	0.6	—	—	β-Thal. trait (7.4)	—	
	Adiwasi	3.5	—	—	β-Thal. trait (5.7)	22.0	
	Sahariya	—	—	—	—	17.5	
	Bhill	7.35	—	—	β-Thal. trait (4.08)	8.16	(21)
Udaipur*	Mina	5.88	—	—	β-Thal. trait (6.47)	15.29	
	Roat	7.27	—	—	β-Thal. trait (9.00)	10.00	
	Bhill	18.0	—	—	—	—	(17)
	Dumaria	1.45	—	—	—	—	
Dungarpur	Koli	7.14	—	—	—	—	
	Damor	4.29	—	—	—	—	(28)
	Sirohi	20.00	—	—	—	—	(17)
Tonk	Gamit	3.45	—	—	—	—	
	Meena	—	—	—	HbD-Thal. (0.2)	—	(22)

Place of district	Ethnic/ caste groups	Hb phenotypes			Types of Thalassaemia (%)	G <sup>d</sup>	Reference No.
		Hb-AS	Hb-SS	Hb-AD			
<i>B. Scheduled castes</i>							
Udaipur Region	Regar	0.68	—	1.08	β-Thal. trait (1.85)	5.55	(14, 15, 29)
	Khatik	0.85	—	0.68	β-Thal. trait (1.66)	3.33	
	Bhangi	0.98	—	—	β-Thal. trait (2.88)	7.69	
	Mochi	2.58	—	—	β-Thal. trait (1.65)	7.83	
	Meghwal	4.2	—	—	β-Thal. trait (1.88)	15.09	
	Ganchha	4.2	—	—	β-Thal. trait (3.12)	—	
	Nagarchi	2.5	—	—	—	7.40	
Udaipur Region*	Salvi	3.7	—	—	—	10.00	(21)
	Harijan	1.79	—	—	β-Thal. trait (3.57)	2.68	
	Khatik	1.19	—	2.38	β-Thal. trait (1.19)	—	
	Meghwal	3.27	—	—	β-Thal. trait (1.96)	9.15	
	Mochi	2.82	—	0.70	β-Thal. trait (2.11)	3.52	
	Regar	1.47	—	2.94	—	—	
<i>C. Other caste groups</i>							
Udaipur District	Bohra -Muslim	—	—	—	β-Thal. trait (3.7)	17.60	(30, 31)
	Hindu	—	—	0.09	β-Thal. trait (0.76)	1.10	
	Muslim	—	—	—	β-Thal. trait (2.35)	5.68	
	Christian	—	—	—	—	—	
	Sikh	—	—	—	—	—	
	Parsee	—	—	—	—	9.80	
Udaipur region	Jangid	4.71	—	—	β-Thal. trait (0.00)	5.12	(32)
	Mevali	1.28	—	0.64	β-Thal. trait (7.05)	15.49	
	Vishwakarma	5.98	—	—	β-Thal. trait (5.82)	13.26	
Udapur*	Brahmin	—	—	—	—	—	(33)
	Jain	2.38	—	0.95	—	11.42	
	Rajput	—	—	—	—	1.51	

Place of district	Ethnic/ caste groups	Hb phenotypes			Types of Thalassaemia (%)	G <sup>d</sup> Reference No.
		Hb-AS	Hb-SS	Hb-AD		
	Sikh	—	—	—	—	—
	Bohra -Muslim	1.16	—	—	—	13.95
	Non-Bohra Muslim	—	—	—	—	7.36
Udaipur	Jain	—	—	One case	—	— (23)
Jaipur	Sindhi	—	—	—	HbD-Thal. (one case)	— (24)
	Christian	—	—	Hb-DD (one case)	—	— (25)
Jaipur and Jodhpur	Not specify	—	—	—	β-Thal. (8 cases)	— (34)
Dungarpur	General castes	—	—	—	α-Thal. (0.9)	— (35)
District	Scheduled- tribes	—	—	—	α-Thal. (5.0)	—
	Scheduled- castes	—	—	—	α-Thal. (2.3)	—
	Muslims	—	—	—	α-Thal. (2.2)	—
	Bohra -Muslims	—	—	—	—	—

\*Study in Children

6.13% in G.C. groups. The frequency of G<sup>d</sup> in various ethnic groups of S.T., S.C. and G.C. is demonstrated in Table 1. G<sup>d</sup> individuals were also screened on electrophoresis but no evidence of interaction with mutant haemoglobins was found.

### Discussion

Basically Hb-S and Hb-E are reported in Indian tribes. However, a few cases of Hb-D can also be observed in tribes. The Hb-E gene is endemic in the Eastern and Southern regions whereas Hb-S is endemic in the Western and Middle regions of India [16,36]. Evidence of the Hb-S gene was first observed by Negi [17] who reported an incidence of 20.00 to 31.14% in Garasia and 3.45 to 14.67% in Gamit tribes of Udaipur district of southern Rajasthan. Today these findings cannot be considered reliable since he did not have the modern technique of electrophoresis — the most reliable for the identification of mutant haemoglobins — at his disposal. Which is the most reliable for the identification of mutant haemoglobins. However, Jain et al. [18–20] performed electrophoresis and reported 1.1 to 5.7% incidence of Hb-S gene in tribes of Udaipur district (Table 2). Later on, Choubisa [21] screened the tribal children of the Udaipur region with electrophoresis and reported 5.88–7.35% incidence of Hb-S in trait form. Hb-D gene together with β-thalassaemia was first observed in a Meena tribal girl of Tonk



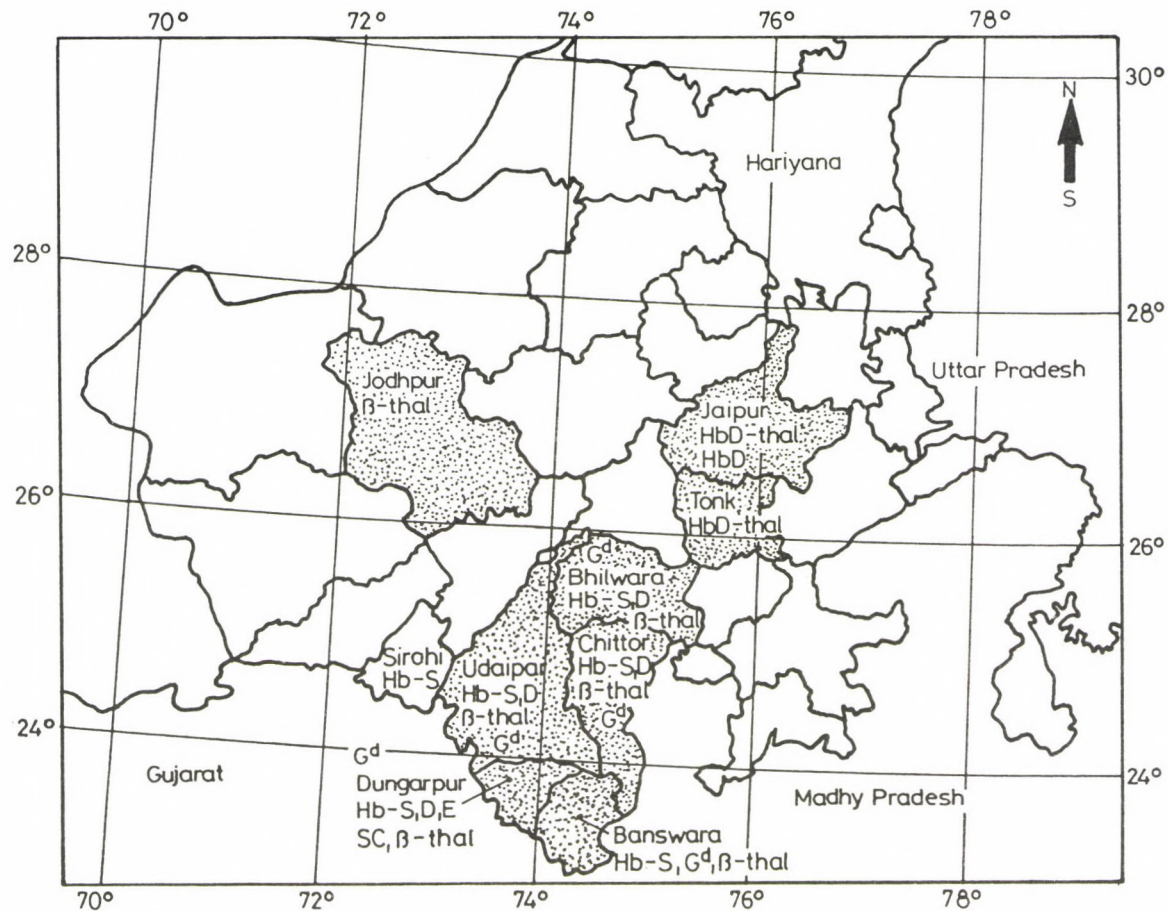


Fig. 4. Map of Rajasthan State (India) showing distribution of abnormal Hbs, thalassaemia and G<sup>d</sup> in different districts

district [22]. In the present series overall incidence of Hb-AS (5.69%), Hb-SS (0.80%) and Hb-AD (0.35%) in tribal population has been observed. Hb-D gene in heterozygous form also reported in isolated forms in the Jain community of Udaipur district [23], and the Sindhi and Christian communities of Jaipur [24, 25]. This means this gene is endemic in Rajasthan. Besides these genes (Hb-S and D), Hb-E and Hb-SC genes are also endemic and more surveys are still required for the tracing of the latter genes in G.C. communities of Rajasthan state. The distribution of mutant haemoglobins in different districts, and ethnic groups is shown in Fig. 4 and Table 2, respectively.

In our present survey,  $\beta$ -thalassaemia traits and  $G^d$  genes occurred in all except a few ethnic groups. The maximum incidence of these genes was observed in the tribal population as compared to S.C. and G.C. (Table 1). These are also higher in this district as compared to other districts of Rajasthan (Table 2) due to hypermalaria endemicity and sexual contacts as well as different environmental conditions. But incidence of Hb-S, Hb-D,  $\beta$ -thalassaemia traits, Hb-S-thalassaemia and  $G^d$  genes is higher in the tribal populations of Dungarpur district since they reside in the hilly areas where the hyper endemicity of malaria (12% incidence of *Plasmodium* infection) and sexual contacts between inter and intra tribal groups are more common. Another possible reason may be the migration of the sickle cell gene through sexual contacts and/or marriage factor of the tribes of Gujarat and Madhya Pradesh states with a high incidence of the sickle cell gene, 15.53 to 31.40% and 24.64 to 35.51%, respectively [16]. The geographical location of the Dungarpur district is such that Gujrat shares its northern border while Madhya Pradesh shares its western border (Fig. 4). However, the maximum chances of the Hb-S gene migration are from the Gujrat State, since its Panch Mahal district (tribes having 15.53 to 31.40% of Hb-S gene) is much nearer to the Dungarpur district. The situation is similar in the S.C. communities. However, the incidence of these genetic disorders in ethnic groups of G.C. is relatively low, but the number of mutant genes such as Hb-S, D, E, SC, HbD-thalassaemia, and HbS-thalassaemia are higher, this is perhaps due to the hypoendemicity of malaria and comparatively higher sexual contacts between individuals of the same ethnic group, inter ethnic groups and the individuals of S.C. and S.T. communities, respectively. This is further supported by the high incidence of venereal and infectious diseases in this region (unpublished data). The significance of the present study is that it reports for the first time, the existence of Hb-D and HbS-thalassaemia genes in tribes, Hb-E and SC in G.C., and comparative findings on erythrocyte genetic disorders in S.T., S.C. and G.C. To the best of this author's knowledge, he has reviewed all the up-to-date data (Table 2) related to erythrocyte genetic disorders reported by workers from different districts of Rajasthan state, Western India.

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## **Molecular Genetics – New Horizons in Haematology**

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The unprecedentedly swift developments in molecular genetics has opened up a new era in biology and medicine. The powerful methods of recombinant DNA (rDNA) technology are fast moving into the fields of diagnostics and therapy since the newly found ability to define physiological and pathological cell functions at a molecular level. The centerpiece of molecular genetics is the possibility to map and determine the fine structure of human genes and to define in molecular terms how each gene controls all the enzymes of energy metabolism, structural proteins of cells, the membrane proteins, including transport proteins and receptors, the plasma proteins and those proteins which participate in the synthesis of complex lipids, carbohydrates, lipoproteins and glycoproteins.

The fundamental change of emphasis in cellular and clinical research in medicine started in haematology. No wonder, since the circulating blood cells, the bone marrow, and lymphoid tissue cells are easily available for investigation and most of the haematological diseases are well defined entities. This review will try to present the increasing depth and broadening spectrum of molecular haematology by arbitrary chosen examples:

- 1) Molecular regulation of cell specific gene expression and of age specific switch of the globin genes,
- 2) Revisiting the haemolysis of paroxysmal nocturnal haemoglobinuria (PNH) cells – the phosphatidylinositol-glycan (PIG) anchored membrane proteins,
- 3) Molecular genetics of the ABO and Rh blood group specificity,
- 4) The regulation of stem cells and the multistep process of their malignant transformation.
- 5) The impact of gene technology on diagnostics, prevention and therapy in haematology,
- 6) The present state of art and future possibilities of the treatment of genetic diseases.

**Keywords:** gene technology, LCR, PCR, growth factors, gene transplantation, molecular genetics

### **1. Molecular Regulation of Cell Specific Gene Expression and of Age Specific Switch**

Haemoglobins (Hb-s) played an important role in the development of molecular genetics. Over forty years ago, Pauling [1] revealed that the most widespread genetic disease, sickle cell anaemia, is caused by a single amino acid substitution in the  $\beta$ -globin chain. The human globin chains were among the first mammalian genes to be cloned and sequenced. Identification of mutations in genes coding for different abnormal haemoglobins and for the defect of globin chain synthesis in the thalassaemia syndromes provided evidence for the near-complete description of the molecular mechanisms underlying inherited diseases.

Mammalian globins are encoded in small  $\alpha$ - and  $\beta$ -like gene clusters. The human  $\alpha$ -globin locus occupies approximately 30 kb on the short arm of chromosome 16, while the  $\beta$ -globin locus spans roughly 60 kb on the short arm of chromosome 11. The order



of genes in both clusters correspond to the time sequence of their expression during ontogenesis (Fig. 1). Expression of globin genes is restricted to erythroid cells and is developmentally regulated in such a way that only a subset is transcriptionally active at any particular developmental stage.

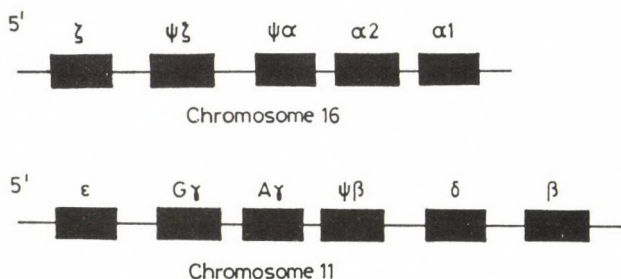


Fig. 1. The human globin gene loci

The pattern of inheritance of one of the  $\alpha$ -thalassaemia syndromes, HbH disease, remained a riddle for a long period of time. It could be, however, easily explained after the finding of three  $\alpha$ -chains ( $\alpha$  J Buda,  $\alpha$  G Pest and normal- $\alpha 2$ ) in a Hungarian patient and his brother [2]. The demonstration of the complete identity of the gene products of the two  $\alpha$ -genes is still a unique example in human genetics [3], its cause remains for the time being, unanswered, just as the question of why the two  $\gamma$ -genes ( $G\gamma$  and  $A\gamma$ ) differing only in a single amino acid, remain both parallelly expressed. The level of expression of both the duplicated  $\alpha$ - [4, 5] and  $\gamma$ -genes differ. The difference between the level of the two  $\gamma$ -gene products undergoes even a programmed change during ontogenesis [6]. There are many more unsolved riddles pertaining to the control of globin gene expression and red cell development. It is still unknown how the haemopoietic stem cells, capable of differentiating into all blood lineages, commit themselves to erythroid development. We are just at the beginning of understanding some factors participating in the activation of globin genes and the regulation of the programmed switch from embryonic to foetal to adult globin synthesis during human ontogenesis. Research to answer these questions brought the globin genes into the fore of interest since detailed disclosure of the regulatory factors participating in the activation and maintenance of cell and age-specific gene expression are indispensable for designing improved strategies for the management of genetic diseases, including reactivation of foetal genes and gene transplantation.

Investigations of the rare genetic deletions in thalassaemias have first pointed out that a region of DNA located far upstream of the human globin loci is critically involved in the regulation of the two globin gene families. The functional activity of erythroid-specific DNA-ase I hypersensitive sites in these regions [7] has been tested directly by ligating them to human  $\alpha$ - and  $\beta$ -globin genes and assaying their expression in transgenic mice [8] and cultured erythroid cells [9, 10]. The investigation of the "humanized" mice rendered evidence of sequences that confer position independent expression of linked genes which have been termed either the determinant control region (DCR) or locus activating region (LAR) and are called locus control regions (LCR-s) since 1990.

The  $\beta$ -globin-LCR contains 4 prominent hypersensitive sites (HS-s) designated 5' HS-1 through HS-4, residing upstream of the embryonic  $\epsilon$ -gene (Fig. 2). Of the four principle  $\beta$ -LCR 5' HS-s, HS-2 accounts for roughly 50% of the total LCR activity. In the course of ontogenesis, genes in the closest vicinity of LCR-s are expressed first. The  $\epsilon$ -gene which is expressed during the yolk sac erythropoiesis is being silenced by a specific repressor protein in the liver. LCR effects are then suggested to be recruited by the downstream  $\gamma$ - and  $\beta$ -genes which remain in a continuous competition for the strong stimulating influence of the LCR [11]. During intrauterine life, the higher  $O_2$  affinity of foetal red cells is advantageous. In contrast, under physiological conditions, the  $O_2$  supply is abundant during extrauterine life. As a consequence, it is not the  $O_2$  uptake, but the  $O_2$  release to the tissues that has to be ensured primarily. The  $\beta$ -globin by its

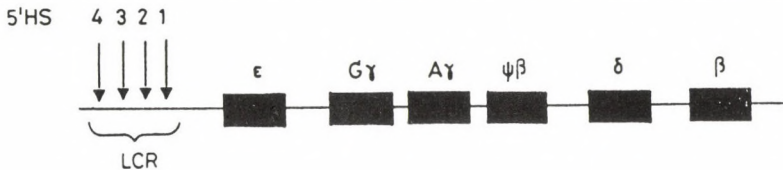


Fig. 2. Erythroid DNA-ase hypersensitive sites (HSs) in the locus control region (LCR) of the human globin gene complex

2,3-DPG binding ability stabilizes the Hb molecule in the low affinity deoxy-conformation, that is probably the way by which the  $\beta$ -genes gain advantage over the  $\gamma$ -genes. The switch from  $\gamma$ - to  $\beta$ -gene expression may be achieved, however, both through this competition or via more direct silencing (Fig. 3).

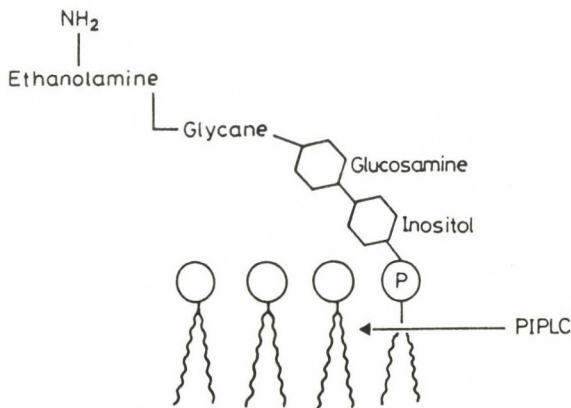


Fig. 3. The phosphatidylinositol glycan (PIG) anchor. PIPLC = phosphatidylinositol specific phospholipase C

An LCR element for expression of the  $\alpha$ -like globin genes was identified more than 30 kb upstream of the embryonic zeta-gene by Higgs [cited in 11]. This LCR was found within the intron of an ubiquitously expressed gene transcribed in the opposite direction from the  $\alpha$ -globin locus.



In the 1960s, we were involved in the investigation of the reactivation of foetal Hb (HbF) and showed that in the O<sub>2</sub> poor environment of hibernation, a foetal type Hb is being reactivated in bats and frogs [12]. Our clinical investigations have rendered evidence that during enhanced "stress erythropoiesis" parallel to the reactivation of HbF synthesis, other characteristics of foetal erythropoiesis are also reactivated [13, 14]. The most recent findings substantiate these suggestions on a molecular level by the evidence of the presence of binding sites for an erythroid DNA-binding protein (now designated GATA-1) in cis-elements of the vast majority of globin and non-globin, erythroid specific genes of all vertebrate species so far examined [15]. GATA-1 is implied to function together with other cellular factors as a coordinating transcriptional initiator of several erythroid expressed genes (i.e. erythropoietin receptor, pyruvate kinase and glycophorin B) to establish lineage-specific patterns of gene expression [16].

In the LCR domain of the  $\beta$ -globin cluster, the DNA sequence within 5' HS-1, HS-2 and HS-3 is highly conserved. This is a remarkable feature for a non-coding region. The DNA-ase footprinting of these minimal domains revealed the consistent presence of three motifs: GATA, TGAGTCA and GGTGG (or CACCC). The same motifs are represented in the  $\alpha$ -LCR providing strong support for their functional importance.

The GATA-1 (earlier designated as ERYF-1, NF-E1, GF-1) recognizes a consensus GATA motif in cis elements expressed in erythroid specific globin and non-globin genes. GATA-1 is highly restricted to erythroid cells at all developmental stages. It is, however, expressed in megakaryocytes and mast cells as well. This fact points to the possibility that GATA-1 is first activated in a multipotential stem cell that limits its subsequent maturation to the erythroid lineage. Within the erythroid specific promoters, GATA elements appear to cooperate with CACCC boxes in directing specificity. In the DNA-binding domain of GATA-1, the two zinc finger structures of the protein cooperate to achieve high stability, sequence specific interactions. The tips of these fingers make direct contact with the major groove of the DNA, alternate fingers binding on opposite sides of the helix [17]. Targeted mutations at the GATA-1 locus block erythroid differentiation in transgenic mice resulting in severe, frequently in utero fatal anaemia. From the two other GATA-binding proteins, GATA-2 is expressed at a low level in erythroid and non-erythroid cells. GATA-3 mRNA was found to be present at a low level in erythroid cells and at significantly higher levels in brain and T-lymphoid cells. Since GATA motifs have been found in T cell receptor enhancers by cell-specific DNA-ase footprinting, GATA-3 has been suggested to be a transcription factor for T-cell specific gene expression.

In addition to GATA-1, a newly described erythroid factor, NF-E2 has a prominent role. It is suggested that these two factors cooperate to establish the erythroid programme, while additional stage- or gene-specific factors provide a finer level of regulation. It is speculated that another recently revealed erythroid factor, NF-E4, which binds to the adult  $\beta$ -chain promoter and 3' enhancer, may mediate the switch to  $\beta$ -gene expression, while the PAL red cell protein which binds to a palindromic sequence upstream of the  $\beta$ -gene would be responsible for the shutoff of globin expression in mature erythroblasts.

It is a core problem of gene regulation how the interaction of LCR-s with individual genes influence the chromatin structure and maintain an open configuration. The current view of eukaryotic gene transcriptional regulation is based on the finding that a variety of cis-acting DNA sequences are able, both singly or in concert, to enhance or inhibit



initiation of productive transcription. These regulating interactions are also affected by the site specific DNA-binding of different constitutive and tissue-specific regulatory trans-acting factors [18, 19]. Hb has been again the working horse for the elucidation of many newly revealed factors in the regulation of cell-specific gene expression and stage-specific switching.

## **2. Revisiting the Haemolysis of PNH-cells – Phosphatidylinositol-Glycan Anchored Membrane Proteins**

The molecular basis of increased haemolysis was amply studied in a vast number of Hb- and red cell enzyme variants and in mutants of the proteins of the erythrocyte cytoskeleton. This review will be restricted to the discussion of the molecular defect underlying PNH, the unique acquired intrinsic haemolytic anaemia.

It has been known for over 40 years that this syndrome, characterized by intravascular haemolysis and venous thromboses is caused by an increased susceptibility of the patients' cells towards complement. The deposition of large amounts of C3b has been described in the early 1970s. It was suggested to be due to a greater efficiency of the convertase complex on the PNH membrane [20]. It turned out later that the defect originates from the lack of a protein (decay accelerating factor, DAF CD55) that accelerates the disassembly of the bimolecular convertase complexes C3bBb and C4b2a. The deficiency of an other protein, protectin, the membrane inhibitor of reactive lysis (MIRL, CD59) turned out to be even more important in the pathogenesis of PNH. CD59 is missing totally or in part on PNH erythrocytes. The lack of a third protein (C8 binding protein = homologous restriction factor) was added later. Difficulties in the characterization of this last protein led to the suggestion that it might be a polymeric or otherwise altered form of CD59. It has been shown unequivocally that CD59 inhibits the C5b-8 catalyzed insertion of C9 into the lipid bilayer of normal human red cells [21, 22, 23]. The mechanism by which a somatic mutation in the stem cells of PNH cells can induce the lack of CD55 and CD59 was solved by revealing a new type of protein anchoring into the lipid bilayer of the plasma membrane. Most proteins span the cell membrane and are bound by stable interactions between a sequence of 23 to 26 hydrophobic amino acids and the lipid moieties of the bilayer. This very characteristic transmembrane sequence of membrane proteins is preceded by a hydrophilic, charged extracellular part and succeeded by another hydrophilic cytoplasmic tail of the molecule. The recently described alternative way of anchoring proteins to the lipid bilayer involves the attachment of the protein through the charged headgroup of a phosphatidylinositol moiety that is inserted into the lipids of the membrane by the fatty acids of a diacyl glycerol [24]. The carboxyl end of the attached protein is fixed via an ethanolamine-glycan bond to the invariable non-acetylated glucosamine, which is an ultimate member of a series of sugars attached to the phosphatidylinositol moiety. This phosphatidylinositol-glycan (PIG), also called glycoposphatidylinositol (GPI) linkage of proteins renders them an increased lateral mobility in the plane of the membrane. PIG-linked proteins can also be easily removed by phosphatidylinositol specific phospholipases, most frequently phospholipase C (PIPLC). The defect in PNH cells was shown to be posttranslational and to reside in their inability to synthesize the PIG anchor [25, 26]. The defect or defects of

synthetizing the complete anchor is not likely to be absolute in most PNH patients [27, 28], since the presence of the so-called PNH II cells, having an intermediate sensitivity to complement is a characteristic feature of many cases. The increased spectrum of PIG-linked proteins (Table 1) include in addition to the complement regulatory proteins, a number of immune regulatory proteins, adhesive proteins and membrane enzymes. The lack of these membrane proteins explains the increased susceptibility of PNH patients to infections. It explains also two other very characteristic diagnostic features: the decreased level of acetylcholinesterase in the red cells and the low score of alkaline phosphatase in the granulocytes of PNH patients.

The frequent thrombotic events of PNH patients can now be explained by the lack of CD59 which results in the insertion of polymeric C9 into the platelet membrane. This leads to a process by which they are removed in external vesicles without lysis of the platelets. The acidic phospholipids are not kept internalized in these vesicles and become sites of prothrombinase formation with strong procoagulant activity [29, 30].

PNH is a good example of how the disclosure of the molecular defect can explain the protean clinical signs and symptoms of an old known disease entity.

Robinson [31] argues that other symptoms of PNH, like the relatively frequent occurrence of bone marrow aplasia of different degrees and the very rare occurrence of subsequent leukaemia cannot be explained by the absence of PIG-linked proteins. My personal opinion is that these last two features are rather due to a relatively more frequent incidence of the somatic mutation causing PNH in preleukaemic patients with aplastic anaemia or myelodysplasia.

Table 1

Phosphatidylinositol-glycan anchored human membrane proteins of functional importance

*Complement regulatory proteins*

decay accelerating factor (DAF)  
membrane inhibitor of reactive lysis (MIRL)  
C8 binding protein

*Membrane bound enzymes*

acetylcholinesterase (AChE)  
alkaline phosphatase  
alkaline phospho-diesterase  
lymphocyte 5'-ectonucleotidase

*Other*

LFA-3  
proteoglycan sulphate  
FCγ RIII (on neutrophils) CD16, NA-1, NA-2 Ag-s  
CD14 (on monocytes)  
neural cellular adhesion molecule (NCAM)  
carcino-embryonic Ag (CEA)  
prion protein (PrP<sup>Sc</sup>)  
placental p 34 growth factor  
folate binding protein



Endogenous PIG-anchored proteins are apically polarized. This, together with their increased lateral mobility led to the suggestion that they could act as a "dominant" apical signal to determine the final destination of the extracellular domain of a given membrane protein [32]. PIG-anchored proteins like Fc-receptor, DAF, Th-1 are localized to the anterior pole of locomotor lymphocytes. Neutrophils undergo polar reorganization of the membrane proteins as a response to the f-Met-Leu-Phe sequence of chemoattractants. PIG-anchored proteins may act as sorters in the functional reorganization of membrane microdomains. Another possible significance of PIG-anchored proteins in the lymphocyte membrane is to provide Ag-independent costimulatory signals [reviewed in 31].

### 3. Molecular Genetics of the ABO and Rh Blood Group Specificity

The ABO blood groups were discovered by Landsteiner over 90 years ago. The blood group O designated the lack of A and B antigens before H antigen was recognized to be characteristic of blood group O and to be the precursor of both A and B antigens. A, B and H antigens differ from each other in a single sugar residue at the non-reducing end of oligosaccharide chains on glycoproteins and glycolipids. A, B and AB individuals express glycosyltransferases which convert the H antigen into A or B antigens. Blood group O (H) individuals lack such activity. With the cloning and sequencing of the cDNAs for the human A and B glycosyltransferases, and the corresponding cDNAs from cells of H antigen specificity, the differences in the expression of glycosyltransferases in individuals with differing blood groups could be explained in molecular terms. It was shown, that the A and B genes differ in some single-base substitutions and the resulting change in four amino acid residues is responsible for the differing blood group specificity between A and B cells. There is a critical single base deletion in the O gene. This nonsense mutation results in an inactive protein, incapable of modifying the H antigen [33]. It is predicted that a few amino acid substitutions beyond those that are distinguishing the A and B genotypes could be responsible for the known subgroup polymorphisms.

The ABH antigen structures are not confined to red cells, but show differing patterns of expression in various tissues and secretions. In fact, phylogenetic and ontogenetic investigations revealed that the ABH antigens evolved earlier on epithelial cells than on blood cells. It has been recently suggested to be designated as ABO(H) histo-blood group specificity [33]. The function of these antigenic oligosaccharide antigens is unknown, but search for their relevance was stimulated by renewed interest to explain their marked changes during differentiation and oncogenic transformation. In stratified squamous epithelia, A and B cell-surface antigens are acquired at a defined stage of differentiation, and deleted or reduced in malignant or premalignant cells [34, 35].

The RH system is a very complex blood group specificity with multiple antigenic variants. From the simplified scheme of C/c, D/d, E/e alleles, D is the major Rh antigen. The antithetical d is not immunologically detectable and is not expressed, hence "Rh negative". Colin et al. [36] conclude from the results of their most recent investigations that the d protein does not exist at all.



The Rh polypeptides proved to be highly unusual. In contrast to all known blood group antigens and virtually all mammalian proteins, the Rh polypeptides are non-glycosylated. The amino acid composition of Rh polypeptides contain approximately 37% hydrophobic residues. This, together with the finding that RhD antigenic reactivity is lost after RBC membranes are solubilized in detergents show that most of the molecule resides between the leaflets of the phospholipid bilayer. Rh polypeptides contain an extracellular free sulfhydryl that is crucial for Rh specificity. Several intracellular sulfhydryls appear to be palmitylated. Molecular cloning of the genes coding Rh polypeptides was realized by isolating an Rh cDNA using probes derived from the cytoplasmic N-terminal amino acid tail, the sequence of which had been determined by classical sequencing methods and by the use of the polymerase chain reaction (PCR). Rh recombinants were found in low abundance in preparations from adult erythroblasts, HEL and K562 erythroid cell lines and the MEG O1 megakaryocytic line, but not in adult human liver, kidney, Jurkat lymphoblastic or HL60 promyelocytic cell lines [37, 38]. This is in agreement with the previous findings that Rh specificity is restricted to the erythroid cells.

The open reading frame of the cloned Rh cDNA encodes 416 amino acid and corresponds probably to the Rh C/c or E/e sequences. Search of existing data banks did not reveal sequence homology with any known protein. Modelling suggests 13 bilayer-spanning domains with only very short connecting regions extending outside of the cell or protruding into the cytoplasm (Fig. 4). The multiple bilayer spanning structure of the molecule is reminiscent of known membrane transporters. The Rhc D and E polypeptides proved to be closely related, but distinct proteins. Theoretical genetic analyses suggested two separate, but closely linked loci coding for D and CcEe polypeptides which could be the products of a duplicated ancestral gene. According to recent restriction fragment length polymorphism analysis, individuals with Rh D positive red cells seem to have two Rh polypeptide genes, whereas the Rh D negatives have only one gene [39]. It is surmised that the D gene represents an ancient genetic accident during which an ancestral Rh gene was imperfectly duplicated. The evolutionary selection of the D gene originates probably from the reproductive advantage of the Rh D positive mothers. This becomes especially obvious in some genetically isolated populations, such as Japan, where the incidence of the Rh D positive phenotype is 99%.

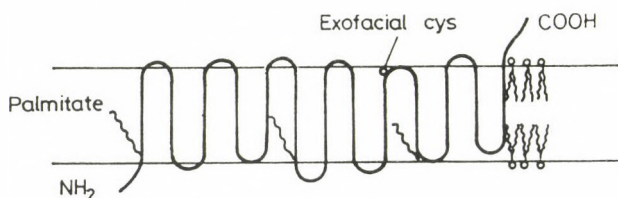


Fig. 4. The Rh polypeptide resides within the membrane lipid bilayer

Rh D is only immunogenic for Rh D negative individuals. The only exception is in the course of "spontaneous" or drug induced autoimmunity. The very rare Rh<sub>null</sub> individuals suffer from a mild or moderate chronic haemolytic anaemia, their red cells

are polymorphic with slight stomatocytosis and sphaerocytosis. The red cells have hyperactive membrane ATP-ases, the cation and water content is reduced, there is a relative deficiency of membrane cholesterol and the phospholipid composition is abnormal. The glycophorin content of the membrane of the Rh<sub>null</sub> cells is reduced to approximately 30% of the normal level and in addition to the Rh antigens some other blood group antigens (Duffy, U, Duclos) are also missing. This all argue for an important role of the Rh polypeptides in membrane integrity. Rh polypeptides appear to be needed for the membrane expression of several glycoproteins. It was also speculated that Rh polypeptides may function as an ATP-dependent phosphatidylserine translocase (PS flippase). This latter function of the Rh polypeptides is now debated [39].

In conclusion, our knowledge of the structure and function of ABO and Rh blood group antigens has increased manifold in the present molecular era of haematology.

#### **4. The Regulation of Stem Cells and the Multistep Process of Their Malignant Transformation**

The most relevant results of recent leukaemia and lymphoma research furthered our understanding of the regulation of normal cell growth and differentiation. It resulted in a deeper insight into mechanisms of cell-cycle regulation, transcription and cellular transformation. No wonder, since malignant cells reflect clonal selection of a cell type frozen in an early, normally infrequent and transitory phenotype [40]. In the development of leukaemias a haemopoietic cell clone escapes from physiological regulation and enters into an infinite growth having lost the ability to differentiate into functioning terminal cells with a programmed cell death.

Malignant cells are characterized by a high level of proliferation and block of differentiation. In preleukaemias there is only one of these two criteria present. In the myeloproliferative disorders there is an expansion of cell growth (proliferation) without the inhibition of differentiation. In contrast, the myelodysplastic syndromes (MDS) are characterized by a clonal inhibition of cell differentiation without increase in cell proliferation. Both preleukaemic syndromes can progress into the definitive malignancy of acute leukaemia (Table 2). Changes in clinical signs and symptoms together with the changing cytogenetic and morphologic pattern of the cells reflect already the multi-step process of malignant transformation. The different steps are initiated by structural changes (mutation, deletion, chromosomal translocation) of a respective oncogene involved in normal cell proliferation, differentiation or the regulation of any other important normal function (i.e. signal transduction). Most of the oncogene mutants (i.e. *myc*, *fos*, *jun*, *myb*, *ras*, etc.) will induce instead of the physiological transient stimulation dominant, permanently increased autocrine production of a growth factor (GF) or constitutive, permanent activation of a GF receptor (GF-R). In contrast to this, the tumor suppressor genes like the retinoblastoma sensitivity gene (RB), p53 and *krev-1* inhibit the development of malignant transformation. They can be inactivated by deletions, mutations, chromosomal translocations. Tumor suppression is a recessive process. A single normal (wild-type) gene can inhibit cancer development. Some oncogene mutants (*bcl-2*, *krev-1*) further the malignant transformation by inhibiting programmed cell death (apoptosis).



Viral proteins may also induce constitutive stimulation of the haemopoietic cells of the infected host by binding as pseudoligands to GF-Rs (molecular mimicry). Virus-mediated transformation is induced, i.e. by the tax gene of HTLV-1 in infected individuals following a 20 to 30 year incubation.

Table 2

Multi-step development of the Malignant transformation in myeloproliferative syndromes and myelodysplasia

Preleukaemia	Molecular Genetic Defect		Definitive Malignancy
	Proliferation	Differentiation	
Myeloproliferative syndromes	increased	normal	acute leukaemia
Myelodysplasia	normal	blocked	acute leukaemia

Advancement in the isolation and characterization of the pluripotent haemopoietic stem cell (HSC) has furthered our understanding of its related malignancies. The definition of a stem cell is still based on its capacity for extensive self-renewal and retention of multilineage differentiation potential. The finding that populations enriched for CD34 surface marker have the ability to form a variety of colonies in semisolid media in response to different lineage specific and non-specific GFs enabled the investigation of the regulation of normal haematopoiesis and its disruption in the development of leukaemias. The primitive HSCs are characterized in addition to the very high expression of CD34 by dull staining with rhodamine 123, low or no expression of HLA-DR and CD71 and relative insensitivity to 4-hydroxyperoxycyclophosphamide [41].

Eighteen distinct haemopoietic GFs have now been cloned and characterized. These control the production, differentiation and functional activity of the eight major blood cell lineages generated by the ancestral HSCs. It was shown recently that more than one factor controls cells in any one lineage and most factors are active on cells of more than one lineage [42]. Just to mention two examples: a) neutrophil production can be stimulated, at least in vitro, by the colony-stimulating factors, G-CSF, GM-CSF and IL-3 (multi-CSF) and by interleukin-6 (IL-6) and the stem cell factor (SCF), also called c-kit ligand and Steel factor, b) eosinophil production can be stimulated by IL-5, GM-CSF and IL-3.

This redundant and pleiotropic action of GFs originates from a subunit promiscuity among their receptors [43], the molecular basis of which resides in the fact that most of the haemopoietic GF-Rs or at least one of their subunits belong to the same superfamily [44]. They share conserved amino acid sequence elements in their ligand-binding extracellular domains and are suggested to have similar double  $\beta$ -barrel steric structure [45]. The human independent specific binding chains, ( $\alpha$ -subunits) for GM-CSF, IL-3 and IL-5 have been cloned and shown to bind only to their respective ligands with a low affinity. Coexpression of each  $\alpha$ -chain with a distinct new member of the growth factor superfamily, KH-97 (the  $\beta$ -chain) results in the formation of high affinity receptors. A cross-competition for the formation of high-affinity receptors (depending on the relative



affinity of each  $\alpha$ -chain for the common  $\beta$ -chain) has been proposed [43]. The suggestion of Kitamura et al. [46] that cell signaling is mediated by the common  $\beta$ -chain could easily explain the common biological actions of haemopoietic growth factors.

The development of reproducible methods of continuous bone marrow culture and techniques for the study of single cell interactions between haematopoietic and stromal cell in vitro enabled to detect by the use of a variety of molecular probes the expression of relevant haemopoietins, cell surface receptors and lineage specific differentiation markers for both haemopoietic and stromal cells in the bone marrow [reviewed in 47]. Cells are present in the normal bone marrow at a concentration high enough ( $2 \times 10^9$  cells/mL) to permit physical contact between developing and other haemopoietic cells and between haematopoietic and stromal cells. Cell-cell contacts and specific effects of the extracellular matrix are involved in the regulation of cells by binding GFs to signal molecules on the surface of the cells. Recent results of several research groups identified a novel growth factor produced by bone marrow stromal cells and other fibroblastic cell lines. It has a pleiotropic regulatory activity involved in haematopoiesis, gamatogenesis and melanogenesis. The protein has been named according to its various functions as mast cell growth factor (MGF) haemopoietic stem cell factor (SCF) and growth factor kit ligand, (KL). C-kit is a member of the tyrosine kinase receptor class of proto-oncogenes and has a decisive role in development of primordial germ cells. The binding of SCF to C-kit induces potent stimuli for growth and development of different haemopoietic cell lines [48, 49]. The gene coding for human SCF (kit ligand, KL) has been cloned and mapped to chromosome 12q 22–24. It was found active in both membrane bound and soluble form. The two forms result from different splicing [50, 51]. The effects of the soluble SCF are primarily manifest in combination with other haemopoietic GFs. The concentration of GFs required for colony formation by haemopoietic progenitors is reduced by 1 to 2 orders of magnitude in the presence of recombinant SCF. The ability of SCF to affect primitive cells distinguishes it from previously described GFs. Preliminary reports indicate that SCF improves the radiation tolerance of mice and may enhance engraftment in bone marrow transplantation [52]. C-kit-R expressing human HSCs could be isolated from bone marrow, peripheral blood and foetal liver by using immunoadherence (panning) to an Ab against human SCF [53].

More and more is learned about factors regulating the overall cellularity of the bone marrow and the "cell-sociology" of defined zones, the physical "niches" for stem cell replication. There is a growing literature on specific negative regulators of the stem cell and the lineage committed precursors, like the transforming growth factor  $\beta$  (TGF- $\beta$ ) [54, 55], small peptides [56, 57] and the inhibition of stem cells by large granular lymphocytes [58].

Following several decades of frustrating clinical trials to induce in vivo differentiation of malignant cells, the impressive therapeutic effects of cis-retinoic acid (cis RA) and all-trans retinoic acid in acute promyelocytic leukaemia (APL) gave rise to new hopes for a cure of malignant diseases. The first observations of Huang et al. [59] had been fully supported by the results of French clinical trials [60]. Seventy to 80% of APL patients have been reported to attain complete remission within 30 to 90 days or more of oral treatment with cis-RA. Complete remission was induced without the imposition of bone marrow hypoplasia, the high risk consequence of the present cytotoxic therapy. Many responses lasted only some months, but several lasted a year or more. The t(15;17)



(q22;q11-q21) translocation, a characteristic cytogenetic defect in APL has recently been shown to disrupt the retinoic acid- $\alpha$  receptor (RAR $\alpha$  or RARA) gene localized on band 17q 21, a region close to the generally accepted chromosome break-point localization. This cytogenetic translocation was found in 20 of 21 patients with APL before treatment and disappeared during complete remission.

RAR $\alpha$  belongs to the steroid/thyroid nuclear receptor family, the members of which are related to the viral oncogene *erb-A*. It has been recently suggested that *v-erb-A* oncogene function in neoplasia correlates with its ability to repress retinoic acid receptor action by promiscuously interfering with a retinoid-mediated differentiation process [61]. Attempts to demonstrate the role of the retinoic acid receptor subunits (RAR $\alpha$  and RAR $\beta$ ) gave controversial results. RAR $\alpha$  appears to be expressed in most human leukaemic cells regardless of the type of biologic response to retinoic acid [62]. Serum inhibitors of retinoids found in normal and leukaemic sera [63] may modulate the antileukaemic activity of retinoids.

There are still a lot of question marks in the anti-leukaemic action of retinoic acid. To get a better insight into this process, further studies are needed. It has even been suggested that APL may turn out to be another pseudo-leukaemia, just like pernicious anaemia had been thought to be once [64].

Although complete remissions accomplished by oral transretinoic acid treatment may be brief and relapses do occur in most patients, it still gives a ray of hope for more effective and causal treatment of malignancies by overcoming the block of differentiation of the malignant clone without toxic effects on other proliferating cells of the organism. Based on this hope, a number of new protocols combining physiological inducers with different GFs have been started, i.e. the combination of retinoic acid with G-CSF, or that of vitamin D3 with M-CSF. Terminal differentiation of an acute myeloid cell line (AML-193) could be achieved *in vitro* by these combinations [65].

New therapeutic perspectives have been opened up by experiments on transgenic mice in which the malignant process could be contained by the transplantation of tumor suppressor gene. To stop the constitutive activation of a dominant oncogene will be a much more difficult task. The potent rDNA technologies provide, however, a number of possibilities to achieve this goal, i.e. the use of site-directed mutagenesis, modification of the oncogene expression by the transfection of anti-sense oligonucleotide information, inhibition of the development of the functional steric structure of the activated oncogene product (like inhibition of dimerization). Experiments with transplantation of tox genes and drug-sensitivity genes into tumor cells are of great importance, too.

## 5. The Impact of Gene Technology on Diagnostics and Therapy in Haematology

The use of gene probes and other rDNA methods have and will have a decisive impact in haematological diagnostics, in tissue typing, in the detection of oncogene activation and in disclosing subtle changes which cannot be detected by cytogenetic methods. It has most important clinical applications in carrier and antenatal diagnostics of inherited defects.

From the wealth of new possibilities just two will be mentioned to demonstrate the power of rDNA methods:



a) *The polymerase chain reaction (PCR)* can detect the presence of a single malignant cell amongst a million normal ones. This has a crucial importance in the detection of the so-called minimal residual disease and the early detection of a leukaemic relapse. This can enable an earlier, more targeted, and hence more effective application of therapeutical interventions. The use of this technique is also a powerful aid to monitor the effectiveness of therapy and to adjust it to the real needs of the patient.

The use of PCR was most widely studied in CML, where i.e. in the follow-up of patients treated with IFN $\alpha$  or following allogeneic transplants, PCR could detect bcr-abl transcripts in a number of those patients who had complete cytogenetic response. The method is also powerful in monitoring the efficacy of purging bone marrow for autologous transplantation.

In the newly developed CDR III PCR generic oligonucleotide primers are used to amplify DNA regions which are frequently rearranged in Ig regions in B cell malignancies and in T cell receptor domains in T cell leukaemias and lymphomas. Consensus sequences of the patients are sequenced and patient specific oligonucleotide probes are developed. CDR III PCR allows not only a most sensitive detection of malignant cells, but in addition enables the quantification of the clonal cells in relation to normal B or T cells.

b) *New approaches for the production of monoclonal antibodies (mAbs)* open up new horizons in haematological diagnostics and therapy. As a result of gene technology, Ab genes can now be altered to order. The ready manipulation of Ig genes by cutting and joining restriction fragments or by the use of site-directed mutagenesis enabled the construction of new antibody reagents and fine-mapping of Ab structure-function relationships.

In the most recent approaches, the hybridoma technique can be bypassed. In "classical" Ab engineering, hybridomas of known specificity have provided the raw material for cloning the rearranged V<sub>H</sub> and V<sub>L</sub> genes. However, the use of "universal" primers and the PCR enables the rescue of V genes. By building restriction sites into the primers, the amplified DNA can be cloned directly for expression in mammalian cells or bacteria. Hybridoma technology can immortalize the Ab-producing hybrid cells, gene technology can immortalize their genes.

Human mAb can be produced in transgenic mice. Gene technology offers new ways for "humanizing" specific rodent Ab-s by transplanting the Ag-binding loops to human Ab-s [66].

*The genetic engineering of human proteins* has enriched the treatment of haematological diseases by a number of new possibilities. Most of the clinically important proteins are already cloned and a number of them, like the recombinant plasminogen activator, Factor VIII, interferon  $\alpha$  and  $\gamma$  (IF $\alpha$  and IF $\gamma$ ), interleukin 2 (IL-2), tumor necrosis factor (TNF) and the haemopoietic growth factors: erythropoietin, G-CSF, GM-CSF and IL-3 are already in clinical application. Hairy cell leukaemia was the first indication field of IF $\alpha$  in haematology. The clinical experience of the last few years rendered evidence of its efficacy in the treatment of chronic myeloid leukaemia and it is under trial in other myeloproliferative disorders, as well as in myeloma and in progressive haemangiomatosis. GM-CSF alone or in combination with IL-3 and in some trials with IL-6 can shorten significantly the aplasias following high dose chemotherapy and irradiation in the treatment of malignancies or bone marrow transplantation. This is



of paramount importance since most patients die as a consequence of severe infections and/or bleeding during these aplasias. The rSCF will most probably have a number of important clinical uses as a mediator of early haematopoietic cell proliferation: 1) to shorten the critical interval between myelosuppressive therapy and haematological reconstitution, 2) to facilitate the harvesting of blood cell progenitors for transplantation, 3) to recruit early progenitors into cell cycle to make them more transducible targets for retrovirus-mediated gene transfer, 4) in combination with other growth factors to magnify the decreased clinical effects and reduce the dose-related toxicities of these other cytokines by reducing their required dose, 5) in disease caused by defects of the early progenitor cells SCF in combination with different growth factors could enable the induction of cellular responses tailored to the individual patient's need in these very protean clinical syndromes and 6) since SCF can expand early T and B cell populations, it may have a role in the treatment of diseases caused by lymphocyte depletion, among others in HIV infection.

It is also hoped that studies with the rSCF will yield further insight into interactions between HSCs and their microenvironment and further a better understanding of the development and function of melanocytes, mast cells, germ cells and neural tissues.

## **6. The Present State of Art and Future Possibilities of the Treatment of Genetic Diseases**

By now, more than 4000 different inherited gene defects are known. The vast majority of them cannot be cured today. It follows that the main efforts in clinical medicine have to be directed towards the prevention of inherited diseases. This can be achieved by early diagnosis, the detection of carriers and by establishing up-to-date well-organized programmes for the prenatal diagnosis of genetic disease in high risk groups of populations. Major programs have to include adequate public education. Such well-organized programs for the prevention of thalassaemias have rendered evidence of a remarkable reduction in the birth of severely affected homozygotes [67, 68].

The treatment of genetic diseases is still restricted to the prevention of clinical symptoms by well organized programs for patient care and replacement of the deficient gene product in defects where this is feasible (i.e. insulin, FVIII, FIX, etc.).

Research programs targeted to the reactivation of foetal genes i.e. in sickle cell anaemia and in some forms of thalassaemias are still in early experimental phase.

Bone marrow transplantation proved to be effective in defects residing in HSCs. The application of this type of treatment is, however, still restricted, due a.) to the lack of suitable donors, b.) to the high incidence of sometimes fatal complications and c.) to its very high costs.

No wonder that attempts to treat genetic diseases by introducing healthy genes into the body aroused great public interest. Different attempts have been made in animal experiments to transfer genes directly by physical methods. These routes of gene transplantation turned out to be ineffective. The only promising approach so far is the use of retroviral vectors. Recombinant infectious viruses are able to express the transferred gene after integration into the host cell genome. The potential of generating replication-competent virus has to be excluded. This problem seems to be effectively



solved with improved vector and packaging design [69]. Retroviral vectors have, however, two other disadvantages. They can only be produced from living cells and thus may carry unwanted contaminants that are difficult to remove. In addition, the capacity of retroviral vectors is limited, up to about 7 kb, which creates additional problems in case of large genes. Random insertion of new genes carries also the risk of either activation or inactivation of important genes at the site of integration, which could result in cell death, oncogenic transformation or other alterations in function. To date, no major problems have been observed in experimental studies, still substantial further work is needed to assess the relative risks and benefits of gene transplantation.

Gene should be transferred only into somatic cells. Germ-line therapy: the introduction of genes into fertilized eggs raise severe ethical problems since germ cells will pass on the experimentally introduced genes to future generations.

The results of experiments showing that self-renewing and pluripotent stem cells can be transformed [70] are very encouraging. It turned out, however, that by using a variety of promoters for the transfected gene although excellent expression can be obtained in haemopoietic cell lines and fibroblasts only a very low level of expression can be achieved in primary murine haemopoietic cells. When bone marrow cells are transfected, the pluripotent stem cells make up only a small proportion and most of the haemopoietic stem cells are out of cycle and this hinders successful retroviral transfection. The results have been improved by the addition of IL-1, IL-3 and IL-6, but it did not completely solve the problem. The recent discovery of major regulatory elements which direct high level tissue specific expression of globin genes and those involved in the regulation of stem cell proliferation have raised new perspectives to overcome these difficulties.

Taking into account the aforementioned still unresolved problems, the following prerequisites have been set for the first human gene transplantation trials:

- the gene should be cloned
- it should not need cell specific regulation, i.e. the gene should be present in most cells (it should be a so-called "house-keeping" gene)
- even low level gene products should function efficiently
- the target cell to be infected should be able to survive the manipulation
- it should have a fairly long lifespan
- it should be easily removed from and returned into the body

The best candidate for the first human therapeutical gene transplantation has been the adenosine deaminase deficiency. This enzyme is present in virtually all cells, but its deficiency causes severe functional defect only in lymphoid cells, primarily in immature T cells, eliciting severe combined immunodeficiency (SCID). A matched bone marrow transplantation is curative for ADA-deficient SCID, while the success rate of mismatched bone marrow transplants is disappointingly low. The first human gene therapy experiment was approved by the National Institutes of Health Recombinant DNA Advisory Committee in early autumn of 1990. The gene transplantation was carried out in a child suffering from SCID caused by ADA-deficiency. The lymphocytes of the child were removed by lymphocytapheresis. The ADA-deficient T cells were activated by OKT3 and grown in culture in the presence of IL-2. After one day in culture, the cells were transduced with a retroviral vector, LASN, which carries a copy of a normal human ADA gene. The gene converted T cells are then expanded to about  $2 \times 10^{10}$  cells

and reinfused to the patient. Due to the limited life span of the lymphocytes, the procedure is being repeated monthly. In addition, the child also receives PEG-ADA injections (the polyethylene glycol increases the half life of the enzyme). At present (December, 1991) two children are being treated at NIH (Bethesda, MD, USA) on the ADA gene therapy protocol. There have been no significant side effects from the treatment. T cells expressing ADA were isolated from the peripheral blood of the patients. Marked improvement in the clinical and immune function status has been reported [71].

This first approved therapeutical gene transplantation was based on the methods and results of the first approved human experiment which was aimed to follow the path and the survival of tumor infiltrating lymphocytes (TILs) in terminally ill cancer patients [72]. It is forecasted that the next approval for therapeutical gene transplantation will be given to those experiments in pipeline which are aimed to integrate the genes for "anticancer" cytokines (TNF, IL-2+TNF, IL-2+IL-4+TNF) into TILs [73].

The successful retroviral transfer of genes into keratinocytes, hepatocytes, endothelial cells and fibroblasts opened up new ways for the treatment of genetic disorders. Fibroblasts are especially attractive candidates because they are easily accessible and the altered cells can be implanted into the dermis. They can secrete the initially lacking substances into the blood and can also easily be removed if necessary. By using retrovirus mediated transfer, human fibroblasts have been shown to make biologically active ADA, glucocerebrosidase, purine nucleoside phosphorylase, low density lipoprotein (LDL) receptors and factor IX, even though they do not synthesize these latter proteins. Experimental results have also demonstrated the feasibility of retrovirus mediated transfer of human FVIII gene and its amplification to increase expression levels [74]. These experiments also gave evidence that skin fibroblasts can be engineered to produce those proteins that require post-translational modification and secretion for activity [75].

The advantage of vascular endothelial cells as targets for gene therapy resides in their direct access to circulation. It is planned to insert genes coding for anticlotting agents into the endothelial lining of vascular grafts to inhibit local thrombosis [76].

The presently applied retrovirus-based gene delivery vehicles have a number of inherent disadvantages (unforeseen deleterious side effects of cancer induction or virus infection in a small percentage of patients, immune response against the intrinsic retroviral antigens, and the irreversibility of the procedure in the case of undesirable side effects). This together with the difficulties in the manufacturing scale-up initiated research to find ways for the direct delivery of purified genes *in vivo*, and their application as drugs without the use of retroviruses [77].

## 7. Conclusions and the Horizons of Progress

This review was aimed to demonstrate by arbitrary chosen examples the fundamental impact of molecular genetic research in haematology. The number of examples had to be limited and therefore could not encompass such very fast moving fields as the molecular basis of lymphocyte, granulocyte and platelet function and the molecular



mechanisms underlying the processes of haemostasis. The exciting field of how cells talk to each other has recently been reviewed in this journal [78]. In this review, six quickly expanding fields of molecular genetics are discussed:

- 1.) the regulation of the expression of tissue and age specific genes,
- 2.) a newly disclosed type of membrane anchoring of proteins,
- 3.) the molecular basis of blood group specificity,
- 4.) the regulation of haemopoietic stem cells in health and disease,
- 5.) examples for the unprecedentedly fast advances in diagnostics, prevention and treatment of haematological diseases, and
- 6.) the present state of art and the future horizons of the therapy of genetic diseases.

1.) The recent progress in the understanding of haemoglobin regulation and switching has provided fundamental insight into a core problem of eukaryotic biology: the long distance influence of LCR elements on chromatin structure and gene expression. The disclosure of critical transcriptional regulators of gene expression in erythroid cells forms the basis for analysis of other cell specific and developmentally regulated gene loci. These recent results are absolute prerequisites for the development of new therapeutic strategies in haematology and oncology.

2.) The recent finding that certain cell surface proteins undergo post-translational modification to form a PIG-anchor as their only means of attachment to cell membranes has opened a new exciting field of research. Recent evidence has suggested that PNH is caused by a defect in the synthesis of this anchor. The PIG-anchored proteins occur in a wide range of eukaryotic cells. They may play an important function in the polar reorganization of membrane proteins, i.e. as a response to ligand binding in lymphocytes and as an effect of chemoattractants in granulocytes. Another possible significance of PIG-attached proteins in the lymphocyte membrane is to provide Ag-independent co-stimulating signals.

3.) Our knowledge of the structure and function of the ABO histo-blood group and the red cell specific Rh antigens has increased powerfully in the present molecular era of haematology. Four single base differences between the A and B genes are causing the differences in the A and B glycosyltransferases that convert H antigen to A and B. The critical single base deletion in the O gene results in a protein incapable of modifying the H antigen. The function of ABO(H) antigens is unknown. Their changes during ontogenesis and oncogenesis attract great interest.

The Rh polypeptides have been shown to have a highly unusual structure. They contain no carbohydrates. Most of the polypeptide resides between the leaflets of the phospholipid bilayer. Its multiple bilayer spanning domain structure is similar to known membrane ion transporters. The Rh polypeptides are probably required for normal membrane integrity and for the membrane expression of several glycoproteins.

4.) Recent advances in molecular haematology helped to define pluripotent stem cells and their regulation. Cell cycling of haemopoietic stem cells (HSCs) has to be tightly regulated. Recent data suggest a finely tuned regulation in which previously described growth factors and the recently cloned and characterized stem cell factor (SCF) act in combination with each other in a concert with other positive and negative regulators adapting proliferation and differentiation to the actual need of the organism.



The malignant transformation of HSCs and progenitor cells is a multistep process. The different steps are initiated by mutations, deletions and chromosomal translocations of respective cellular oncogenes involved in normal cell proliferation, differentiation, signal transduction, etc. Most of the oncogene mutants will induce dominant autocrine production of growth factors or constitutive activation of a growth factor receptor (GF-R). Other oncogenes act as recessive tumor suppressors. Some of these further the malignant transformation by inhibiting the programmed cell death (apoptosis). Viral proteins may also induce constitutive stimulation of the haemopoietic cells of the infected host.

The malignant process could be contained by transplantation of a suppressor gene to transgenic mice. It is hoped that the potent rDNA technologies will provide possibilities to stop the dominant constitutive activation of mutant oncogenes as well.

The impressive results of achieving complete clinical, haematological and cytogenic remission in acute promyelocytic leukaemia patients by oral treatment with retinoic acid without imposition of bone marrow hypoplasia gave rise to new hopes for overcoming the block of differentiation of the leukaemic clone by combining retinoic acid with different GFs.

5.) The use of the polymerase chain reaction (PCR) and the tailor to order production of mAbs by advanced gene technology represent infinite possibilities to refine methods for diagnostics, prevention and treatment of haematological diseases. A growing number of agents of potential or already proven clinical use have been produced by rDNA technology. In this review, the molecular basis of GF regulation and horizons of the clinical use of haemopoietic GFs are discussed. Special attention is drawn to the paramount importance of the recently cloned SCF and to the future possibilities of its clinical use, especially in combination with other GFs.

6.) Due to the lack of cure of genetic diseases, medical efforts have to be concentrated on their prevention by well-functioning combination of antenatal diagnostics with educational programs. The present state of the treatment of genetic diseases is restricted to the prevention of clinical symptoms by multidisciplinary patient care and by the replacement of the deficient protein where this is feasible. Bone marrow transplantation proved to be effective in defects residing in the HSCs. Its use is and will be restricted by the lack of matching donors, by the risk of severe, often fatal complications and by the very high cost of the procedure.

Retroviral mediated gene transfer has been demonstrated to be an efficient method of inducing gene sequences into mammalian cells. The potential risk of generating replication competent viruses seems to be largely excluded with improved vector and packaging design. The use of retroviral vectors carries, however, other inherent risks. Substantial work is still needed to assess the relative risks and benefits of this type of gene transplantation. HSCs have to be recruited into cell-cycle to achieve their successful retroviral mediated transfection. The addition of IL-1, IL-3 and IL-6 improved the results, but could not completely solve the problem. It is hoped that the combination of rSCF with these or other GFs will bring success.

The recent discoveries of the major regulatory elements which direct high level tissue specific expression of globin genes and those involved in the regulation of stem cell proliferation raised new perspectives to overcome the difficulties of effective gene transplantation into erythroid precursor cells and HSCs.



The present prerequisites for approval of human gene transplantation and the first approved therapeutic human gene transfer is described. The successful transfer of genes into keratinocytes, hepatocytes, endothelial cells and especially into fibroblasts opened up new horizons for the treatment of genetic disorders. Fibroblasts are easily accessible, the transfected cells can be transplanted into the skin and can easily be removed if needed. There is experimental evidence that fibroblasts can be engineered to produce a number of enzymes, LDL-Rs and FIX, while transfected endothelial cells are able to produce FVIII, and the level of the expression of the transfected gene can be amplified. Most recent experimental research is focussed to find ways for the direct delivery of purified genes in vivo and their application as drugs without the use of retroviruses.

In spite of the challenging perspectives opened up by experimental research in clinical trials, the top priority has to be given to the assessment whether it is feasible to correct a monogenic disease with complete safety. The horizons of gene transplantation surpass over and above this goal. Animal experiments are in way to insert genes for anticlotting agents into the endothelial lining of vascular grafts and to transplant genes for LDL receptors into liver cells to reduce cholesterol level.

I am convinced that this review is not a light diet for those who work in clinical practice of haematology. My only excuse is that the recent explosion in the knowledge of human genetics increases further in an exponential way. At the same time, many of the research results have already proven to be of clinical value. The diagnostic reagents and the drugs to be used will include in the near future a knowledge of the primary sequences of the organism's genes, together with the results of all previous deductions of that sequence. For the past 15 years the DNA databases have grown by 60 per cent annually and the sequence data for the total human genome is expected to be available in the near future. The flood of this knowledge poured across computer networks will definitely change the everyday medical practice. As a consequence, we have to learn nilly-willy the language and the most important principles of molecular genetics to be able to synthesize the laboratory data with the clinical signs and symptoms of our patients and to be able to use the best and most reliable methods for their treatment.

Molecular biology is unraveling the intricate architecture and complex dynamics of the operation of the exquisitely refined networks regulating the functions of the organism in health and disease. At the moment, we have only a partial picture. Many details of the landscape are cloudy, but the tips of the icebergs are clearly visible and permit correlations on a much higher level between molecular genetics and pathogenesis of a number of diseases. We are in the fortunate position of having the majority of the tips of icebergs in the field of haematology.

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## Abstracts

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*Activation of intracellular calcium-activated neutral proteinase in erythrocytes and its inhibition by exogenously added inhibitors.* M. Hayashi, M. Inomata, Y. Saitok, H. Ito and S. Kawashima (Department of Biochemistry, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan). *Biochim. Biophys. Acta* 1094, 249 (1991).

Intracellular calcium-activated neutral proteinase (CANP) in rabbit erythrocytes was activated by an influx of  $\text{Ca}^{2+}$  into the cells. The catalytic large subunit changed from the original 79 kDa form to the 77 kDa and 76 kDa forms on activation just in the same manner as occurs in the autolytic activation of purified CANP *in vitro*. The activation required both extracellular  $\text{Ca}^{2+}$  and A23187, and was accompanied by the degradation of some membrane proteins and morphological changes in erythrocyte shape from discocytes to echinodisks, echinocytes, and spherocytes. Exogenously added Cbz-Leu-Leu-Leu-aldehyde inhibited the activation of intracellular CANP as well as the degradation of membrane proteins and the morphological changes indicating that the latter two processes are due to the action of CANP. Leupeptin and E64d were without effect on intracellular CANP.

B. Sarkadi

*Protein kinase C phosphorylates the carboxyl terminus of the plasma membrane  $\text{Ca}^{2+}$  ATPase from human erythrocytes.* K.K.W. Wang, L.C. Wright, C.L. Machan, B.G. Allen, A.D. Conigrave and B.D. Roufogalis (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada). *J. Biol. Chem.* 266, 9078 (1991).

Purified  $\text{Ca}^{2+}$ -stimulated,  $\text{Mg}^{2+}$ -dependent ATPase ( $\text{Ca}^{2+}$ -ATPase) from human erythrocytes was phosphorylated with a stoichiometry of about 1 mol of phosphate/mol of ATPase at both threonine and serine residues by purified rat brain type III protein kinase C. In the presence of calmodulin, the phosphorylation was markedly reduced. Labeled phosphate from [ $\gamma$ - $^{32}\text{P}$ ]ATP was retained on an 86-kDa calmodulin-binding tryptic fragment of  $\text{Ca}^{2+}$ -ATPase but not on 82- and 77-kDa non-calmodulin-binding fragments. Similarly, fragmentation of the phosphorylated  $\text{Ca}^{2+}$ -ATPase by calpain I revealed that calmodulin-binding fragments (127 and 125 kDa) retained phosphate label whereas a non-calmodulin-binding fragment (124 kDa) did not. The calmodulin-binding domain, located about 12 kDa from the carboxyl terminus of the  $\text{Ca}^{2+}$ -ATPase, was thus located as a site of protein kinase C phosphorylation. A synthetic peptide corresponding to a segment of the calmodulin-binding domain ( $\text{H}_2\text{N-R-G-L-N-}$

R-I-Q-T-Q-I-K-V-V-N-COOH) was indeed phosphorylated at the single threonine residue within this sequence. The additional serine phosphorylation site was carboxyl terminal to the calmodulin domain. Phosphorylation by purified type III protein kinase C (canine heart) antagonized the calmodulin activation of the  $\text{Ca}^{2+}$ -ATPase, particularly at lower  $\text{Ca}^{2+}$  concentrations (0.2–1.0  $\mu\text{M}$ ). By contrast, a purified but unresolved protein kinase C isoenzyme mixture from rat brain stimulated the activity of  $\text{Ca}^{2+}$ -ATPase prepared in aolec-tin, but not glycerol, by more than 2-fold in the presence of the ionophore A23187, without increasing its  $\text{Ca}^{2+}$  sensitivity. The results clearly indicate that human erythrocyte  $\text{Ca}^{2+}$ -ATPase is a substrate of protein kinase C, but the effect of phosphorylation on the activity of the enzyme depends on the isoenzyme form of protein kinase C used and on the lipid associated with the  $\text{Ca}^{2+}$ -ATPase.

B. Papp

*Influence of associated lipid on the properties of purified bovine erythrocyte acetylcholinesterase.* C.P. Nichol and B.D. Roufogalis (Laboratory of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, B.C., Canada). *Biochem. Cell Biol.* 69, 154 (1991).

Acetylcholinesterase has been isolated from bovine erythrocyte membranes by affinity chromatography using a m-trimethyl-ammonium ligand. The purified enzyme had hydrophobic properties by the criterion of phase partitioning into Triton X-114. The activity of the hydrophobic enzyme was seen as a slow-moving band in nondenaturing polyacrylamide gels. After treatment with phosphatidylinositol-specific phospholipase C, another form of active enzyme was produced that migrated more rapidly toward the anode in these gels. This form of the enzyme partitioned into the aqueous phase in Triton X-114 phase separation experiments and was there-

fore hydrophilic. The hydrophobic form bound to concanavalin A in the absence of Triton X-100. As this binding was partially prevented by detergent, but not by  $\alpha$ -methyl mannoside, D-glucose, or myo-inositol, it is in part hydrophobic. Erythrocyte cell membranes showed acetylcholinesterase activity present as a major form, which was hydrophobic by Triton X-114 phase separation and in nondenaturing gel electrophoresis moved at the same rate as the purified enzyme. In the membrane, the enzyme was more thermostable than when purified in detergent. The hydrophobic enzyme isolated, therefore, represents a native form of the acetylcholinesterase present in the bovine erythrocyte cell membrane, but in isolation its stability becomes dependent on amphiphile concentration. Its hydrophobic properties and lectin binding are attributable to the association with the protein of a lipid with the characteristics of a phosphatidylinositol.

B. Papp

*Monomeric erythrocyte band 3 protein transports anions.* S. Lindenthal and D. Schubert (Max Planck Institute of Biophysics, D-6000 Frankfurt am Main, F.R.G.). *Proc. Nat. Acad. Sci.* 88, 6540 (1991).

The anion transport system of the human erythrocyte membrane was reconstituted in egg phosphatidylcholine membranes by using either the unmodified transport protein, band 3, or covalently crosslinked band 3 dimers. Unilamellar vesicles of a diameter of  $32 \pm 3$  nm were then isolated from the sample by passage through a French press and subsequent gel filtration. According to sedimentation equilibrium measurements, around 85% of the vesicles were devoid of protein. The remaining 15% contained either a single band 3 monomer or, when crosslinked band 3 protein was used, a single band 3 dimer. Vesicles containing either single monomers or single dimers showed a rapid, inhibitor-sensitive



sulfate efflux, and the turnover numbers of band 3 for the inhibitor-sensitive flux component were identical in both systems. This shows that monomeric band 3 protein is able to transport anions and that dimerization of the protein does not change its transport activity.

B. Sarkadi

*Okadaic acid inhibits activation of K-Cl cotransport in red blood cells containing hemoglobins S and C.* E.P. Orringer, J.S. Brockenbrough, J.A. Whitney, P.S. Glosson and J.C. Parker (Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA). *Am. J. Physiol.* 261, 0591 (1991).

The sensitivity of red blood cells containing hemoglobins S and C to activation of K-Cl cotransport by osmotic swelling and acidification was reduced by okadaic acid, a specific protein phosphatase inhibitor. The dose-response curve for okadaic acid suggests its action is on a type 1 protein phosphatase. Okadaic acid has been previously shown to inhibit swelling-induced activation of K-Cl cotransport in red blood cells from rabbits, normal humans, and dogs. The present work confirms the observation that okadaic acid blunts the stimulation of K-Cl cotransport by cell swelling. The new information is that okadaic acid reduces the effects of hemoglobins S and C on the volume and pH sensitivity of K-Cl cotransport. Thus the influences of cell volume, pH and mutant hemoglobins may all be mediated via a common mechanism that affects the phosphorylation state, either of the K-Cl cotransporter itself or of a protein that regulates its function.

B. Sarkadi

*Reconstitution of  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  transport in erythrocyte membrane vesicles requires a cytoplasmic protein.* R.B. Moore,

M.V. Mankad, S.K. Shriver, V.N. Mankad and G.A. Plishker. (Comprehensive Sickle Cell Center, University of South Alabama, Mobile, Alabama, USA). *J. Biol. Chem.* 266, 18964 (1991).

Calcium-dependent potassium transport in erythrocytes requires the participation of a cytoplasmic protein. Activation of calcium-dependent potassium transport causes an increase in the membrane-bound levels of this protein which is dependent on the calcium concentration and which is highly correlated ( $r = 0.791$ ,  $p < 0.0001$ ) with the loss of potassium. Reconstitution of this transport pathway in sonicated erythrocyte membrane vesicles was achieved only in vesicles containing the cytoplasmic protein indicating a causal relationship in this transport system. The protein is found in high levels within the cytoplasm of erythrocytes (5.6 mg/ml red blood cells) and yet less than 1% of the protein located in the cytoplasm is required to bind to the membrane in order to initiate the potassium efflux. The analysis of rat organ homogenates demonstrated that this protein is located in most tissues with particular enrichment in adrenal glands, brain, lung and blood. These results demonstrate that there is a cytoplasmic protein, herein named calpromotin, which is a necessary and sufficient cytoplasmic component of calcium-dependent potassium transport in erythrocytes and perhaps other tissues.

B. Sarkadi

*Red cell volume regulation: the pivotal role of ionic strength in controlling swelling-dependent transport systems.* R. Motais, H. Guizouarn and F. Garcia-Romeu (Laboratoire Jean Maetz, Département de Biologie Cellulaire et Moléculaire du C.E.A., Villefranche-sur-Mer, France). *Biochim. Biophys. Acta* 1075, 169 (1991).



A volume increase of trout erythrocytes can be induced either by  $\beta$ -adrenergic stimulation of a  $\text{Na}^+/\text{H}^+$  antiport in an isotonic medium (isotonic swelling) or by suspending red cells in a hypotonic medium (hypotonic swelling). In both cases cells regulate their volume by a loss of osmolytes via specific pathways. After hypotonic swelling several volume-dependent pathways were activated allowing  $\text{K}^+$ ,  $\text{Na}^+$ , taurine and choline to diffuse. All these pathways were fully inhibited by furosemide and inhibitors of the anion exchanger (DIDS, niflumic acid), and the  $\text{K}^+$  loss was mediated essentially via a " $\text{Cl}^-$ -independent" pathway. After isotonic swelling, the taurine, choline and  $\text{Na}^+$  pathways were practically not activated and the  $\text{K}^+$  loss was strictly " $\text{Cl}^-$ -dependent". Thus cellular swelling is a prerequisite for activation of these pathways but, for a given volume increase, the degree of activation and the degree of anion-dependence of the  $\text{K}^+$  pathway depend on the nature of the stimulus, whether hormonal or by reduction of osmolality. It appears that the pattern of the response induced by hormonal stimulation is not triggered by either cellular cAMP (since it can be reproduced in the absence of hormone by isotonic swelling in an ammonium-containing saline) or by the tonicity of the medium in which swelling occurs since after swelling in an isotonic medium containing urea, the cells adopt the regulatory pattern normally observed after hypotonic swelling. It is demonstrated that the stimulus is the change in cellular ionic strength induced by swelling: when ionic strength drops, the cells adopt the hypotonic swelling pattern; when ionic strength increases, the isotonic swelling pattern is activated. To explain this modulating effect of ionic strength a speculative model is proposed, which also allows the integration of two further sets of experimental results: (i) all the volume-activated transport systems are blocked by inhibitors of the anion exchanger and (ii) a  $\text{Cl}^-$ -dependent, DIDS-sensitive  $\text{K}^+$  pathway can be activated in static volume trout red cells (i.e. in the absence

of volume increase) by the conformational change of hemoglobin induced by the binding of  $\text{O}_2$  or CO to the heme.

G. Gárdos

*Photodynamic action of merocyanine 540 on erythrocyte membranes: structural perturbation of lipid and protein constituents.* J.B. Felix, G.J. Bachowski and A.W. Girotti (Biophysics Section, Department of Radiology, Medical College of Wisconsin, Milwaukee, USA). *Biochim. Biophys. Acta* 1075, 28 (1991).

Merocyanine 540 (MC540) is a membrane-directed photosensitizing dye with anti-leukemic and antiviral properties. In this study, biophysical and biochemical techniques have been used to examine MC540-sensitized photooxidative damage in the lipid and protein compartments of a test membrane, the human erythrocyte ghost. Irradiation of MC540-sensitized ghosts with white light resulted in oxidative damage to proteins, as manifested by (i) loss of sulfhydryl groups; (ii) intermolecular cross-linking of major polypeptides; and (iii) loss of  $\text{Mg}^{2+}$ -ATPase and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activities. Photooxidation also produced a rapid and progressive increase in general protein motion, as measured by electron paramagnetic resonance spectrometry (EPR) with the sulfhydryl spin label MAL-6. In addition to these effects, ghosts exposed to MC540 and light underwent lipid peroxidation. EPR with two lipophilic spin probes, 5-doxylstearate and 16-doxylstearate, showed that lipid peroxidation is accompanied by a progressive decrease in bilayer fluidity (motional freedom). At a given dye concentration, structural perturbations of proteins were detected at much lower light fluences than those of lipids. When photoreactions were carried out in the presence of ascorbate and iron, there was a strong stimulation of lipid peroxidation (attributed to free radical chain reactions), with a concomitant

greater decrease in lipid mobility. Thus, the deleterious effects of photoperoxidation on lipid structure and motional freedom were greatly exacerbated by ascorbate and iron. Membrane damage similar to that described here may play a role in the phototherapeutic activity of MC540.

G. Gárdos

*Influence of phloretin and alcohols on barrier defects in the erythrocyte membrane caused by oxidative injury and electroporation.* B. Deuticke, P. Lütke-meier and B. Poser (Institut für Physiologie, Medizinische Fakultät, Rheinisch-Westfälische Technische Hochschule, Aachen, Aachen, FRG). *Biochim. Biophys. Acta* 1067, 111 (1991).

Oxidative damage by diamide, periodate and oxygen-derived reactive species, but also exposure to electroporation induce in the erythrocyte membrane dynamic, presumably fluctuating, defects having the properties of aqueous holes with definable radii and selectivities. These leaks, which can be quantified by measuring tracer fluxes or rates of colloid-osmotic lysis, are here shown to be inhibited by phloretin and a small number of related phenol compounds (phenolphthalein, hydroxyacetophenones, nitrophenol), while a host of other "membrane-active" agents is not effective in this respect.  $I_{50}$  values range from about 200  $\mu\text{M}$  for phloretin and phenolphthalein to about 10 mM for 4-nitrophenol. Inhibition by phloretin is reversible, not competitive and not related in its extent to the extent of leakiness. In contrast, the enhancement of transbilayer mobility of amphiphilic lipid probes, which invariably goes along with leak formation of the type described, is not affected by phloretin. Aliphatic alcohols (hexanol, butanol) have an amplifying effect on leaks induced by oxidative damage but do not affect leaks induced by electroporation. The alcohol-amplified leaks maintain the properties of aqueous holes as indicated by a low

activation energy of leak fluxes. Since both, inhibition and stimulation of leak fluxes do not go along with appreciable changes of the apparent radii of the aqueous holes, changes in the dynamics (opening and closing) of the defects are proposed to underly the effects of phloretin and alkanols. The membrane lipid domain is likely to be the site of the leaks and of their modulation.

G. Gárdos

*Effect of tumour-promoting phorbol ester on calcium homeostasis in human platelets.* M. Lucas and F. Solano (Departamento de Bioquímica, Hospital Universitario Virgen Macarena, Facultad de Medicina, Sevilla, Spain). *Int. J. Biochem.* 23, 1261 (1991).

The more interesting features of the effects of PMA on  $[\text{Ca}^{2+}]_i$  and ATP release were the following: (1) preincubation with PMA inhibited thrombin-evoked calcium transients; (2) PMA stimulated slightly the release of calcium and ATP whereas it inhibited calcium and ATP pools sensitive to thrombin; (3) A23187 reversed the inhibitory effect of PMA; (4) subsaturating thrombin concentrations gave results similar to PMA on thrombin-induced calcium and ATP release but not on  $[\text{Ca}^{2+}]_i$ .

G. Gárdos

*Electrophysiological evidence that glycoprotein IIb-IIIa complex is involved in calcium channel activation on human platelet plasma membrane.* T. Fujimoto, K. Fujimura and A. Kuramoto (Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Hiroshima, Japan). *J. Biol. Chem.* 266, 16370 (1991).

The involvement of platelet glycoprotein (GP) IIb-IIIa complex in calcium channel activity on the plasma membrane was investigated using an electrophysiological



approach. Plasma membrane vesicles were prepared from thrombin-stimulated platelets and incorporated into planar lipid bilayers. Voltage-independent  $\text{Ca}^{2+}$  channel currents with a conductance of about 10 pS (in 53 mM  $\text{Ba}^{2+}$ ) were observed in membranes derived from thrombin-stimulated, but not unstimulated platelet membranes. These channel activities were markedly reduced by exposure of membranes to EGTA at 37°C. This reduction was specifically related to the dissociation of the GPIIb-IIIa complex since preincubation of the membranes with a monoclonal antibody to the GPIIb-IIIa complex (AP-2) could protect the channel activities from the effect of EGTA. Thrombasthenic platelets, which lack the GPIIb-IIIa complex, showed impaired channel activities characterized by decreased open probability and lowered conductance states. Furthermore, when platelets were stimulated by thrombin in the presence of EGTA, AP-2, or the synthetic peptide RGDS, to prevent fibrinogen binding to the GPIIb-IIIa complex, open probabilities of the channel currents in these membrane vesicles were also decreased. These results suggest that the GPIIb-IIIa complex is involved in platelet  $\text{Ca}^{2+}$  channel activation and that ligand binding to the complex during platelet

activation may modify the activation of  $\text{Ca}^{2+}$  channels.

B. Papp

*Solution structure of kistrin, a potent platelet aggregation inhibitor and GP IIb-IIIa antagonist.* M. Adler, R.A. Lazarus, M.S. Dennis and G. Wagner (Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA). *Science* 253, 445 (1991).

The structure of kistrin, which is a member of a homologous family of glycoprotein IIb-IIIa (GP IIb-IIIa) antagonists and potent protein inhibitors of platelet aggregation, has been determined by two-dimensional nuclear magnetic resonance (NMR) spectroscopy. The 68-residue protein consists of a series of tightly packed loops held together by six disulfide bonds and has almost no regular secondary structure. Kistrin has an Arg-Gly-Asp (RGD) adhesion site recognition sequence important for binding to GP IIb-IIIa that is located at the apex of a long loop across the surface of the protein.

B. Papp



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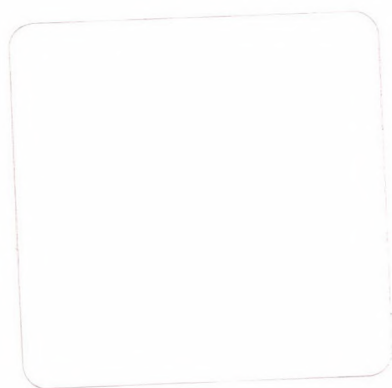
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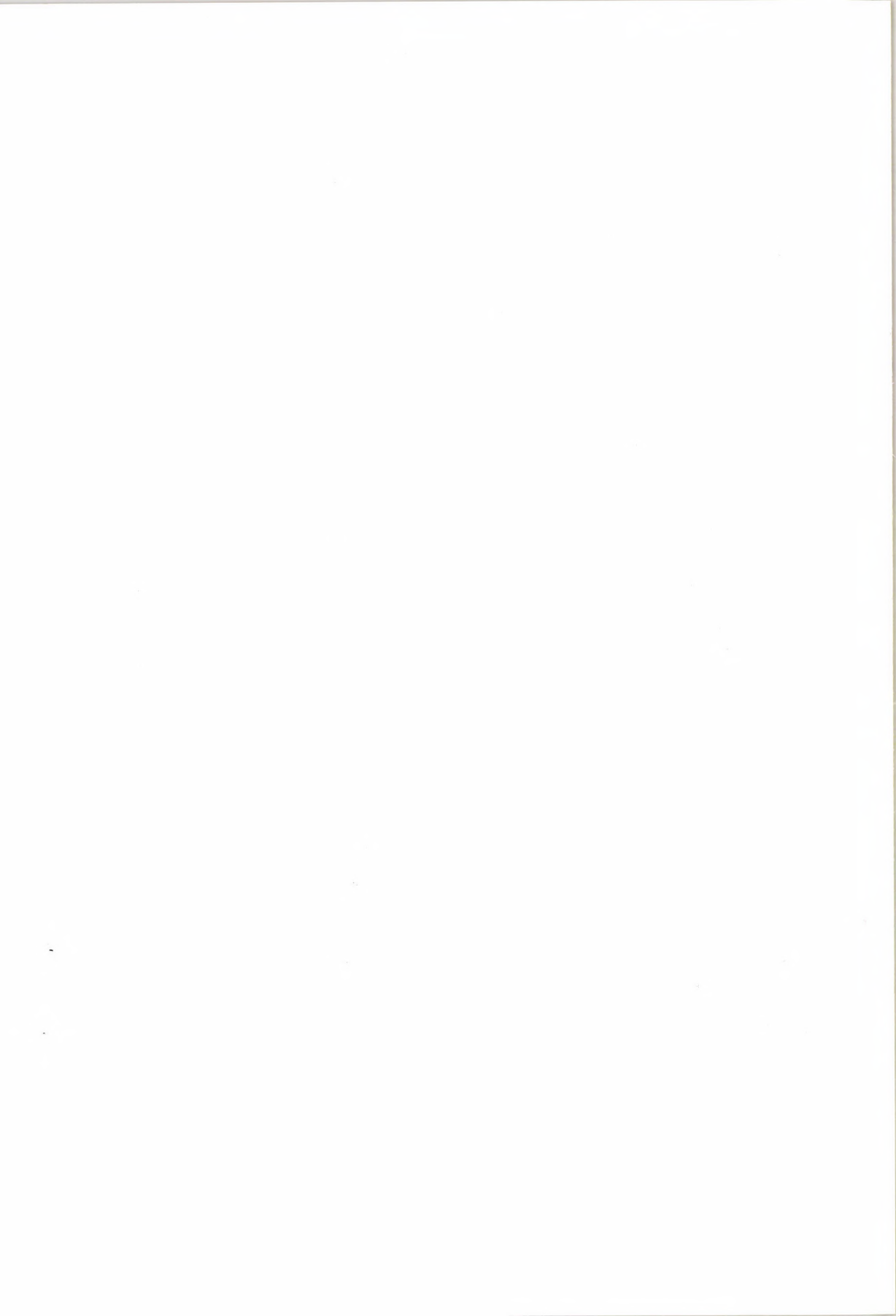
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## **Preventing Transfusion-Transmitted Infections: Issues Related to Migrating Populations and Increasing World Travel**

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Blood donations by emigrants or other travelers from distant lands require special attention for the prevention of transfusion-transmitted infections. The human immunodeficiency virus, type 1 (HIV-1) is only one of several bloodborne viruses that may be introduced into non-epidemic areas by transfusion and spread further by sexual intercourse, needles shared by drug users or mother-child transmission. Although transfusion-transmitted parasites, such as *Trypanosoma cruzi*, do not spread further in non-endemic areas in the absence of specific vectors, such unlikely infections may cause serious disease before a diagnosis is made. The challenges posed by transfusion-transmitted infections in a shrinking world require specific preventive strategies and enhanced clinical awareness for timely diagnosis and treatment.

**Keywords:** blood transfusion, Chagas disease, human immunodeficiency virus, human T-lymphotropic virus, malaria, public health, syphilis, transfusion-transmitted infections, viral hepatitis

### **Introduction**

Only a few years ago, efforts to prevent transfusion-transmitted infections were directed, almost exclusively, to indigenous diseases, such as viral hepatitis and syphilis. As a consequence of the acquired immunodeficiency syndrome (AIDS) epidemic and heightened concern about the risks of bloodborne infections, there has been an ever-widening focus and many relatively uncommon infectious diseases have been added to the list of possible complications of blood transfusion. Some of these infections may be the consequence of blood donations by emigrants or other travelers from distant endemic areas and, therefore, preventive strategies require a global perspective and increased awareness of the epidemiology and infectious diseases. Some bloodborne infectious agents, such as human immunodeficiency viruses (HIV) or human T-lymphotropic viruses (HTLV) are a double concern. First, for the recipient of an infectious blood product, they may be the cause of considerable morbidity and mortality. Second, once introduced in a non-endemic area by transfusion, they may spread further by sexual transmission, needles shared by drug users or mother-child transmission. Unexpected non-indigenous diseases, for example, Chagas disease (American trypanosomiasis) occurring in Europe or North America, pose special problems because of their rarity in non-endemic areas and the difficulty of establishing a diagnosis before the disease has

progressed. The following review addresses strategies for preventing transfusion-transmitted infections, particularly by non-indigenous agents, and provides guidelines for reducing the risks of such complications.

## Human Immunodeficiency Viruses

### *Human Immunodeficiency Virus, Type 1 (HIV-1)*

As of November 1991, the World Health Organization (WHO) estimated that there were more than 1.5 million cases of the acquired immunodeficiency syndrome (AIDS) in more than 150 different countries worldwide [48]. There are an estimated 9–11 million people infected by HIV-1 and this number is expected to increase to 30–40 million by the year 2000. More than 7 million people are infected in Africa and at least 1 million are infected in South and South-East Asia. Worldwide, HIV-1 is spread, primarily, by sexual intercourse, mother-child (maternofetal) transmission and shared needles by drug users. While blood transfusions account for only a small percentage of the total AIDS/HIV epidemic — in fact, only 2% of all reported cases of AIDS in the United States are attributed to transfusions [9] — vigorous donor education and laboratory testing programs are essential to reduce the risk of this complication of blood transfusion.

In non-endemic countries, the approach to preventing transfusion-transmitted HIV-1 infection has been based on donor screening to exclude persons with risk behavior for HIV-1 infection, and laboratory testing of donated blood for anti-HIV-1 [2]. Also, educational programs for physicians, other healthcare workers and patients have an important role in increasing awareness of alternatives to homologous blood transfusion, such as autologous and directed blood donations [36]. Retrospective studies have documented the effectiveness of donor education and self-deferral, particularly in the years immediately before immunoassays for anti-HIV-1 were available [8]. These preventive measures continue to be the foundation for efforts to reduce the risks of transfusion-transmitted HIV-1 infection. The key elements of such programs are educational materials to inform potential donors about HIV-1 risk behaviors, opportunities for donors to self-defer and avoid donating blood, availability of after-hours telephone call-in service for donors who reconsider their qualifications, and a donor deferral register that lists all previously disqualified blood donors.

Laboratory testing of donated blood for anti-HIV-1 is a very important, although, secondary, preventive measure. Every effort must be made to maintain a high quality laboratory testing program using the most sensitive anti-HIV-1 immunoassays available. The HIV-1 seronegative window period will persist as long as the objective of laboratory testing is detection of anti-HIV-1, i.e., a measurable humoral response in the infected host. The addition of HIV-1 p24 antigen as a supplemental test for HIV infectivity will not appreciably improve the current detection rate because p24 antigen is likely to be detected only 5 to 10 days before anti-HIV-1 by conventional enzyme immunoassays [25].



Table 1  
Epidemiologic patterns of HIV-1 and HIV-2 in African countries\*

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*Pattern A: HIV-2 low to absent. HIV-2 entering with travelers from HIV-2 endemic areas.*

Algeria	Gabon	Somali
Angola	Ghana	South Africa
Benin	Kenya	Sudan
Botswana	Lesotho	Swaziland
Burundi	Lybia	Tanzania
Cameroon	Madagascar	Togo
Central African Republic	Malawi	Tunisia
Chad	Mauritania	Uganda
Congo	Morocco	Western Sahara
Djibouti	Namibia	Zaire
Egypt	Niger	Zambia
Equatorial Guinea	Nigeria	
Ethiopia	Rwanda	

*Pattern B: HIV-2 present in 1–5% of general population; less HIV-1.*

Cape Verde	Mali
Gambia	Senegal
Liberia	Sierra Leone

*Pattern C: HIV-2 highly endemic: 5–10% of general population infected; less HIV-1.*

Gambia  
Guinea Bissau

*Pattern D: HIV-1 and HIV-2: 1–10% of both viruses in general population.*

Burkina Faso  
Ivory Coast  
Mozambique

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\*Adapted from PJ Kanki [22].

New technologies are being investigated to further reduce the risk of transfusion-transmitted HIV-1 infections. The polymerase chain reaction (PCR) detects DNA sequences in early HIV-1 infection and offers the promise of identifying potentially infective persons during the seronegative window period [7, 39]. Also, several laboratories are actively pursuing the development of viral inactivation methodologies that could, potentially, render cellular blood components free of all transmissible infectious agents [37, 44]. Unfortunately, both PCR screening and viral inactivation, even if they prove to be effective and technically feasible, will be expensive and not available for routine operations for several years. Clearly, the most important and cost-effective component in a program to prevent transfusion-transmitted HIV-1 infection is donor screening.

### *Human Immunodeficiency Virus, Type 2 (HIV-2)*

HIV-2, a second human immunodeficiency virus that causes AIDS, has a more limited geographic distribution and, possibly, a different natural history compared with HIV-1 [22]. HIV-2 is spread by the same routes as HIV-1, and strategies to prevent transfusion-transmitted HIV-2 infection are based on the same principles, namely, exclusion of donors with risk behavior for infection and laboratory testing of donated blood. Since more than 99% of all persons, worldwide, who are infected by HIV-2 are residents of, or emigrants from, West Africa, questioning potential donors to identify and exclude persons with this geographic risk factor is the key prevention strategy. Secondary spread has infected travelers returning from Portugal, France, Belgium, United States and, probably, other countries. Therefore, it is appropriate to question all potential blood donors about travel to HIV-2 endemic areas and exclude those who may have acquired HIV-2 infection by sexual contact, shared intravenous or other needle exposure or blood transfusion.

In the United States, all persons who were born in, or have emigrated from, sub-Saharan countries, are deferred unless the donated blood is tested by a specific anti-HIV-2 assay that has been licensed by the Food and Drug Administration. A summary of the epidemiologic patterns of HIV-1 and HIV-2 is presented in Table 1.

Presently, blood donor services have three options for testing donated blood for anti-HIV-2. First, commercially manufactured enzyme immunoassay kits are available that are specific for anti-HIV-2. Second, combination anti-HIV-1/anti-HIV-2 enzyme immunoassays are available. Third, significant sensitivity for detecting *anti-HIV-2* is achievable by testing blood with whole virus lysate-based enzyme immunoassays for *anti-HIV-1*. There is considerable genomic homology between HIV-1 and HIV-2 which results in serological cross-reactivity between anti-HIV-2 and HIV-1 antigens in enzyme immunoassays in more than 80% of samples tested.



## Human T-Lymphotropic Viruses

### *Human T-Lymphotropic Virus, Type I (HTLV-I)*

One of the first infectious disease testing programs for donated blood that was implemented, specifically, to prevent the introduction of a non-indigenous virus in a new geographic area was the American Red Cross' HTLV-I testing program [33]. By 1986, it was known that HTLV-I was a bloodborne infection associated with adult T-cell leukemia-lymphoma and tropical spastic paraparesis (TSP)/HTLV-I-associated myelopathy (HAM) in endogenous areas of Japan, the Caribbean basin, Latin America and Africa — and HTLV-I posed a potential risk to transfusion recipients [32]. Although relatively few cases of HTLV-I-associated disease had been identified and none were linked to transfusions, it was estimated that without excluding HTLV-I infected blood, 2,800 transfusion recipients each year in the United States would become infected by HTLV-I [46]. Since HTLV-I is spread, also, via shared intravenous needles, sexual intercourse and from mother-to-child, secondary spread would further establish this agent in the non-endemic area [34]. It has been estimated that a person infected with HTLV-I has a lifetime risk of 2–5% for developing adult T-cell leukemia-lymphoma [16] and approximately 1% for developing TSP/HAM [21]. As of February 1, 1992, there are no published case reports of adult T-cell leukemia-lymphoma attributed to transfusion-transmitted HTLV-I, although there are several reported cases of transfusion-transmitted HTLV-I infection that have resulted in TSP/HAM [17]. In these cases, overt clinical symptoms of TSP/HAM have been detectable less than 2 years after transfusion. Therefore, it seems possible that the relatively large dose of HTLV-I in an infected transfusion and/or other unidentified factors may result in a shorter latency period from the time of infection to onset of disease. Strategies to prevent transfusion-transmitted HTLV-I infection have focussed on laboratory testing using enzyme immunoassays or hemagglutination assays to detect anti-HTLV-I. Some degree of prevention is probably achieved, coincidentally, by donor screening for AIDS risk behavior (e.g. exclusion of persons using shared needles for drug use and geographic exclusions for HIV-2). Additional criteria for HTLV-I-specific geographic exclusions would be difficult to implement, at least in the United States, because of the large number of persons with origins in, or who have traveled to, endemic geographic areas in the Caribbean basin, Latin America, Japan and Africa.

### *Human T-Lymphotropic Virus, Type II (HTLV-II)*

An early, unexpected finding of testing donated blood for anti-HTLV-I in the United States, was the identification of a significant number of donor-carriers with HTLV-II infection [20, 35]. Since HTLV-II and HTLV-I have a high degree of genomic homology, routine testing for anti-HTLV-I detects a certain proportion of HTLV-II infected persons whose anti-HTLV-II cross-reacts with HTLV-I antigens in the immunoassay [12].



HTLV-I has an established association with adult T-cell leukemia-lymphoma and TSP/HAM, but as of early 1992, there are no diseases recognized to be associated with HTLV-II infection [23]. Therefore, it is important that all blood samples testing reactive for HTLV-I by enzyme immunoassay be serotyped to distinguish HTLV-I and HTLV-II infections. While such viral typing previously required PCR analysis of proviral DNA, the availability of synthetic peptides that are specific for HTLV-I or HTLV-II epitomes has made HTLV serotyping considerably more accessible [13].

It is reasonable to ask whether HTLV-II infected persons should be excluded as blood donors, since no disease has been associated with HTLV-II infection. Only recently, an apparent natural reservoir of HTLV-II has been identified in Native American Indians [17, 19]. Most HTLV-II infections in the United States are associated with drug users who share needles and, therefore are presumed to have been infected for a relatively short duration [23]. Given the wide spectrum of diseases associated with retroviral infections in monkeys, cows, sheep, cats, mice and other animals [31], it seems only prudent to exclude blood for transfusion from all persons identified to be HTLV-II carriers in the course of routine testing for HTLV-I infection.

## Hepatitis Viruses

### *Hepatitis A Virus*

Although the hepatitis A virus (HAV) has a worldwide distribution, transfusion-transmitted hepatitis A occurs very rarely. There is no chronic asymptomatic carrier state for hepatitis A virus and, therefore, transfusion-transmitted HAV infection occurs only when blood is donated during the early viremic phase. Since HAV-infected persons not only have high fever during viremia, but are also symptomatic, routine blood donors who feel well and are not febrile are very unlikely to be infected by HAV.

### *Hepatitis B Virus*

Transfusion-transmitted hepatitis B virus (HBV) infection continues to be an important complication of blood transfusion worldwide. There are several reasons why donor screening and laboratory testing for the hepatitis B surface antigen (HBsAg) have not eliminated this hazard. First, some persons infected by HBV develop a long-term, asymptomatic carrier state. While only 0.1% to 0.5% of potential blood donors in the United States and Western Europe are HBsAg carriers, some donor services in China, Southeast Asia and sub-Saharan Africa have reported carrier frequencies as high as 5–15% [28]. Second, although highly sensitive, third generation enzyme immunoassays for HBsAg are widely available, some potentially infectious donated blood with low levels of HBsAg ( $< 0.5$  nanograms/mL) may elude detection. Some HBsAg-negative, HBV-infective units of blood may be detected if additional laboratory tests, such as alanine aminotransferase (ALT) or antibody to hepatitis B core antigen (anti-HBc), are used. Even when all three tests (HBsAg, ALT, anti-HBc) are incorporated in donor

screening, there is still a small risk of transfusion-transmitted HBV infection [18, 49]. The most effective preventive measure for susceptible (e.g. HBV seronegative) transfusion recipients is immunization with hepatitis B vaccine which is strongly recommended for all persons anticipating long term treatment with blood products (e.g. persons with hemophilia A or B, thalassemia or sickle cell anemia).

### *Hepatitis C Virus*

Prior to 1989, most cases of hepatitis following blood transfusion were named non-A, non-B hepatitis (NANBH) which was diagnosed by excluding known causes of hepatitis including HAV, HBV, cytomegalovirus, Epstein-Barr virus and drugs. In 1989, the RNA genome of the NANBH agent was identified and the cDNA molecularly cloned [11]. Utilizing the new cloning technology, an HCV-derived recombinant polypeptide (c100-3) was derived which serves as the "capture antigen" for the first generation enzyme immunoassays for anti-HCV. Commercially manufactured test kits for anti-HCV are now widely used by blood services to test donated blood and identify potentially infectious HCV carriers. Surprisingly, relatively small differences in the seroprevalence of HCV have been observed in different donor populations worldwide. Seroprevalence rates have been reported from Canada (0.3%), northern Europe (0.6%), United States (0.6%), southern Europe (1.2–1.5%) and the United Kingdom (0.06%) [3, 24]. Prior to the implementation of donor screening for anti-HCV, the risk of NANBH was estimated to be 1 in 1,000 to 1 in 200 per unit of blood transfused [37]. There have been no published results of prospective studies to determine the actual impact of anti-HCV testing programs, but an updated estimate of the risk of posttransfusion NANBH 1 in 3,000 to 1 in 5,000 per unit of blood transfused may be reasonable. Improved, second generation anti-HCV enzyme immunoassays have been developed that are based on additional recombinant DNA-derived HCV polypeptides [1]. Preliminary studies indicate that these improved second generation enzyme immunoassays detect additional HCV carriers in blood donor populations and earlier seroconversion in serial blood samples from persons recently infected by HCV [1]. Prior to the availability of anti-HCV enzyme immunoassays, blood donor services in the United States, Japan and several European countries began testing for ALT and anti-HBc as surrogates for NANB infectivity. Although there is reasonably good correlation between the results of these surrogate tests and anti-HCV, there remains the likelihood that some HCV-infected donors, who are not detected by the anti-c100-3 HCV enzyme immunoassay, will be detected by ALT or anti-HBc testing. For this reason, donor testing for ALT and anti-HBc is likely to continue, pending the outcome of studies of the new second generation anti-HCV enzyme immunoassays. It seems possible that at least ALT testing will continue in the long-term because of the relatively late appearance of anti-HCV in the natural history of HCV infection.



### *The Delta Agent ("Hepatitis D Virus")*

The "delta agent", first identified in 1977 in Italy in persons who were also infected by HBV, has been variously described as a "defective virus" or the "delta hepatitis virus" [30]. The delta agent is a novel subviral pathogen and the only one with circular genomic RNA known to infect mammals. Its structure most closely resembles that of viroids or plant RNAs. The genomic RNA of the delta agent contains approximately 1,700 bases and, therefore, is considerably smaller than the genome of any conventional animal virus [6].

Delta hepatitis is a severe, often fulminant, disease occurring worldwide. Outbreaks have been reported in southern Europe, the Middle East, West Africa, Brazil and certain islands in the South Pacific. The prevalence of the delta agent is highest in tropical and subtropical areas and serosurveys have detected rates as high as 90% in persons living in the Amazon River basin and South Pacific islands [6].

In Italy, the United States, Scandinavia, Japan and Australia, delta hepatitis has been spread via blood transfusions and shared intravenous needles. Since delta agent infection requires actual HBV replication, preventative measures are directed to elimination of blood donation by HBsAg carriers and other persons infected by HBV. Testing donated blood for HBsAg is the most widely used strategy for preventing transfusion-transmitted delta agent infection. Additional levels of safety are achieved if blood is tested for anti-HBc and ALT, and susceptible persons are immunized with hepatitis B vaccine. Although commercially marketed diagnostic test kits for detecting the delta antigen are available, testing donated blood for the delta agent is not a routine procedure in blood services.

### *Hepatitis E Virus and Other Non-A, Non-B Hepatitis Viruses*

Most, but not all, cases of non-A, non-B posttransfusion hepatitis have been identified by retrospective testing using PCR and second generation enzyme immunoassays for anti-HCV to be the result of HCV infection [3]. Nevertheless, a small number of cases — estimated to be less than 10% — of non-A, non-B posttransfusion hepatitis test negative for anti-HCV and there remains the possibility of infection by additional, as yet unidentified, bloodborne hepatitis viruses.

Outbreaks of waterborne non-A, non-B hepatitis have been reported from India, Pakistan, Bangladesh, Nepal, Burma, Borneo, Egypt, Algeria, Somalia, Sudan, Ivory Coast, Mexico, China and the Soviet Union [14]. An RNA virus has been identified as the etiologic agent and named the hepatitis E virus (HEV) [29]. Although HEV is not a likely candidate for explaining the apparent existence of HCV-seronegative cases of non-A, non-B posttransfusion hepatitis, application of the molecular cloning technology used to determine the HCV and HEV genomes may be useful in resolving this issue.



## Non-Viral Bloodborne Agents

### *Trypanosoma cruzi*

Preventing transfusion-transmitted *T. cruzi* infection in North America is a pertinent example of the problem of preventing infections by bloodborne agents in non-endemic areas. There are an estimated 16–18 million persons in Central and South America who are infected by *T. cruzi*, the etiologic agent of Chagas disease (American trypanosomiasis) [15]. For a variety of social and economic reasons, emigration from endemic areas of Latin America has been active in recent years, and, perhaps as many as 100,000 persons chronically infected by *T. cruzi* now live in the United States [40]. *T. cruzi* is a common transfusion-transmitted infection in Brazil, Bolivia, Chile, Argentina and other endemic areas of South America [38]. Although data are scanty, there are reports of seropositivity rates of 14–18% in transfusion recipients in Argentina, Brazil and Chile, and up to 49% in Bolivia [38]. Transfusion-transmitted *T. cruzi* has been diagnosed in the United States and Canada and traced to asymptomatic emigrants from endemic areas of Latin America. A case of congenital *T. cruzi* infection in a 5-year old child in Sweden illustrates the potential for spread of the infection by immigrants in non-endemic areas [26].

In endemic areas, measures for preventing transfusion-transmitted *T. cruzi* infections are often limited by economic factors and availability of standardized diagnostic reagents. Many hospital blood banks avoid the expense of testing donors and discarding their positive units by adding crystal (or gentian) violet, a trypanocidal agent, directly to blood containers and transfusing the blood without special testing [38]. Crystal violet appears to be effective in killing *T. cruzi*, but there are minimal data on the effect that such chemoprophylaxis may have on red cell function, or more importantly, on long-term toxicity or carcinogenesis.

In non-endemic areas, the risk of transfusion-transmitted *T. cruzi* infection is increasing in proportion to the number of emigrants from endemic areas [40]. Several strategies for reducing this risk have been proposed. The cost of routinely testing all donated blood for *T. cruzi* antibodies is high and given the relatively low specificity of current diagnostic reagents, such a program would require discarding many units of blood because of falsely positive test results. An alternative approach would be to defer all potential blood donors who have ever lived in an endemic area, not only Latin American immigrants, but also residents of non-endemic areas who have lived and/or worked in rural areas of endemic countries [38]. When improved diagnostic reagents become available, a more practical two-stage screening procedure could be considered. In such a scenario, blood donated by persons identified to have a history of residence in an endemic area would be collected and quarantined pending the results of laboratory testing. These blood units would be tested selectively by improved assays for *T. cruzi* antibodies and issued for transfusion only if they test negative [40].

## Malaria

For many years, malaria has been the prototypic infection for which blood banks in non-endemic areas implemented special screening questions to identify travelers donating blood after returning from endemic areas. Although there has been an average of only 3 cases of transfusion-transmitted malaria annually in the United States in recent years [40], trends of increasing malarial infections worldwide require careful questioning and enforcement of deferred policies to prevent a parallel rise in the number of cases of transfusion-transmitted malaria. From 1978 to 1982, 5,024 cases of malaria were imported into the United States, representing citizens of the United States (26%), South East Asian refugees (33%) and other non-residents mostly from India, Haiti and Latin America (41%) [45].

The strategy for preventing transfusion-transmitted malaria, as well as other parasitic infections differs from that for preventing most bloodborne virus infections. In the absence of natural hosts, parasitic infections will not spread secondarily in the new population, except in rare cases of maternofetal transmission.

The American Association of Blood Banks recommends the following standard for preventing transfusion-transmitted malaria in the United States [47]:

1. Travelers may donate blood 6 months after returning from endemic areas if they have been free of symptoms and have not taken antimalarial drugs.

2. Persons who have had malaria or who had been taking chemoprophylaxis shall be deferred from donating blood for 3 years after either becoming asymptomatic or stopping therapy or chemoprophylaxis.

In endemic areas for malaria where deferral of blood donors at risk for malarial infection is not practical, testing donated blood by one of several new serological tests may be a reasonable alternative [45].

## *Leishmania tropica*

All military and other persons who participated in the Persian Gulf War are temporarily deferred as blood donors in the United States. This new requirement is another example of a strategy to prevent potential transfusion-transmitted infections by transients returning from an endemic area. The preventative measure was initiated by the United States Department of Defense after 22 cases of *Leishmania tropica* infection in military personnel were diagnosed, including 7 cases of visceral leishmaniasis. No cases of transfusion-transmitted *L. tropica* have been reported, but reports of at least 5 cases of *L. donovani* (kala-azar) spread by blood transfusion, suggest that the potential for such infection exists.

The American Association of Blood Banks has recommended a deferral until January, 1993 for all persons who traveled to, or visited in, Saudi Arabia, Kuwait, Iraq, Oman, Yemen, Quatar, Bahrain or the United Arab Emirates after August 1, 1990.



### *Treponema pallidum*

Probably the most widely used — and in many ways least effective — laboratory test for preventing transfusion-transmitted infections is the serological test for syphilis (STS). Like other assays that detect the infected host's humoral response, the STS is ineffective in detecting blood infected for syphilis that was donated during the early seronegative window period. For syphilis, in contrast to HIV, HTLV and HCV, even a reactive test result for antibody is a poor predictor of infection. By the time a person's STS has become reactive, the spirochetemic phase has typically passed and blood is no longer infective. Thus, *T. pallidum* is more likely to be *present* during the early seronegative phase of STS testing and *absent* during the subsequent seropositive phase [5]. For this reason, the screening of donated blood by serological tests for syphilis cannot be regarded to be the primary reason why transfusion-transmitted *T. pallidum* is uncommon.

The primary factor responsible for the low prevalence of transfusion-transmitted *T. pallidum* infection is the spirochete's highly fastidious growth requirements and its inability to survive in refrigerated blood. More than 50 years ago, studies demonstrated that *T. pallidum* added to conventionally collected donor blood units were no longer viable after 72 hours storage in blood bank refrigerators (4–6°C) [5, 43]. Consistent with these findings is the fact that the only well-documented case of *T. pallidum* infection in recent years was attributed to a unit of platelet concentrate that had been stored at room temperature (22°C) [10]. Further complicating the issue of testing donated blood for syphilis infectivity is the problem of nonspecificity of screening assays. Standard nontreponemal serological tests for syphilis (rapid plasma reagent/RPR, VDRL) are highly sensitive, but because they are based on cross reactivity with nonspirochetal cardiolipin antigens, they are notoriously non-specific. When STS-reactive blood donor samples are retested for specificity by confirmatory assays (fluorescent treponemal antibody absorption FTA-ABS), as many as 80–90% may be determined to be nonspecific biological false positive (BFP) reactions [4].

Why then do blood banks continue to test donated blood for syphilis infectivity? At least in the United States, the answer lies in an understanding of the strategies for preventing HIV infection. To the extent that both HIV and *T. pallidum* are sexually transmitted agents, a reactive STS may serve as a surrogate for HIV infection in persons at high risk for AIDS [27]. Thus, syphilis testing of blood donors has become the subject of renewed importance in recent years as part of the strategy to identify donors at risk for HIV/AIDS. Although testing donated blood for STS has little measurable impact on reducing the already very low risk of transfusion-transmitted *T. pallidum* infection, this test is unlikely to be eliminated in the foreseeable future.

### Other Transfusion-Transmitted Infections

An even-increasing number of bacteria, parasites and viruses have been identified as etiologic agents in transfusion-transmitted infections. Among the more important infectious agents not yet mentioned in this review are *Yersinia enterocolitica*, *Toxoplasma gondii*, *Borrelia burgdorferi* (Lyme disease), *Babesia microti*, Epstein-Barr virus



(infectious mononucleosis) and cytomegalovirus. Since the objective of this review is to highlight *selected* bloodborne infections that may result from blood donations by migrants and other travelers returning from endemic areas, the reader is referred to recent texts on the subject for a comprehensive listing and discussion of these other causes of transfusion-transmitted infections [41, 42].

## Conclusion

Special alertness is required to prevent uncommon transfusion-transmitted infections by migrants and other travelers donating blood in geographic areas where their infections are unexpected. Of particular concern are the sexually transmissible viruses, HIV-1, HIV-2, and HTLV-I, which not only cause serious disease in recipients, but also may spread secondarily in previously non-endemic areas. Diagnostic acuity is required for early diagnosis and treatment when blood transfusion results in non-indigenous infections, such as Chagas disease or malaria in North America.

Strategies for preventing such transfusion-transmitted infections must be individualized by geographic area and be economically feasible. To the extent that blood donor screening and laboratory testing programs are preventive measures, they should receive appropriate prioritization within the framework of national public health policy.

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## A Two-Step Liquid Culture — A Novel Culture Procedure for Studying Erythroid Cell Development

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The red blood cell (RBC) has a major contribution to biological research. For example, the basic knowledge on the structure and function of the plasma membrane and the structure, function and genetics of proteins came from studies of RBC and hemoglobin (Hb) [1–3]. The reasons why the RBC has become such a useful experimental tool is due to several facts: (a) peripheral blood (PB) is readily available; (b) pure populations of RBC can be easily isolated from other blood cells; (c) RBC can be maintained *in vitro* for weeks under simple conditions (physiological salt solution at 4°C) and finally, (d) the mature RBC is a rather simplified cell that lost many cellular structures and functions as a result of its extreme specialization.

When studying the developmental aspects of erythroid cell differentiation and maturation, one faces a more complicated situation. Like other hemopoietic cells, the origin of RBC is in precursors in the bone marrow (BM). However, unlike PB, BM is not readily available and it consists of a mixture of cells of various types and stages of differentiation, which makes the purification of a particular cell population quite difficult.

### *In vitro established erythroid cell lines*

One approach to overcome this difficulty is to study *in vitro* established, immortalized, erythroid cell lines. These include cells derived from virus-infected erythroleukemic mice [4] and cells derived from patients with chronic myeloid leukemia at blast crisis [5]. These cells grow continuously in suspension liquid culture as proerythroblasts. They maintain their immature phenotype as manifested morphologically, immunologically and biochemically, and their leukemogenicity as demonstrated by injection into suitable experimental animals. Various chemical agents, including dimethyl sulfoxide, hexamethylene bisacetamide and butyric acid, can induce these cells to undergo further maturation [4, 6–7]. This maturation process has many similarities to normal erythropoiesis, including typical morphological changes, accumulation of globin mRNAs, synthesis of globin chains and heme, formation of Hbs, the appearance of RBC-specific membrane antigens and cessation of cell division [8].

Although these established cell lines serve as convenient experimental models, because of their leukemic origin and long history in culture they do not recapitulate all aspects of erythropoiesis. For example, most cell lines are not inducible by erythropoietin (EPO), the normal physiological inducer of erythropoiesis. In addition, most human cell lines do not synthesize adult Hb and do not undergo terminal cell division [9].

### *Erythroid colony development in semi-solid medium*

An alternative approach to the study of erythropoiesis is cloning of BM or PB cells in semi-solid medium and studying the development of erythroid colonies in the presence of EPO [10]. Since each colony is the outcome of the proliferation and differentiation of one particular progenitor, the quantity and quality of the progenitors in the hemopoietic tissue can be evaluated. Based on the time of maturation and the proliferative potential (size of the colonies), progenitors at different developmental stages, the early burst forming units (BFUe), and the late colony forming units (CFUe), could be distinguished [11].

This assay system is valuable for determining pathological progenitor subpopulations, e.g. in polycythemia vera (PV), where 2 subpopulations, one derived from the abnormal clone and another belonging to normal clones, could be identified based on their response to EPO [12]. The colony assay could also be adapted to serve as a bioassay for quantitation of EPO levels in patients' sera [13].

The fact that the cloned cells are immobilized in the viscous medium results in several disadvantages: (a) The yield is low ( $<10^5$  cells/ml culture), thus affording it technically difficult to carry out quantitative analysis of growth kinetics as well as biochemical, molecular and immunological characterization of the developing cells; (b) It is a one-step continuous culture, thus making it difficult to determine the effects of various factors (i.e., cytokines), at different maturation stages, since it is difficult to add or subtract components to/from the culture; (c) In addition, since the cells are immobilized, cell-cell interactions are limited.

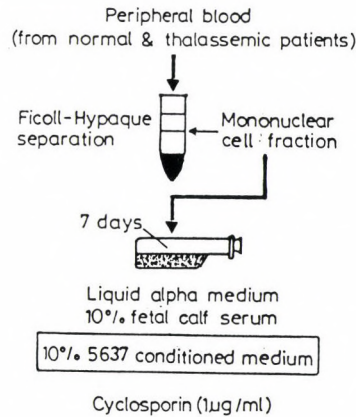
### *The two-step liquid culture*

These disadvantages can be overcome using a liquid suspension culture, recently described by us, that supports the growth and maturation of human erythroid progenitors [14]. In this procedure, the culture was divided into 2 phases; an EPO-independent phase and EPO-dependent phase (Fig. 1). PB mononuclear cells, depleted of most platelets, monocytes and lymphocytes, were cultured for one week in liquid medium supplemented with various hemopoietic growth factors, but no EPO. Prior to the second phase, cells were washed thoroughly to remove the growth factors and then recultured under conditions conducive to the development of erythroid cells, including the presence of EPO and incubation under low oxygen pressure. This resulted in the exclusive development of erythroid cells (Fig. 2). During the first 5 days of the second phase, the total cell number decreased, but the proportion of erythroid cells increased. Hb-containing cells, as determined by the benzidine cytochemical staining [15], started to appear 3–5 days following exposure to EPO. A maximum number and percentage of benzidine-reactive cells were achieved after 10–12 days, when cell concentration reached 3–5 million per ml. At this stage, 90% of the cells were identified morphologically as orthochromatic normoblasts and the rest as enucleated RBC.



# PROCEDURE FOR GROWING ERYTHROID CELLS IN LIQUID CULTURE

## A) EPO-independent phase



## B) EPO-dependent phase

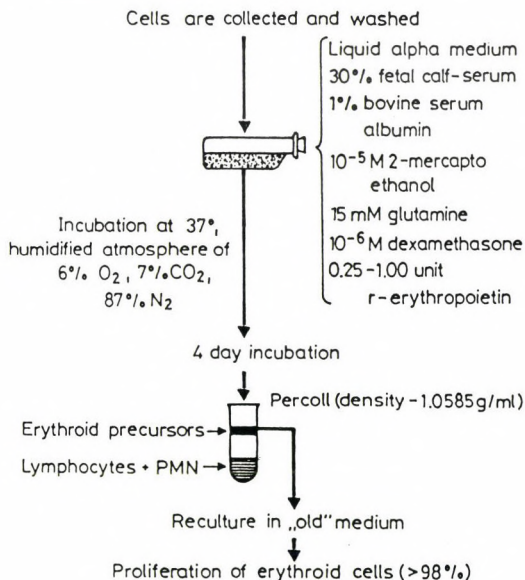


Fig. 1 Procedure for growing erythroid cells in liquid culture.

A. EPO-independent phase. Mononuclear cells, isolated from PB by centrifugation through Ficoll-Hypaque gradient, were cultured for one week in liquid medium supplemented with cyclosporin A and medium conditioned by bladder carcinoma cell line — 5637, which contains various hemopoietic growth factors, but no EPO.

B. EPO-dependent phase. Prior to the second phase, cells were washed and recultured under conditions conducive to the development of erythroid cells, including the presence of EPO and incubation under low oxygen pressure. Lymphocytes were removed after 4 day incubation by sedimentation on Percoll gradient. This procedure resulted in the exclusive development of erythroid cells



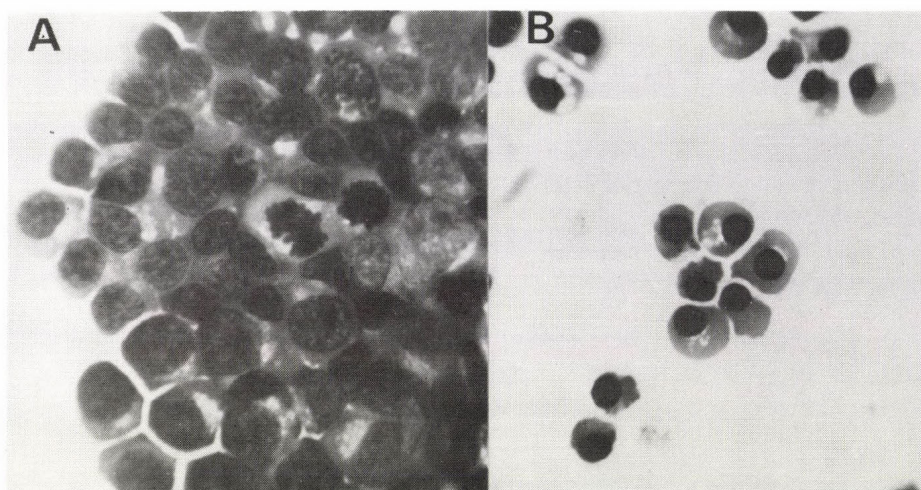


Fig. 2 Erythroid cells in phase II of the liquid culture.

A. Cultures 10 days after addition of EPO, demonstrating proerythroblasts, some of which are in mitosis.

B. Cultures 20 days after addition of EPO, demonstrating orthochromatic normoblasts

PB cells have been used in these procedures for the following reasons: (a) the availability of PB from normal individuals and patients; (b) the homogeneity of the circulating erythroid progenitor population, namely the early BFUe, as opposed to the BM which contains progenitors at various developmental stages [16].

Initially, we used cells derived from patients with  $\beta$  thalassemia, since, due to the state of chronic anemia, their PB contain an increased number of BFUe [17]. Recently, we have modified the procedure to allow large scale cultures of erythroid cells derived from normal donors [18].

In order to increase the yield of erythroid cells, and since normal blood contains a low frequency of erythroid progenitors, each culture was initiated with the buffy coat fraction derived from a whole blood unit. This starting material is readily available as it is routinely separated by blood banks during the procedure of preparation of packed RBC and the platelet-rich plasma. The mononuclear cells (MNC) could be easily separated from the buffy coat fraction by adding a Ficoll/Hypaque solution directly into the transfer bag, spinning and collecting the interphase layer. Monocytes could be depleted by adherence; however, since they do not interfere with the development of erythroid progenitors, they were not removed prior to culturing. Lymphocytes, which do interfere with erythroid development (manuscript in preparation), were previously [14] removed by complement-mediated cytolysis following treatment with rat anti-lymphocytic monoclonal antibodies (CAMPATH-1) [19]. When large volumes of blood had to be treated, the use of antibodies was quite expensive. Instead, the lymphotoxic drug, cyclosporin A, was included in the culture medium. This drug interferes with lymphocyte activation and lymphokine production [20], but at the concentration used (1  $\mu\text{g/ml}$ ) had no effect on erythroid cell development. The decrease in lymphocyte number was gradual, and by day 17 (day 12 of the second phase), when erythroid cells reached maximum number and full maturation, they represented only a minor (5%) contamination-



tion of the erythroid cultures. In order to collect pure populations of erythroid cells at early maturation stages, when lymphocytes constituted a considerable proportion of the population, they could be removed by separation from the much larger erythroblasts by sedimentation on Percoll gradient.

When the two-step liquid culture was compared to semisolid culture, erythroid cell yield was found to be superior in liquid culture in several aspects:

A) The two-step liquid culture supported almost exclusively the growth of erythroid cells, providing uniform erythroid cell populations. In contrast, the EPO-supplemented semisolid culture supported the growth of myeloid, as well as erythroid colonies, and pure populations of erythroid cells could be obtained only by picking up individual colonies.

B) The yield of erythroid cells per MNC cultured was significantly higher in liquid compared to semisolid culture. This was mainly due to the longer period of proliferation, resulting in more cell divisions in liquid culture. Proliferation stopped in semisolid culture after 14 days, while in liquid culture it continued until day 17. Assuming that the same number of BFU-e are capable of developing in both culture systems, these results imply that in liquid culture each BFU-e yields more progeny. This suggests that colonies grown in semisolid culture do not represent the full growth potential of the progenitors.

C) Even more impressive was the advantage of the liquid culture in terms of erythroid cell concentration per ml culture. It was about 10-fold higher than in semisolid culture. Colony formation (number and size) in semisolid cultures is cell-dose dependent at low seeding concentrations, but it reaches plateau and even decreases at high seeding cell concentration due to overcrowding effect. Seeding  $5 \times 10^5$  PB MNC/ml semisolid culture was optimal for colony growth. In liquid culture, initial cell concentration could be ten times higher, without reducing the final cell yield, which could reach up to  $7.5 \times 10^6$  erythroid cells/ml culture.

D) Moreover, the thickness of the semisolid layer that permits cell growth is limited, only 0.1–0.2 cm, as compared to 2 cm (in 80 cm<sup>2</sup> flasks) of liquid medium. Therefore, in liquid culture, less surface area (culture vessels) is required to set up the same volume of culture. For example, we were able to obtain  $4.2 \pm 1.7 \times 10^8$  cells from one unit (400 ml blood) in two 75 cm<sup>2</sup> flasks each containing 50 ml culture. In comparison, to get the same culture volume using the methylcellulose medium, 800 dishes (of 35 mm diameter) would be required.

In addition to the better yield, purity and convenience of the liquid culture procedure, it affords several additional advantages in studying erythroid development, including separation of the EPO-independent and EPO-dependent phases, permitting the study of the effect of various factors in each phase specifically and manipulation of culture conditions and components in various stages without terminating the culture.

#### *The development of erythroid progenitors in phase I*

The developmental stages and the role of growth factors in the primary phase of the culture could be best analysed by a combination of liquid and semi-solid cultures (Fig. 3). An aliquot of PB cells was cloned directly in semi-solid medium supplemented with EPO. In parallel, another aliquot was first precultured in liquid medium and then

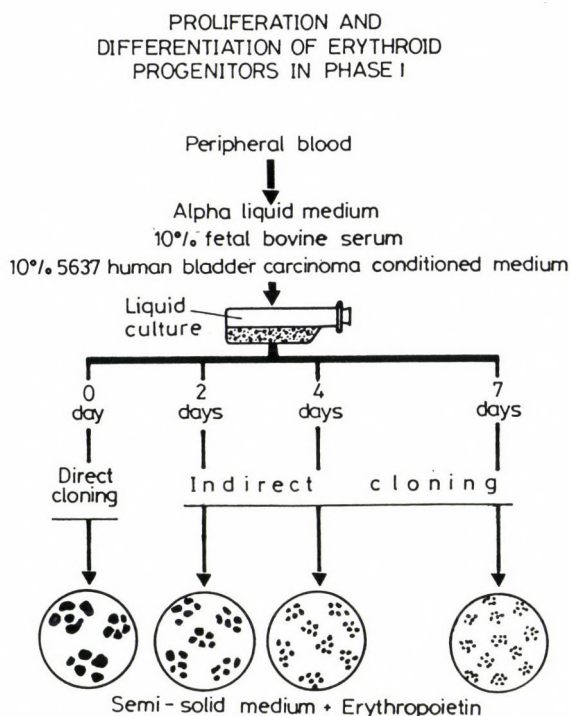


Fig. 3 Proliferation and differentiation of erythroid progenitors in phase I of the liquid culture.

The developmental stages and the role of growth factors in the primary phase of the culture could be best analysed by a combination of liquid and semi-solid cultures. PB cells were precultured in liquid medium. On day 0 (direct cloning), and on subsequent days (indirect cloning), aliquots were washed and cloned in EPO-containing semi-solid medium. The extent of proliferation in the liquid culture was calculated by comparing the number of colonies that developed following direct and indirect cloning

cloned in EPO-containing semi-solid medium (indirect cloning). The extent of proliferation in the liquid culture was calculated by comparing the number of colonies that developed following direct and indirect cloning.

Colonies which developed following direct cloning were the classical bursts, consisting of thousands of cells distributed in several sub-colonies and reaching full hemoglobinization and terminal differentiation after about 2 weeks. Following indirect cloning, colonies reached full maturation after 1 week and were much smaller, similar to CFUe-derived colonies. These results suggest that during the first phase, the BFUe, present in PB, proliferated as well as differentiated, into CFUe [14].

This primary phase was considered EPO-independent. To rule out the possibility that trace amounts of EPO present in the serum used for the culture have an effect, the proliferation of the erythroid progenitors was determined either in the absence of EPO, addition of anti-EPO antibodies at titer enough to neutralize EPO in serum or addition of 0.5 U/ml EPO. The results indicated no effect of EPO during the first week in liquid culture.



The role of other growth factors at this stage was determined in medium supplemented with only 2% serum to avoid serum-derived endogenous growth factors. The results indicated an effect of GM-CSF and IL-3, but no effect of G-CSF or IL-6 during the first phase. Other cytokines, such as the c-kit ligand, and combinations are presently under study.

### *Erythroid progenitors in Polycythemia Vera*

This approach was also utilized to study erythroid progenitors in polycythemia vera (PV) [21]. The abnormality of the erythroid series in PV has been shown to be associated with an increased responsiveness of the progenitors to EPO [22,23]. A basic question in this clonal stem cell disorder is at what developmental stage this abnormality of the PV clone is phenotypically expressed. We have studied this question by comparing the development of EPO-dependent and EPO-independent CFUe from PB BFUe of a PV patient, during the BFUe to CFUe transition in the liquid culture. Because of the wide heterogeneity in the response of cells derived from different patients, rather than making a comparison between cells derived from normal individuals with those of PV patients, we compared the behaviour of EPO-dependent and EPO-independent progenitors coexisting in the PB of each PV patient [24,25]. The proportion of EPO-independent BFUe was first determined by direct cloning of PB cells in either the presence or absence of EPO, and then the proportion of EPO-independent CFUe was similarly determined by indirect cloning. We assumed that if the abnormality takes place at the BFUe-CFUe transition stage, EPO-independent CFUe will be generated preferentially in the liquid culture.

The results indicated that both types of CFUe are generated and that in all cases tested, the ratio of EPO-independent progenitors at both the BFUe and CFUe stage was similar, indicating no preferential development of EPO-independent CFUe. These results strongly suggest that the abnormality of the PV erythroid progenitors is expressed only at the CFUe level. Moreover, since the liquid culture did not contain EPO, the results also support the conclusion that BFUe do not require EPO for proliferation or differentiation into CFUe.

### *Erythroid cells in phase II*

Experiments were also performed to study the effect of EPO and other cytokines in the second phase of the culture. The results indicated that EPO was required continuously, throughout the second phase, mainly for stimulation of cell proliferation. Withdrawal of EPO at any stage of this culture caused almost immediate cessation of cell proliferation. In contrast, following initial exposure to EPO for 4–6 days, cell maturation could proceed in the absence of EPO, indicating that normal erythroid progenitors undergo an irreversible commitment step beyond which the presence of EPO is not required. Other growth factors had no effect on the erythroid development at this stage.

The cell cycle status of human erythroid precursors generated in the secondary phase of the liquid culture was studied by a double labelling flow cytometric technique [26]. In the presence of optimal concentrations of EPO (2 U/ml), a high percentage (>40%) of cells were found in the S phase of the cell cycle until day 10. Then, as a result of maturation, the proportion of cells in S gradually decreased, reaching by day 21 less than 2%. At this time, the culture consisted of >95% Hb-containing, non-proliferating, orthochromatic normoblasts. Cell cycle analysis of this normoblast population demonstrated a bimodal distribution; while the majority of the cells had a diploid (2C) DNA content, i.e., cells in G1 (or G0) phase, a sizeable fraction were tetraploid (4C) corresponding to cells in G2. In contrast, in cultures stimulated with physiological concentrations of EPO (50 mU/ml), all the terminally differentiated cells were arrested at the G1 phase. These results support the notion that EPO is an essential growth-promoting factor for erythroid precursors, but suggest that supra-physiological concentrations, such as present *in vivo* in severe anemia (e.g. aplastic anemia) or after EPO administration, may be associated with development of normoblasts with abnormal DNA content.

Maturation in these cultures was associated with acquisition of a variety of erythroid specific features and the kinetics and temporal relationship between the various parameters could be measured (manuscript in preparation). These included the transcription of globin genes, the accumulation of globins' m-RNAs, synthesis of globin chains and the formation of Hbs, the increased uptake of iron and increase followed by a decrease in intracellular accumulation of ferritin, and the expression of membrane proteins and antigens.

The presence of ABH antigens was studied by the indirect immunofluorescence technique using monoclonal antibodies and flow cytometry [27]. No fluorescent cells were observed during the EPO-independent phase, where early progenitors differentiated into late ones. Transfer of the cells into EPO-containing medium resulted in sequential maturation into pronormoblasts and normoblasts. Antibody binding was confirmed by fluorescence in the developing cells 2 days after exposure to EPO, before any Hb could be detected by the benzidine cytochemical staining.

These results indicated that ABH antigens are absent or unexposed on erythroid progenitors. Their development depends on exposure to EPO and they first appear at early stages of maturation (pronormoblasts) prior to the accumulation of Hb in the developing erythroid cells.

We have recently studied the detailed kinetics of globin gene expression at the transcriptional level, in adult and newborn erythroid cells in the second phase cultures [28]. The results showed that the m-RNAs for  $\alpha$ ,  $\beta$ , and gamma globins first appeared on day 4 following exposure to EPO. In cultures derived from normal adults, the m-RNA levels for  $\alpha$  and  $\beta$  globin increased throughout most of the culture period, while gamma globin m-RNA remained at a low level. In contrast, high expression of all three globin genes was observed in cultures derived from cord blood.

The two-step liquid culture was recently utilized to study the mechanism underlying Band 3 deficiency in a subset of patients with hereditary spherocytosis [29] and  $\alpha$  spectrin synthesis defect underlies spectrin deficiency in a subset of patients with hereditary pyropoikilocytosis [30].



## Conclusions

The two phase liquid culture procedure was found useful in elucidating various aspects of normal and pathologic erythropoiesis, including:

A. The effects of growth factors on proliferation and differentiation at various erythroid developmental stages.

B. The kinetics of globin mRNAs accumulation during erythroid maturation.

C. The expression of exogenous human  $\beta$  globin gene in  $\beta$ -thalassemic cells as a model for gene therapy.

D. Synthesis of erythroid-specific membrane proteins.

E. The expression of surface antigens: Transferrin receptor, glycophorin, A, B, H, D and I/i antigens.

F. The enhancement of gamma globin chain synthesis by chemical agents.

G. Intracellular iron metabolism in normal and thalassemic erythroid cells and the role of ferritin as an iron donor for heme synthesis.

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## **Ethics and Haematology**

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### **Introduction**

Blood transfusion was applied for the first time on a large scale during the Oriental battles of World War I in the Dardanelles and Salonika. These were times of strong racial prejudice. Following the discovery of the human blood groups by Landsteiner in 1900, it was surmised that each human type corresponded to a particular blood group. In the Dardanelles and at Salonika there were, however, in addition to the French, English, German, Turkish and Bulgarian army soldiers from India as well as Algerian and Senegalese native soldiers. Apart from very small differences in the distribution, the blood groups were the very same in these differing populations. This was the first very strong objective argument rendered by haematology to disprove the racial theories.

Even more hard facts were learned from the history of haemoglobins half a century later. The first descriptions deemed the vigorous population of Northern Europe and America superior to the poor Mediterranean, African population damned by the haemoglobinopathies. The results of Allison's research into the correlations of haemoglobin S and malaria have shown that 1) heterozygosity is advantageous, and 2) there are no inequalities but rather differences amongst people. A characteristic feature of blood may be disadvantageous in a given region and advantageous in another. The demographer Albert Jacquart states the following: "I have two friends, Martin and Mohammed. Martin is white and is my next-door neighbor, Mohammed is black and lives in Dakar. Martin appears to be very close to me, while Mohammed very far. But I am ill. I need a bone marrow transplant. The results of the HLA groups revealed that Martin's cells are very different from mine, while those of Mohammed are almost identical. It is Mohammed's bone marrow that saves my life."

The studies revealing 600 million combinations of the HLA groups have broken down the gregarious concepts in medicine and have shown the unique irreplaceable individuality of each human being. In the whole history of mankind with the exception of identical twins, they have never found and will never find two completely identical persons.

These first fields of haematology have already given valuable models for bioethics and for ethics in general. Such models can be found in several other domains.

### **Ethical Problems of Human Trials**

New therapeutic procedures harbour serious moral problems. These trials are certainly based on solid therapeutical hypotheses, on experimental results and clinical observations. The first trials are, however, very exciting. This was the case when the first attempts to treat acute leukaemias began. Acute leukaemia invariably had a fatal outcome in 1947. Patients frequently died within two months. Physicians deemed this disease absolutely untreatable. The working hypothesis of Marcel Bessis and myself was based on experimental and clinical studies and led us to treat these patients with massive exchange of blood, with the so-called exchange transfusion. There were some frightening questions of responsibility. Up till then, exchange transfusion was applied only in the case of newborns, never for older children or adults. We took the risk. Our working hypothesis seemed reasonable, but we could not know what the result would be in human therapy. The constantly fatal outcome and the cruelty of the disease, along with the intolerable pains during its evolution argued in favor of the trial. It is indubitable that at the beginning of the treatment we only thought about the child whom we were trying to save or at least trying to help. We thought also of all those children who could benefit in the future from the progresses inspired by these first experiments. The exchange transfusion was well tolerated. It was followed by a remarkable amelioration. We achieved complete haematological remission in the peripheral blood and in the bone marrow as well. Unfortunately, the remission did not last long. This first success, however, inspired many research workers and the final trials produced advantageous results.

The ethical questions are different in trials involving healthy volunteers. These ethics are oriented to a great extent on the studies of Jean Dausset. Jean Dausset, after discovering the HLA system, postulated that there is a correlation between the HLA system and the tissue or organ transplants.

Skin transplant is the simplest type of transplantation. Jean Dausset and his coworkers mutually transplanted their own skin from arm to arm. The first results of these experiments were encouraging and seemed to confirm the initial hypothesis. But the arms of some of the persons involved became embroidered with scars. It seemed reasonable to stop the experiment.

At this time, blood donors presented themselves as skin donors. This was a brave, but ignorant attitude. Jean Dausset invited them to evening reunions and informed them in detail. From then on, the experiment could be continued under very favorable conditions. The hypothesis was confirmed with very important consequences as regards bone marrow and different organ transplantations. The method applied is exemplary. Volunteers have to be real, informed volunteers, they should run only a minimal risk, they have to be covered by insurance, they should be unpaid, having no financial or other interest in the trials, and can be compensated only for eventual displacement or loss of time.

Randomized trials for the comparison of treatment results between groups of patients are used widely in haematology especially for the evaluation of treatment protocols for haematological malignancies. This method is morally needed, since drugs cannot be authorized without being assured of their value. It is, however, also immoral, because the patient is not only treated in function of his or her disease, but also in function



of future patients. One has to hope for the development of other methods in the future, but at present these randomized trials had enabled the remarkable progress in the treatment of Hodgkin's disease and the leukaemias. The insufficient recourse to this method explains the persistent uncertainties in the treatment of solid tumors, like that of the breast.

### **Ethical Problems of Organ Transplantation**

It is well known that organ transplantation raised a number of important and difficult ethical questions. And here again in the scope of haematology, bone marrow transplantation is of model value, first of all because it frequently concerns a child. A child is by definition a developing being. Its body changes and biology calculates with these changes. Its mental capacities ripen much quicker than it is considered by the adults and the bio-ethics has to take account of this maturation. The HLA-compatible donor may actually be a brother or a sister of the patient. Is it permissible, without being able to get his consent, to expose the child to the small but not zero risk of general anaesthesia? Yes, it is justified if the parents give their consent. It is less sure from the moral point of view. The French experts of bone marrow transplantation have – after an initial hesitation – accepted the authorization of the parents. In various states of the United States, a child solicitor or a layman is nominated to make the decision. He studies the facts of the problem and decides whether or not to give his consent.

Even more considerable questions are raised when no compatible donor exists either among the brothers and sisters or on the list of voluntary donors. Certain desperate parents may decide to have another child in the hope that it will be compatible. On the one hand, this family may be lucky if the leukaemic child recovers with the help of the bone marrow or cord blood of the new brother or sister. On the other hand, there are the possibilities of deviations, abuse, and termination of the pregnancy if the foetus is incompatible.

Even in the successful cases, however, what will be the fate of such a 'prothesis' infant, a 'medicine' infant as it is called? Thorough ethical and psychological studies are indispensable: "Find a good doctor" said the child cited by Nicole Alby. "It is a good doctor, who does not need me to save my brother." The law of cannibalism has to be a temporary law.

The recipient may face other difficulties. "My heart drives the flow of my brother's blood in my vessels", said a young girl to me last year. She suffered from bone marrow insufficiency and was saved by the bone marrow of her brother. And this is true. This young girl became a chimera. She is certainly not a lion with the head of a unicorn. She harbors, however, in her body, in close neighborhood of her own organs, her own heart together with the blood and bone marrow of her brother.

It is not only the bone marrow. The liver grafts from a dead foetus has also been proposed for the treatment of newborns with severe immunodeficiency. These experiments evoked vivid controversies, request for persecution by people who are afraid that these new perspectives will augment further the number of voluntary interruption of

pregnancies. In France the Consultative Committee for National Ethics – after stating that the foetus has to be considered as a potential human person, – distinguished three cases:

- 1) Authorization, since foetal liver graft is the only method to save the life of a condemned child
- 2) Refuse use foetal tissues for the preparation of various beauty products (cosmetics, etc.)
- 3) Turn to the Ethics Committee in case of any new problems (e.g. pancreas and diabetes).

Recently, the indications of liver graft have been reduced in favor of the bone marrow graft. Scientific development has curtailed the importance of a moral problem.

### **Ethical Problems of Prenatal Diagnosis**

Around the Mediterranean, in the large islands like Sardinia and Cyprus, thalassaemia is very frequent and the cost of treatment of children with thalassaemia major has become unendurable. It burdens heavily the budget and curtails the correct treatment of children who suffer from curable diseases. To limit the costs, the practice of systemic prenatal diagnosis of thalassaemia major at the beginning of the pregnancy is recommended, along with interruption of the pregnancy if the disease is revealed. This is a remarkable decision in regions inhabited by very religious peoples, Catholics and Greek Orthodoxes. The decision is doubly dramatic if we consider the interrupted lives and recall the possibility that an early bone marrow graft can cure a very high proportion (>80%) of the infants. However, this can cost 400.000 to 500.000 FFfr. Thus one enters an ever winding infernal coil of medical facts, biology, ethics, religion and finance.

Is it justified to prohibit the marriage of a couple if each is a carrier of the anomaly which may cause catastrophe in the offsprings? Interdiction represents a severe curtailing of individual liberty. Tolerance, on the other hand, results in the birth of an unfortunate and condemned child. At present the method of counselling is recommended. The blood of young people at the age of marriage are studied. They are informed of the risk.

In addition to the diseases that are inherited strictly according to the Mendelian genetics (e.g. haemoglobinopathies, haemophilia), the study of the HLA system enables in certain cases (like diabetes and polyarthritis) at least the recognition of the morbid predisposition. It is at least needed to underline the importance of the progress originating from the development of the medicine of prediction. It can decrease the misfortune for the individual and the family concerned. It can decrease the health budget for the society.

These progresses, however, raise ethical questions, too. They are of two orders. One concerns the person himself. They are sometimes deeply troubled by the revelation of their fragility. The others concern society itself. Several foreign firms ask for HLA group before employing a new staff member, and have been known to refuse them if they carry an HLA group which is known to be a signal of risk. In France, it is a rule that an employer, public or private, should never be authorized to have information on the HLA group of the person they wish to employ.



There is, however, an exception. It is well known to haematologists that workers exposed to benzene intoxication are injured differently. If one day in the future, the underlying cause of these differences, the reason for the fragility of some is known, it will be better to inform the employer in one form or another to transfer the person in question to a job where benzene is not used.

Apart from the diseases, the progress in genetics enabling the identification of each human individual has caught the attention of moralists and jurists in a double way:

1) it is possible by the investigation of the blood groups and especially by that of the HLA system to affirm paternity with a quasi absolute certainty. According to the estimation of geneticists and demographers, 5 to 15% of the children in Western Europe are illegitimate. This is a high number even if it is limited to the lowest level. There is no end to the possibly hurting questions brought to the fore by modern biology (like those allied to dirty or non-dirty interests to injured vanity and may be hurting in a tragical way the love of parents and children);

2) methods using molecular probes and techniques of amplification enable obtaining from a minimal starting DNA from some cells of an organ, or even from a single cell very important quantities of a specific region of the DNA for the investigation of polymorphism. The DNA can thus be extracted from blood, even from dried blood, from sperm and from the roots of the hair. This method is of great importance for the identification of a victim or a criminal, although its value is not yet completely assured.

What are the consequences?

1) Only a magistrate should be authorized to order a genetic investigation of paternity.

2) Only highly competent laboratories should be authorized to carry out such examinations.

3) Eventual intervention of commercial societies possessing a license concerning these diagnostic methods should be rigorously controlled. It seems desirable to orient the activities of these societies in other directions (zoo-technology, animal biology).

Haematologists concern themselves with severe inherited blood diseases, follow with great interest progresses of the genetic genius and contribute sometimes to its progress.

Research tends to transform the individual entirely. The possibility to change genetic heritage has to be condemned formally. In contrast, the aims to transfer a gene into the cells of an organ, into the so-called somatic cells should be encouraged. It can be reasonably hoped to correct the disorders of the function of these organs by the transfer of these genes. This is the case at present for some immunodeficiencies, and probably soon in metabolic errors, in haemophilia and in haemoglobinopathies. There seem to be no important ethical problems involved in this field of research.

### **Ethical Problems of Voluntary Donation**

The human body should never be sold, it cannot be an object of commerce either as a whole or any part of it.

It is the merit of French haematologists to have had refused soon after World War II the sale of blood, and to have organized the voluntary donation of blood — with great moral and technical success. This ethics of blood donation and not sale was later extended to organs, too. This organization is based on generosity and solidarity. It is unpaid and functions satisfactorily in France and in many other countries.

In some countries, there is, however, a deviation from these principles. The sale of blood leads to the presenting of false identification cards enabling the paid donor to have his impoverished blood taken much more frequently than recommended. There are also cases of small advertisements for the sale of organs in widely-read journals, disputed auction with representatives of rich people seeking to acquire organs from people who had died.

The international developments, the previewed evolutions in Europe following 1993 will raise very important questions. For instance, great vigilance will be needed on the borders of nations where one allows the sale of blood and organs while the other insists on voluntary donation.

The cells of the human haemopoietic organ should not be sold either. A recent American adventure displays the novelty and diversity of these questions.

John suffered from hairy cell leukaemia and was treated by splenectomy. The cultured spleen cells produced 1) interferon and 2) growth factors. As a result of exchange between laboratories of the university, the tubes containing John's cultured cells — emerging from official control — became the possession of a private biotechnology firm which was about to commercialize the products of the cells, the growth factors. By that time John had recovered and contacted the private firm claiming his share of the profit. Three decisions were handed down:

The French Consultative Committee of Ethics reminded the court that the organ-forming cells cannot be objects of sale, thus John should not receive any payment.

In the United States, the court decreed that John should receive his share of the profit gained in selling the products manufactured with his cells.

The Supreme Court, however, overturned the verdict of the court saying that there is no justification in paying John.

### **Conclusion**

This investigation of the bio-ethical questions which turn up as a consequence of the progress of haematology enable the definition of the principles on which these bioethics are based.

The respect for the person has been illustrated by the unique, irreplaceable characteristics of each human being, as it is recognized by the HLA-system. The respect for knowledge with two limitations, deferring in certain cases the authorization of only laboratories with high technical and moral qualifications, refusal of the profits which will be evoked and lastly the responsibility of the researchers. It happens quite often that progress in knowledge will solve the ethical problems that had arisen due to earlier progresses. To support this point, I cite just one example: the discoveries in the field of G6PD have solved the difficult moral problems involved in the prevention of malaria.



All this has to be considered with modesty. In the spring of 1987, an important International Congress of Bio-Ethics was held in Japan. I gave the first lecture. The next speaker was an eminent Buddhist priest. "I have listened to our French colleague with great interest," he said, "but I have to confess that I do not know the dates of my first birth, my second birth, my third birth, neither the forms of the animals that I have assumed in the course of the successive metempsychoses. The diverse questions raised by the artificial procreations, the genetic genius seem to me to be fairly far from my meditations."





## **Report of the First Case of Pyrimidine 5' Nucleotidase Deficiency from Kuwait Detected by a Screening Test. A Case Report**

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### **Introduction**

Pyrimidine 5' nucleotidase (P5'N) deficiency causes congenital non-spherocytic haemolytic anaemia [1, 2]. Physiologically, this enzyme dephosphorylates intracellular Pyrimidine nucleotides. Congenital deficiency of this enzyme causes accumulation of Pyrimidine nucleotides in the red cells [3]. These nucleotides in turn inhibit RNA degradation in the red cells causing prominent basophilic stippling in red blood cells stained by conventional Romanowsky stain.

P5'N deficiency has been described in various populations [4, 5, 6, 7, 8, 9] and is regarded as the third common red cell enzymopathy next to G-PD and pyruvate kinase deficiency [4].

We describe here a P5'N deficiency in a 24 year old Arab woman from Kuwait presenting as congenital non-spherocytic haemolytic anaemia (CNSHA). To the best of our knowledge, this is the first reported case of congenital P5'N deficiency from Kuwait.

### **The Case**

A 24-year old Arab woman presented with yellow discolouration of the eyes and pallor since childhood. On several occasions, she was hospitalized and transfused for her anaemia, which used to become worse, particularly during the pregnancy.

Physical examination revealed an averagely built, well-nourished female with mild pallor, mild icterus, a splenomegaly of 6 cm below the costal margin and just palpable liver. Examination of all the systems were normal. There was no consanguinity among her parents. One of the patient's sisters has suffered from a similar problem since childhood. All the patient's children and her husband are healthy.

Laboratory investigation showed Hb 10.1 Gm/L, reticulocytes 8-16% on different occasions, 3-5% red cells showing prominent basophilic stippling with normal white cell and platelet counts and normal red cell indices, no spherocytes were seen. The patient had normal osmotic fragility, normal G-6PD and normal pyruvate kinase activity in her red cells.

The patient's liver and renal function tests were normal. Serum bilirubin was elevated to 34  $\mu\text{mol/L}$  with 12  $\mu\text{mol/L}$  conjugated bilirubin. She had normal serum iron, lead and copper levels. Her Ham's test was negative and she had a normal leukocyte alkaline phosphatase score. Investigations on immuno-haemolytic anaemia were negative. Haemoglobin electrophoresis was normal with 2% foetal Hb and 2.3% HbA2. HbH inclusions were not demonstrable. The patient's serum haptoglobin level was very low, but caeruloplasmin level was 0.48 Gm/L (Normal: 0.18–0.45 G/L). Haematological investigation on her affected sister showed a similar picture with Hb 9.6 Gm/L, reticulocytes 7% and with all of the above investigations being normal or negative. Peripheral smear showed strong basophilic stippling in 6% of red cells. The red cell nucleotide content was measured [10] in the patient, her affected sister and other available members of the family as well as in 45 haematologically normal controls, by extracting the nucleotides from red cells with 4% perchloric acid and measuring the absorbance of perchloric acid extracts at wave lengths 260 nm and 280 nm. High pyrimidine (i.e. cytidine and uridine) nucleotide residue by this procedure is indicated by the low value

Table 1

Red cell nucleotide ratios, basophilic stippling and reticulocyte counts in the patient, family members and healthy haematologically normal control subjects

Cases	Complete Blood count (CBC)	Peripheral smear for basophilic stippling	Reticulocyte count (%)	Red cell nucleotide ratio (absorbance at $\frac{260 \text{ nm}}{280 \text{ nm}}$ )
Patient (24 yr)	Nonspherocytic haemolytic anaemia	+++	8.0–16.0	2.12
Sister (21 yr)	Nonspherocytic haemolytic anaemia	+++	7.0	2.05
Husband (48 yr)	Normal	–	1.0	3.16
Son (9 yr)	Normal	++	1.0	3.37
Daughter (6 yr)	Normal	–	1.0	3.39
Son (4 yr)	Normal	++	1.0	3.27
Daughter (2 yr)	Normal	–	1.0	3.34
Daughter (8 month)	Normal	–	1.7	3.49
Normal controls (n=45)	Normal	–	0.4–1.6	3.05 $\pm$ 0.20 (m $\pm$ IS.d)
				(Range — 2.61–3.49)



of the ratio of absorbance at 260 nm and 280 nm (i.e. 260 nm–280 nm). The normal values ( $m \pm$  I.S.d and range) and those in the patients and her available family members are presented in Table 1. Red cell extracts were made within 2 hours of collection of blood and the absorbance were read on the same day.

Bone marrow aspirate from the patient showed erythroid hyperplasia with moderate megaloblastic erythropoiesis and mild dyserythropoiesis. She had normal iron stores. No other abnormal cells were present.

### Discussion

The patient described here presented clear evidences of a chronic persistent haemolytic anaemia since childhood. The red cells also revealed a significant amount of coarse basophilic stippling for which no other cause could be detected. Biochemical estimation showed increased intraerythrocytic accumulation of pyrimidine nucleotides indirectly indicating reduced activity of P5'N [3]. Relevant clinical and laboratory investigations ruled out the possibility of other causes of this enzyme deficiency such as lead poisoning [11], myeloproliferative or lymphoproliferative disorders [12] and a  $\beta$  thalassaemia trait [13]. To establish the diagnosis of congenital P5'N deficiency, a mere demonstration of reduced enzyme activity is not sufficient as this enzyme could be deficient in many other acquired conditions. For the diagnosis of congenital P5'N deficiency, the enzyme deficiency must be accompanied by elevated pyrimidine nucleotides in the red cells [4]. P5'N is an age-dependent red cell enzyme [14], hence its level is expected to be high when there is reticulocytosis. The presence of increased pyrimidine nucleotides in the red cells with increased reticulocytes suggests a severe deficiency of this enzyme, as in the heterozygote state of this enzyme deficiency, red cell pyrimidine nucleotides are not increased [13].

The familial nature of this disease was apparent by the fact that one of the patient's sisters had a similar clinical and biochemical illness, and two of her own children also revealed persistent punctate basophilia without any evident cause(s) for this morphological anomaly.

The absence of clinical manifestation may suggest that they represent the heterozygous state of this deficiency. The patient's affected sister — like her — showed an abnormal red cell nucleotide ratio and prominent basophilic stippling in the red cells.

The haematological and clinical manifestations of red cell P5'N deficiency are known to vary considerably. Our patient had a mild, persistent haemolytic anaemia which could be managed by folic acid supplementation alone. However, her haemoglobin dropped substantially during pregnancy resulting in the need for blood transfusion.

A mild degree of mental retardation and seizure have been reported in some patients with deficiency of this enzyme [9]. These manifestations were absent in our case. Since this genetic disorder appears to be transmitted by an autosomal recessive gene [10], the incidence and prevalence of this disorder is likely to be significantly affected by consanguinity in the population.

Several mechanisms have been proposed for the molecular basis of haemolysis in P5'N deficiency, e.g. increased Heinz body formation, altered phospholipid composition of the red cell, increased sensitivity to oxidant stress resulting from the inhibitory effect of uridine and cytidine nucleotides on hexose monophosphate shunt and inhibition of glycolysis in the red cell [11, 15].

One of the postulated mechanisms which can explain most of the above mentioned red cell defects involves entrapment of  $Mg^{++}$  by excess of nonutilized pyrimidine nucleotides inside the red cell [15]. Entrapment of this cation which is required in optimum concentration in several biochemical reactions like glycolytic pathway, nucleotide metabolism, hexose monophosphate shunt, membrane transport system in all cells including red cells may adversely affect some of these important biochemical reactions and may produce additional secondary metabolic defect inside the red cell. It remains to be studied in vitro as well as in vivo whether  $Mg^{++}$  in a suitable form can be transported at an acceptable rate inside the red cell to correct the above mentioned metabolic defects in the red cells of congenital P5'N deficiency.

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## **Skin Manifestation of Extramedullary Relapse in Adult with Acute Lymphoblastic Leukaemia**

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In the group of 75 ALL patients treated between 1980 and 1989, two women (ages 25 and 17, both FAB-L<sub>2</sub> and CALLA +, after 56 and 34 months of continuous CR, respectively) were found to have a relapse manifested as a local infiltration of skin, with involvement of the adjacent lymph nodes in one patient. The leukaemic character of the skin infiltration was confirmed by skin needle aspiration and tissue biopsy expressing CD10 and CD24.

Both patients were given intensified systemic chemotherapy and local X-ray irradiation. Complete remission was obtained with the disappearance of the skin infiltrations followed after 13 and 8 months, respectively, followed by a marrow relapse with symptoms of CNS involvement in one case.

Both patients died because of treatment resistance (the overall survival time equalled 70 and 44 months, respectively).

**Keywords:** acute lymphoblastic leukaemia, relapse, skin infiltration

### **Introduction**

As chemotherapy of ALL patients has become more aggressive, the likelihood of the longer duration of complete remissions has increased. However, the patterns of relapse have begun to change. Isolated skin infiltration has been seen recently in our Clinic as the first recurrence of leukaemia. Reviewing the recent literature describing rare manifestations of extramedullary relapse in (ALL) [1,6], we have not found any similar cases. Amongst the total number of 75 ALL patients treated between 1980 and 1989, there were two patients who developed skin infiltrations during hematologic remission.

### **Case History**

#### *Patient 1*

At the time of initial diagnosis in May 1980, the patient was a 25 year old woman. She presented with fever, weakness and mild enlargement of the axillary and cervical nodes (1.0×1.0 cm). There was no evidence of hepatosplenomegaly. The blood count showed a hemoglobin count of 74.0 g/L and a WBC count of  $0.5 \times 10^9/L$  with 2% blasts and platelets of  $34.0 \times 10^9/L$ .

Bone marrow examination demonstrated 97% lymphoid L<sub>2</sub>-FAB, PAS-positive blast-cells. Immunophenotyping using monoclonal antibodies was not done at that time of the disease. The cerebro-spinal fluid was normal. Treatment for ALL was begun according to the protocol including vincristine, adriamycin and prednisone in induction phase. Complete remission (CR) was achieved after one induction cycle. Then, the patient was given prophylaxis for meningeal leukaemia consisting of 6 doses of intrathecal methotrexate along with systemic consolidation including also L-asparaginase. CR was maintained with administration of vincristine, adriamycin, prednisone, 6-mercaptopurine and methotrexate. Additionally, during the initial course of remission, the patient received immunotherapy with REH blasts every two months. During each adriamycin administration, hair loss was prevented by cooling of the scalp with ice bags.

The patient remained in continuous CR for 56 months. During that time, peripheral blood count, bone marrow and cerebral fluid were completely normal but we found cells with high TdT activity in the peripheral blood in two consecutive examinations particularly in the second one ( $240\text{--}1960\text{ U}/10^6$ ). The patient was well for the next 9 months, but she then developed pain in the neck and the head. Physical examination disclosed swelling and single subcutaneous reddish nodule in the suboccipital region on the left ( $2.5\times 3.0\text{ cm}$ ). The enlarged left cervical nodes ( $1.5\times 1.5\text{ cm}$ ) were observed two months later. The marrow aspirate, spinal fluid and peripheral blood count at that time were normal.

Skin fine needle aspiration biopsy identified lymphoblasts L<sub>2</sub> which were strongly positive for antigen detected with VIL-A<sub>1</sub> (CD 10) and VIB-C5 (CD 24) monoclonal antibodies (from Prof. W. Knapp, Vienna).

Histological examination of the cervical node revealed lymphoblastic leukaemia. The skin infiltration was irradiated with 3.6 cGy and the patient received an intensification regimen. A local regression with 13 months' remission was obtained, followed by marrow relapse with symptoms of CNS involvement.

### *Patient 2*

A 17-year old girl was treated because of ALL classified as L<sub>2</sub> -FAB, CD10+, CD24+. The initial laboratory examination showed hemoglobin 70.0 g/L, a leukocyte count of  $2.3\times 10^9/\text{L}$  with 73% of lymphoid cells and a platelet count of  $20.0\times 10^9/\text{L}$ .

Bone marrow aspirate was of high cellularity and exhibited 97% blasts. Moderate splenomegaly (2 cm below the left costal margin) and slight enlargement of the supraclavicular lymph nodes ( $1.0\times 1.5\text{ cm}$ ) were found. Complete remission was obtained with the same induction regimen that was given to patient No. 1. Six intrathecal doses of methotrexate were given during the consolidation phase, following which the patient received maintenance therapy consisting of vincristine, adriamycin, prednisone, 6-mercaptopurine and methotrexate given at 6-week intervals without immunotherapy and additionally  $650\text{ mg}/\text{m}^2$  cyclophosphamid on day 8.

At the time of induction therapy, the patient suffered widespread extreme pruritic eczema. Dermatologic diagnosis was multiform exudative eczema. The cutaneous therapy combined with systemic antibiotics and steroids resulted in complete clearing



of the skin. Isolated extramedullary skin relapse presenting three painful nodules measuring 3 cm in diameter and involving the skin of the right temple region developed after 34 months of continuous CR.

Histologic examination revealed lymphoblastic leukaemia (CD10+, CD24+). The biopsy specimen showed dermal and subdermal infiltration with blasts especially dense in the perivascular area with epidermis free of invasion. At that time, the peripheral blood count, bone marrow and spinal fluid were normal.

Systemic polychemotherapy according to the consolidation program involving vincristine, adriamycin, methotrexate, 6-mercaptopurine and high dose of Ara-C was given as well as local irradiation with 3.6 cGy. The skin lesion regressed completely within 1.5 months. The girl remained in remission for the next 6 months and later she died because of recurrent systemic leukaemia resistant to therapy (survival 3.5 years).

### Discussion

Long-term remission has been achieved in a significant number of patients with ALL. Extramedullary relapse has appeared as a major clinical problem since chemotherapy has become more effective. Meningeal, testicular and ovarian areas are the most frequent sites of residual disease in ALL [4,6,7,8,9]. Infiltration of the eye (optic nerve, retina, hypopyon) is a remarkably rare manifestation of the extramedullary relapse [3]. Isolated bone involvement has also been observed as the first recurrence of leukaemia [1,2], but skin relapse of ALL patients is an uncommon clinical event. Skin involvement may be present at the onset of ALL or lymphoblastic lymphoma [10,11,12,13,14] and is well-known in the literature but isolated cutaneous manifestation of relapse in long surviving ALL patients is extremely peculiar. On the basis of our observation, we can speculate on the role of the skin as a sanctuary for leukaemic cells and a potential source for relapsing disease.

An autopsy study of 308 ALL patients performed by Barcos et al. has shown that the incidence of residual skin lymphoblastic leukaemia has ranged during the 24-year period from 13% to 2% in recent years [8]. The significant decreases in the rates of skin involvement during the recent study period as compared with earlier ones were achieved mainly by the use of effective chemotherapy. However, our observations indicate that the skin among other organs could be an anatomic "sanctuary" which is not sufficiently penetrated by drugs. Diminished drug penetration due to cooling of the scalp skin might possibly influence the local appearance of relapse in patient No. 1.

An inflammatory process in the skin should be considered as a similar causative factor in patient No. 2. An unexplained chronic inflammatory process or allergic dermatitis with pruritis inducing skin infiltration by leukaemic cells was also observed by other authors [15].

These observations suggest moreover that in some patients the site of relapse may be clinically undetectable for a long time, therefore the notion of a "leukaemia free" status must be viewed with great caution.

It seems that leukaemic relapse may arise from a persisting leukaemic cell population because of reduction but not eradication of these cells. This might be a result of insufficient radical systemic chemotherapy or reduced penetration of the drugs to

sanctuarial regions. On the other hand, the "residual disease" may be caused also by the coexistence of subpopulations with primary refractoriness against the applied cytostatic agents.

Current techniques for evaluation of minimal residual disease include morphology, immunophenotyping with monoclonal antibodies and gene rearrangement analysis [16]. The value of the presence of terminal transferase cells in peripheral blood to detect residual disease is discussed [17]. It is known that TdT positivity likewise CD10 antigen are seen in a small number of cells from normal peripheral blood.

However, patient No. 1 showed high activity of TdT in analysis performed half a year before extramedullary relapse that could have been a result of residual disease.

We have observed that local therapy with intensified chemotherapy was successful but was followed by subsequent systemic relapse.

Finally, we would also like to underline a probable appearance of rare extramedullary relapse into the skin after long-term duration of marrow and spinal fluid remission in ALL patients.

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## **Decreased Platelet Mitogenic Activity in Patients with Diabetes Mellitus**

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Platelet-Derived Growth Factor is involved in the atherogenesis and in the genesis of vascular alterations in diabetes mellitus. Its evaluation in platelet extract, by means of the mitogenic stimulation of BALB/c 3T3 fibroblasts, showed significantly reduced values in ten diabetics compared with ten normal controls. Platelet poor plasma mitogenic activity was also evaluated but showed no significant difference. The release of platelet mitogens into the circulation and a resulting acquired platelet defect are suggested. A hypothetical growth inhibitory activity can be surmised, that could both contribute to angiopathy and wound healing defect typical of diabetes mellitus.

**Keywords:** PDGF, platelets, diabetes mellitus, diabetic angiopathies, atherosclerosis, mitogenic assay

### **Introduction**

Platelet-Derived Growth Factor (PDGF: for review see ref. [1–6]) appears to be involved in vivo in the development of atherosclerotic lesions because of its chemotactic, metabolic, mitogenic and vasoactive effects on vessel wall cells [7, 8]. In vitro, PDGF is the most important serum growth factor for cells of mesenchymal origin, like arterial smooth muscle cells and fibroblasts [1]. BALB/c 3T3 confluent resting cells briefly exposed to PDGF acquire the "competence" to progress through cell cycle. This happens only in the continuous presence of other polypeptides named "progression" growth factors, present in the platelet poor plasma, even after PDGF removal [4].

In diabetic macro- and microangiopathy, an abnormal proliferation of arterial smooth muscle cells and fibroblasts is also observed that may be ascribed to platelet mitogens and first of all to PDGF [9, 10]. In fact, platelet survival is shortened in diabetics [11], probably due to an increased consumption in the circulation of hyperaggregable platelets [12] due to a hypercoagulable background [13]. An abnormal platelet release in vivo and its involvement in the genesis and/or maintenance of vascular lesions seems indirectly confirmed by a delayed onset of diabetic nephropathy found in subjects treated with anti-platelet drugs [14, 15]. Moreover, a reduction in platelet  $\alpha$ -granula content has been described in diabetes [16], even if contradictory observations have also been reported [17].

We tested the platelet extract (PE) as well as the platelet poor plasma (PPP) from ten diabetic subjects and ten normal controls in a "competence" mitogenic assay on confluent BALB/c 3T3 cells with the aim of evaluating a possible PDGF reduction in platelets and increase in plasma suggested by former observations. With this method, a modification of that previously described [18], the sample to be tested (PE and PPP) was incubated during a short period ("competence" phase, 4 hours) with the detector system, constituted of confluent BALB/c 3T3 growth arrested cells, and then removed. Subsequently, all "competenced" cultures were maintained in a serum free medium but in the same permissive concentrations of plasma, to permit their eventual progression through the cell cycle ("progression" phase, 12 hours). At the end of the experiment, DNA synthesis, evaluated by means of  $^3\text{HTdR}$  incorporation, was considered a function of PDGF concentration in the sample.

### Patients and Methods

#### *Subjects*

We studied ten diabetic subjects of varying sex, age, duration of disease, diabetes type, vascular involvement and therapy. The patients, whose main characteristics are reported in Table 1, had not received anti-platelet drugs in the two weeks before testing. They were compared with ten sex and age matched  $\pm 10$  years control subjects with negative clinical history, objective examination and hemocytometric values.

#### *Blood samples*

Platelet Extract (PE) and Platelet Poor Plasma (PPP) were obtained from the same blood sample, collected by venipuncture in plastic syringes containing sodium citrate at a final concentration of  $3.8 \text{ g l}^{-1}$ , transferred to plastic centrifuge tubes and spun at  $250 \times g$  for 15 min at  $22^\circ\text{C}$ . Supernatant platelet rich plasma (PRP) was spun at  $2,000 \times g$  for 15 min at  $22^\circ\text{C}$ , the new supernatant (PPP) drawn off, heated at  $56^\circ\text{C}$  for 30 min, filtered through  $0.45 \mu$  size filters (Millipore) and stored at  $-70^\circ\text{C}$ . The collected platelet pellet was twice resuspended in 5 ml PBS and spun at  $2,000 \times g$  for 10 min at  $20^\circ\text{C}$ , suspended in 1 ml PBS and platelets disrupted by five freezing-thawing cycles, spun again at  $5,000 \times g$  for 30 min at  $20^\circ\text{C}$  and then the supernatant (PE) heated, filtered and stored as PPP.

#### *Cell culture*

Cell culture was routinely managed as previously reported [18]. For assay, fibroblasts were placed into a 96-well, flat bottom plate (Flow),  $10^4$  cells/well, with 200  $\mu\text{l}$  of DMEM containing 10% fetal calf serum (FCS) until confluence. Then medium was



poured off and substituted for 24 h with 200  $\mu$ l of fresh medium containing 0.2% FCS, a concentration that does not enable 3T3 undergoing cell cycle [4], but prevents cells from detachment until mitogenic assay.

### *Mitogenic assay*

At this point, the medium was again poured off and cells incubated for 4 h with new medium containing different concentrations of PE (0%, 1%, 2%) and PPP (0%, 10%, 20%) to be tested ("competence phase"). Subsequently, after removal of sample containing medium, all "competenced" cultures were maintained for 12 h in a 5% normal pooled PPP containing medium to permit cell cycle entry ("progression phase"). All experiments were performed in triplicate.

### *Evaluation of DNA synthesis*

Evaluation of DNA synthesis by means of  $^3\text{HTdR}$  incorporation was conducted as previously reported [19], but results expressed as mean counts per second (CPS) for triplicate wells.

### *Statistical analysis*

Data are expressed as mean CPS obtained with each sample concentration in each tested population and graphed with one standard deviation error bars; Mann-Whitney non parametric test was utilized to compare means of different PE and PPP concentrations between patients and controls.

## **Results**

Platelet extract from diabetic patients produced a mean CPS of 65.48 (SD 28.88,  $n=10$ ) at 1% concentration in culture and of 59.35 (SD 29.32,  $n=10$ ) at 2%, while in ten normal subjects we observed respectively 98.45 (SD 18.97,  $n=10$ ) and 106.35 (SD 46.57,  $n=10$ ). The difference between the two groups was significant ( $P=0.008$  at 1% and 0.004 at 2%). Data are graphed in Fig. 1.

With diabetic platelet poor plasma, we obtained a mean value of 104.88 (SD 62.27,  $n=10$ ) at 10% and 91.59 (SD 52.14,  $n=10$ ) at 20%, while normals showed respectively 118.07 (SD 55.91,  $n=10$ ) and 132.31, (SD 104.31,  $n=10$ ). These differences are not significant. Data are shown in Fig. 2.

Blank controls (only DMEM added during the 4 hours of "competence" test) showed mean count of 4.91 (SD 2.30,  $n=20$ ).

In the last column of Table 1, the ratio between patient 2% PE and normal 2% PE mean is reported (stimulation index).

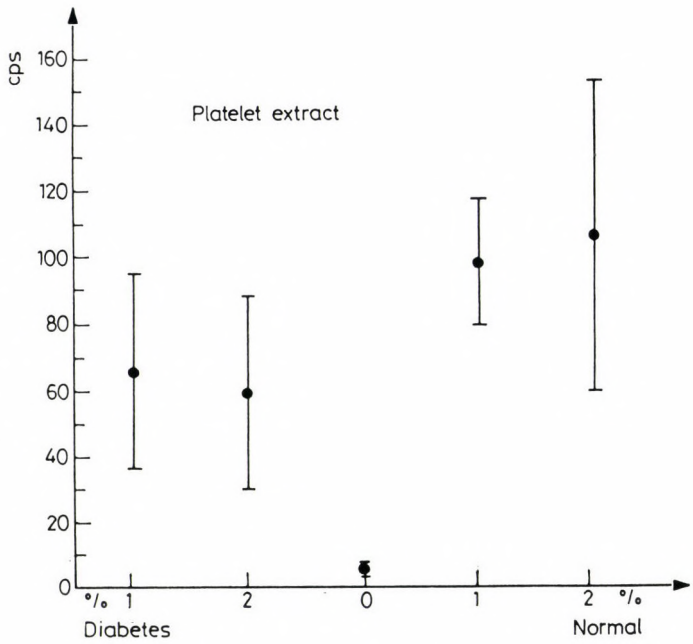


Fig. 1. Mean CPS and SD error bars (vertical axis) obtained with different PE concentrations in culture (horizontal axis) of diabetics (left) and normals (right)

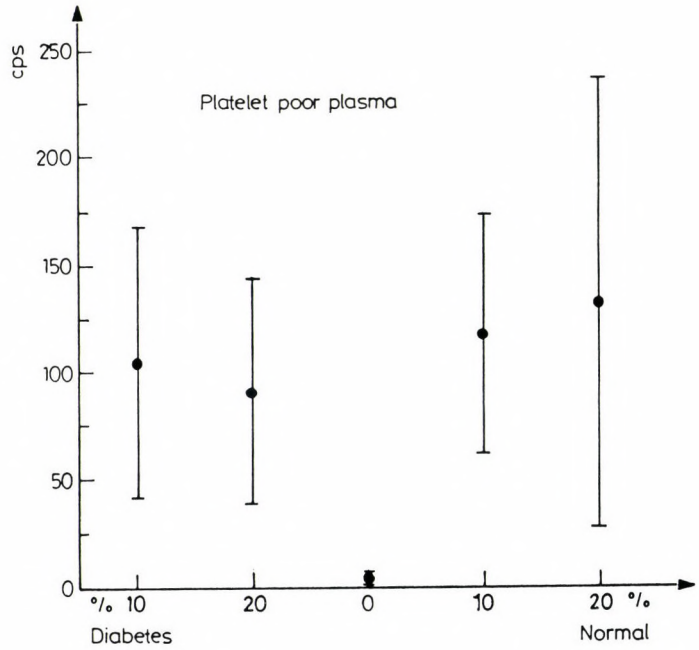


Fig. 2. Mean CPS and SD error bars (vertical axis) obtained with different PPP concentrations in culture (horizontal axis) of diabetics (left) and normals (right)



Table 1  
Main characteristics of patients

Patient	Age Y. rs	Sex	Diabetes Type	Years of Duration	Balance S	Insulin Therapy	Platelets $\times 10^9 \text{ l}^{-1}$	Macroan giopathy	Microan giopathy *	Stimulation Index .
C.C.	84	F	2	7	good	—	259	diffuse	mild	0.34
B.G.	78	F	2	8	good	—	235	diffuse	absent	0.27
V.O.	88	M	2	40	poor	+	160	diffuse	absent	0.32
M.C.	76	F	2	8	good	—	361	cerebral	absent	0.80
D.T.M.	83	F	2	10	good	—	182	cerabral	mild	0.82
M.A.	71	F	2	20	good	+	273	coronatic	mild	0.06
Z.A.	70	F	2	1	poor	—	193	coronatic	absent	0.70
M.G.	47	M	2	1	good	—	200	absent	absent	0.69
V.S.	44	M	1	20	poor	+	177	absent	severe	0.72
S.A.	85	F	1	50	poor	+	281	diffuse	mild	0.81

S) Estimated by fasting plasma glucose, glycosuria, ketonuria, HbA1c

\*) Evaluated on fundoscopy and renal function

.) The patient CPS/normals mean CPS ratio, obtained with 2% platelet extract

## Discussion

The reduced platelet mitogenic activity found in our diabetic population hardly correlates with the features of each diabetic patient when values are singularly considered, probably due to the small number and the heterogeneity of the patient population. Moreover, it is conceivable that multiple and distinct factors concur to different alterations as macro- and micro-angiopathies, making more intricate the interpretation of the individual data. This seems to us a good reason for further investigation in a larger number of patients.

On the whole, the displayed results show a reduced mitogenic competence activity of diabetic platelets for confluent 3T3 cells and suggest a decrease in PDGF, the competence factor *par excellence*. This observation is in agreement with a platelet activation in the circulation and a release of platelet mitogens hypothesized in diabetics [9] causing and/or maintaining angiopathic alterations. On the other hand, a platelet population partly impoverished in its own mitogenic supply could explain the difficult wound healing typical of diabetes, as PDGF is known to have metabolic, mitogenic and chemoattractant actions on many wound healing implied cells [3].

Our results with PE are in line with Guillausseau et al. [16], who found, in a mitogenic assay with 3T3 cells, a platelet PDGF reduction in diabetic type 1 patients, and suggest an increased PDGF release partly responsible for the diabetic angiopathic cell proliferation. Contradictory data are also reported, e.g. Hamet et al., who described an increased growth promoting activity of diabetic platelets, besides some inhibitory effects of plasma and plasma derived serum from diabetics [17,20].

In contrast to a platelet PDGF defect, by release in the circulation, one could expect in theory, an increment in the plasma. Our assay in PPP did not indicate a significant difference between diabetics and normals, but suggested a decreased rather than an increased mitogenic activity. An explanation of such non-increased plasma activity may be given by the presence in plasma of  $\alpha$ -2 macroglobulin that binds and inactivates free PDGF [1]. In fact, PDGF is essentially a local growth factor and circulates whilst only stored in platelet  $\alpha$ -granula [21].

On the other hand, our data obtained with PE and PPP seem to display a different trend in mitogenic activity between the two examined groups: while under normal conditions the activity increases proportionally with PE and PPP concentration, in our diabetic patients it seems to behave in an opposite way (mean counts decrease for increasing concentrations). This could suggest the presence — in the diabetic population — of a component of growth inhibitory activity. For instance, an increase in Transforming Growth Factor  $\beta$  (TGF $\beta$ ) may not be ruled out with certainty, even though its inhibitory activity of proliferation has been described mainly for epithelial cells [22]. We do not know the serum TGF $\beta$  and  $\alpha$ -2 macroglobulin levels of our population, but one can assume a possible circulating increase in antimitogenic factors as a response to cells exaggerated mitogenic activation. So PE and PPP behavior could have multiple origins: a deficit in PDGF and an inhibitory activity. Both might concur to the vascular diabetic alterations, that are characterized by proliferative as well as regressive changes, and to tissue repair defect.



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## Obituary

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### Professor Iván Bernát

(1915–1991)

With the passing away of Professor Bernát we mourn one of the founders of HAEMATOLOGIA. It was he who first suggested the possibility of such a journal, and he supported the journal to the very best of his abilities from the date of founding to the present. Together, we planned and started the multi-lingual Haematologia Hungarica which, in a few years' time turned into an English language journal with an international editorial board.

Professor Bernát was an outstanding expert of internal medicine and haematology. He was devoted to his patients. From the very beginning of his career, he was interested primarily in the field of haematology, and his research work was also connected to this field. In spite of this, his interests remained varied. He "raised" generations of young physicians instilling in them the need for high ethical standards. He was an outstanding lecturer with an excellent knowledge of the ever expanding literature of haematology.

Professor Bernát's research work was aimed primarily at the field of iron metabolism. Already in the 1950s he indicated the untenableness of the theory of the mucosa-block of iron absorption. He demonstrated the role of iron deficiency in ozaena, and studied in detail the disorders of iron metabolism due to severe burns. Prof. Bernát published a number of papers were published Hungarian, English, German and Russian on iron metabolism, iron deficiency and on the results of his research work.

As a young physician, Professor Bernát fought as an active anti-fascist partisan during World War II. For over 40 years he was an army physician, head of department and the army's chief physician of internal medicine. For decades, as a professor of haematology, he taught at the Postgraduate Medical School. He received a number of important awards and commendations for his outstanding work.

In spite of his success, Professor Bernát remained a modest, warm-hearted, humanist. His erudition, fetching personality, exemplary conduct as physician and person will always be remembered by his friends, students and patients.

*Prof. Susan Hollán*





## Abstracts

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*Maintenance and mobility of hemoglobin and water within the human erythrocyte after detergent disruption of the plasma membrane.* I.L. Cameron, L.A. Cox, X.R. Liu and G.D. Fullerton (Departments of Cellular and Structural Biology and Radiology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA). *J. Cell. Physiol.* 149, 365 (1991).

Is an intact plasma membrane responsible for keeping hemoglobin and water within the human erythrocyte? If not, what is responsible? How free is Hb to move about within the erythrocyte? To answer these questions, erythrocytes were taken for phase contrast microscopy, transmission electron microscopy (TEM), determination of water-holding capacity, and proton NMR studies both before and after membrane disruption with a nonionic detergent (Brij 58). Addition of 0.2% Brij to D<sub>2</sub>O saline solution of hemoglobin (Hb) caused particles of Hb to appear and to aggregate. This aggregation of Hb caused the amplitude of the Hb proton NMR spectra to decrease. Thus, the less mobile the Hb the lower the Hb proton spectra amplitude. Erythrocytes washed in D<sub>2</sub>O saline showed proton NMR spectra of relatively low amplitude. Addition of Brij (0.2%) to these erythrocytes caused increased Hb mobility within these erythrocytes. The TEM of fixed and thin-sectioned erythrocytes treated with Brij showed disruption of the

plasma membrane of all erythrocytes regardless of whether or not they had lost Hb. Brij-permeabilized erythrocytes washed in D<sub>2</sub>O saline or in a D<sub>2</sub>O K buffer maintained a higher heavy water-holding capacity upon centrifugation as compared to non-permeabilized erythrocytes. The TEM of Brij-treated and washed erythrocyte "shells" revealed a continuous submembrane lamina but no other evidence of cytoskeletal elements. The water-holding capacity of the erythrocyte can be accounted for by the water-holding capacity of hemoglobin. The evidence favors a relatively immobile state of Hb and of water in the erythrocyte that is not immediately dependent on an intact plasma membrane but is attributed to interactions between Hb molecules and the submembrane lamina.

G. Gárdos

*Orthophosphate-promoted ouabain binding to Na/K pumps of resealed red cell ghosts. Evidence for EP preferentially binding ouabain.* M. Guerra, M. Steinberg and P.B. Dunham (Department of Pharmacology, State University of New York Health Science Center, Syracuse, New York, USA). *J. Biol. Chem.* 267, 1596 (1992).

Modulation of phosphoenzyme forms of the Na/K pump by Na<sup>+</sup> and K<sup>+</sup> was studied by measuring the rate of P<sub>i</sub>-promoted ouabain binding to resealed ghosts made from human

red cells. This system permits distinguishing the effects of the ions at intracellular and external binding sites. Internal  $K^+$ ,  $K_i$ , inhibited the rate of  $P_1$ -promoted ouabain binding, contrary to a prediction based on a current model of the pump. External  $K^+$ ,  $K_o$ , failed to inhibit ouabain binding in the absence of  $K_i$ . However,  $K_o$  enhanced the inhibition of  $K_i$ .  $Na_i$  also inhibited ouabain binding; this inhibition was much less affected by  $K_o$  than was inhibition by  $K_i$ , suggesting that  $K_i$  and  $Na_i$  affect ouabain binding at different internal sites.  $Na_o$  inhibited ouabain binding in the absence of  $K_i$  or  $K_o$ , so  $Na_o$  and  $K_o$  also act at different sites. With  $Na_o$  present,  $K_i$  stimulated ouabain binding. Thus a condition was found in which the predicted stimulation of binding by  $K_i$  was observed. The results of this study are interpreted in terms of three phosphoenzyme forms of the pump:  $E_1P$ ,  $E^*P$ , and  $E_2P$ .  $E^*P$  is the form binding ouabain with highest affinity.  $K_i$  promotes  $E^*P \rightarrow E_2P$ , thereby inhibiting ouabain binding.  $K_o$  binds only to  $E_2P$ , therefore  $K_i$  is required for inhibition by  $K_o$ , and there is little  $E_2P$  present with no  $K_i$ .  $Na_o$  inhibits binding by stabilizing  $E_1P$  whereas  $Na_i$  inhibits by stabilizing  $E_1$ . The stimulation by  $K_i$  with  $Na_o$  present means that  $K_i$  and  $Na_o$  together favor formation of  $E^*P$ . Furthermore,  $K_i$  and  $Na_o$  may bind to the pump simultaneously.  $K_i$  may play a role in the normal pump cycle, binding at allosteric sites to promote  $E^*P \rightarrow E_2P$ .

G. Gárdos

*Palytoxin induces an increase in the cation conductance of red cells.* M.T. Tosteson, J.A. Halperin, Y. Kishi and D.C. Tosteson (Laboratory for Membrane Transport, Harvard Medical School, Boston, Massachusetts, USA). *J. Gen. Physiol.* 98, 969 (1991).

Palytoxin (PTX) isolated from the marine soft coral *Palythoa tuberculosa*, increases the cation conductance of human red cell membranes. In the presence of  $10^{-10}M$  PTX and  $10^{-5}M$  DIDS, the membrane potential approxi-

mates the equilibrium potential for  $Na^+$  or  $K^+$  rather than  $Cl^-$ . Even in the absence of DIDS, the  $Na^+$  and  $K^+$  conductances were greater than the  $Cl^-$  conductance. The selectivity of the PTX-induced cation conductance is  $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+ > choline^+ > TEA^+ > Mg^{2+}$ . Measurements of  $K^+$  efflux revealed two apparent sites for activation by PTX, one with a  $K_{a1}$  of 0.05 nM and a maximum flux,  $v_{max1}$ , of 1.4 mol/liter of cells per h and another with a  $K_{a2}$  of 98 nM and a  $v_{max2}$  of 24 mol/liter of cells per h. These effects of PTX are completely blocked by external ouabain (300 M) and prevented by internal vanadate (100 M). When the PTX channels are open, the Na, K pumps do not catalyze ATP hydrolysis. Upon thorough washout of cells exposed to about five molecules of PTX/pump, the Na, K pump of these cells operates normally. Blockage of the positively charged  $NH_2$  terminus of PTX with a p-bromobenzoyl group reduces the potency of the compound to induce Na and K fluxes by at least a factor of 100, and to compete with the binding of [ $^3H$ ]ouabain by at least a factor of 10. These data are consistent with the conclusion that PTX binds reversibly to the Na,K pumps in the red cell membrane and opens a (10-pS) channel equally permeable to Na and K at or near each pump site.

G. Gárdos

*Purification and reconstitution of phosphatidylinositol 4-kinase from human erythrocytes.* G.H. Jenkins, G. Subrahmanyam and R. A. Anderson (Department of Pharmacology, University of Wisconsin Medical School, Madison, WI, USA). *Biochim. Biophys. Acta* 1080, 11 (1991).

A membrane-bound phosphatidylinositol 4-kinase (PtdIns kinase) has been purified to apparent homogeneity from human erythrocytes. Enzyme activity was solubilized from urea-KCl-stripped, inside-out membrane vesicles by 3% Triton X-100. Purification to apparent homogeneity was accomplished by cation-exchange chromatography on phospho-



cellulose, followed by heparin-acrylamide chromatography. This resulted in a nearly 3900-fold purification of PtdIns kinase activity to a specific activity of 44 nmol min<sup>-1</sup> mg<sup>-1</sup>. The purified enzyme has an M<sub>r</sub> of 59000 on silver-stained SDS-PAGE; however, many preparations also contain 54 kDa and 50 kDa proteins which are related to the 59 kDa protein and have PtdIns kinase activity. Kinetic analysis of the PtdIns kinase indicate apparent K<sub>m</sub> values of 40 and 35 M for phosphatidylinositol and ATP, respectively. The purified enzyme has been reconstituted into phospholipid liposomes and shown to phosphorylate phosphatidylinositol.

G. Gárdos

*Evaluation of platelet calcium ion mobilization by the use of various divalent ions.* Y. Ozaki, Y. Yatomi, and S. Kume (Department of Clinical and Laboratory Medicine, Yamaguchi Medical College, Yamaguchi, Japan). *Cell Calcium* 13, 19 (1992).

Divalent ion mobilization in human platelets was evaluated with Fura-2 fluorescence changes induced by Ca<sup>2+</sup>, Sr<sup>2+</sup>, Ba<sup>2+</sup> and Mn<sup>2+</sup>. Extracellular Ca<sup>2+</sup>, Sr<sup>2+</sup> and Ba<sup>2+</sup> all entered thrombin-stimulated platelets. These divalent ions were also able to refill the intracellular Ca<sup>2+</sup> storage sites which had been depleted of Ca<sup>2+</sup> by ionomycin treatment, and were released from the storage sites upon thrombin stimulation. However, only the refilling of the storage sites with Ca<sup>2+</sup> and Sr<sup>2+</sup>, but not with Ba<sup>2+</sup>, were capable of suppressing the opening state of Ca<sup>2+</sup> channels assessed with Mn<sup>2+</sup> influx. Efflux of intracellularly accumulated divalent ions was observed with Ca<sup>2+</sup> and Sr<sup>2+</sup> but not with Ba<sup>2+</sup>. These findings indicate that there are subtle differences in the Ca<sup>2+</sup>-binding domains of the various systems involved in Ca<sup>2+</sup> mobilization in platelets, some of which discriminate Ba<sup>2+</sup> while accepting Sr<sup>2+</sup>.

B. Sarkadi

*The active site of thrombin is altered upon binding to thrombomodulin. Two distinct structural changes are detected by fluorescence, but only one correlates with protein C activation.* J. Ye, N.L. Esmon, C.T. Esmon and A.E. Johnson (Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma, USA). *J. Biol. Chem.* 266, 23016 (1991).

The association of thrombin with thrombomodulin, a non-enzymatic endothelial cell surface receptor, alters the substrate specificity of thrombin. Complex formation converts thrombin from a procoagulant to an anticoagulant enzyme. Structure-function analysis of this change in specificity is facilitated by the availability of two soluble proteolytic derivatives of thrombomodulin, one consisting of the six repeated growth factor like domains of thrombomodulin (GF1-6) and the other containing only the fifth and sixth such domains (GF5-6). Both derivatives can bind to thrombin and block fibrinogen clotting activity, though only the larger GF1-6 can stimulate the activation of protein C. To ascertain whether the substrate specificity change from fibrinogen to protein C is accompanied by structural changes in the active site of the enzyme, fluorescent dyes were positioned at different locations within the active site. A 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) dye was covalently attached to the active site serine to form dansyl-thrombin, while either a fluorescein or an anilinonaphthalene-6-sulfonic acid (ANS) dye was attached covalently to the active site histidine of thrombin via a D-Phe-Pro-Arg linkage. The environment of the dansyl dye was altered in a similar fashion when either GF1-6 or GF5-6 bound to thrombin, since a similar reduction in dansyl emission intensity was elicited by these two thrombomodulin derivatives (25 and 32%, respectively). These spectral changes, and all others in this study, were saturable and re-ached a maximum when the ratio of thrombomodulin derivative to thrombin was close to 1. The environments of

the fluorescein and ANS dyes were also altered when GF1-6 bound to thrombin because binding resulted in emission intensity changes of -13% and +18%, respectively. In contrast, no fluorescence changes were observed when the fluorescein and ANS thrombin derivatives were titrated with GF5-6. Thus, the structure of the active site was altered by thrombomodulin both immediately adjacent to the active site serine and also more than 15 Å away from it. However, the structural change far from Ser-195 was only elicited by thrombomodulin species that stimulate thrombin-dependent activation of protein C.

B. Sarkadi

*Metabolic rate of membrane-permeant diacylglycerol and its relation to human resting T-lymphocyte activation.* Y. Asaoka, M. Oka, K. Yoshida and Y. Nishizuka (Biological Research Center, Kobe University, Kobe, Japan). *Proc. Nat. Acad. Sci.* 88, 8681 (1991).

D1-1,2-dioctanoylglycerol (1,2-DiC<sub>8</sub>) added to human peripheral resting T lymphocytes was rapidly metabolized to produce octanoic acid and further to small molecules, probably by the action of diacylglycerol lipase and/or nonspecific esterase. Only a small portion was converted to the corresponding phosphatidic acid or was isomerized to 1,3-DiC<sub>8</sub> before being metabolized. The uptake of 1,2-DiC<sub>8</sub> by the cell was apparently fast, and the rate of disappearance of 1,2-DiC<sub>8</sub> was dependent on the cell densities; at a higher density of T lymphocytes 1,2-DiC<sub>8</sub> was removed quickly, whereas at a lower cell density 1,2-DiC<sub>8</sub> remained for a longer period of time. With a fixed amount of 1,2-DiC<sub>8</sub> added, the extent of interleukin 2 receptor  $\alpha$ -subunit (IL-2R) expression was inversely related to the cell density and proportional to the duration of exposure of the cells to 1,2-DiC<sub>8</sub>. Repeated doses of 1,2-DiC<sub>8</sub> potentiated IL-2R expression. In contrast, a single dose of phorbol 12-myristate 13-acetate caused T-lymphocyte activation to similar extents irrespective of the cell density, probably

because the phorbol ester was not metabolized and remained in membranes. The available evidence supports a proposal made in a previous paper and indicates that the sustained activation of protein kinase C for at least the first 3-4 hr is essential for the activation of resting T lymphocytes.

B. Sarkadi

*The ATP<sup>4-</sup> receptor-operated ion channel of human lymphocytes: Inhibition of ion fluxes by amiloride analogs and by extracellular sodium ions.* J.S. Wiley, R. Chen, M.J. Wiley and G.P. Jamieson (Haematology Department, Austin Hospital, Heidelberg, Victoria, Australia). *Arch. Biochem. Biophys.* 292, 411 (1992).

Extracellular ATP is known to increase the membrane permeability of a variety of cells. Addition of ATP to human leukemic lymphocytes loaded with Ca<sup>2+</sup> indicator, fura-2, induced a rise in cytosolic Ca<sup>2+</sup> concentration which was attenuated or absent in NaCl media compared with KCl, choline Cl, or NMG Cl media. In contrast, anti-immunoglobulin antibody gave similar Ca<sup>2+</sup> transients in NaCl and KCl media. A half-maximal inhibition of peak ATP-induced Ca<sup>2+</sup> response was observed at 10-16 mM extracellular Na<sup>+</sup>. Basal <sup>45</sup>Ca<sup>2+</sup> influx into lymphocytes was stimulated 9.6-fold by ATP added to cells in KCl media, but the effect of ATP was greatly reduced for cells in NaCl media. Hexamethylene amiloride blocked 74% of the ATP-stimulated Ca<sup>45</sup> uptake of cells in KCl media. Flow cytometry measurements of fluo-3-loaded cells confirmed that the ATP-induced rise in cytosolic Ca<sup>2+</sup> was inhibited either by extracellular Na<sup>+</sup> or by addition of hexamethylene amiloride. Extracellular ATP stimulated <sup>86</sup>Rb efflux from lymphocytes 10-fold and this increment was inhibited by the amiloride analogs in a rank order of potency 5-(N-methyl-N-isobutyl)amiloride > 5-(N,N-hexamethylene)amiloride > 5-(N-ethyl-N-isopropyl)amiloride > amiloride. ATP-induced <sup>86</sup>Rb efflux showed a sigmoid dependence on the concentration of ATP and



Hill analysis gave  $K_{1/2}$  of 90 and 130 M and  $n$  values of 2.5 and 2.5 for KCl and NaCl media, respectively. However, the maximal ATP-induced  $^{86}\text{Rb}$  efflux was 3-fold greater in KCl than in NaCl media. Raising extracellular  $\text{Na}^+$  from 10 to 100 nM increased ATP-induced  $\text{Na}^+$  influx from a mean of 2.0 to 3.7 nEq/ $10^7$  cells/min, suggesting either saturability or self-inhibition by  $\text{Na}^+$  of its own influx. These data suggest that ATP opens a receptor-operated ion channel which allows increased  $\text{Ca}^{2+}$  and  $\text{Na}^{2+}$  influx and  $\text{Rb}^+$  efflux and these fluxes are inhibited by extracellular  $\text{Na}^+$  ions as well as by the amiloride analogs.

B. Sarkadi

*Induction of members of the IL-8/NAP-1 gene family in human T lymphocytes is suppressed by cyclosporin A.* P.F. Zipfel, A. Bialonski and C. Skerka (Department of Molecular Biology, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany). *Biochem. Biophys. Res. Comm.* 181, 179 (1991).

Human membranes of a family of structurally related cytokines, which play a role as effectors of inflammation, were analyzed for their expression and regulation in T lymphocytes. Members of this gene family include Platelet Basic Protein (PBP); Platelet Factor 4 (PF-4); IL-8/NAP-1; IP-10, a gamma interferon induced protein; GRO; pAT 464 and pAT 744. In resting T lymphocytes the RNAs of the individual genes could not be detected, but all genes were induced upon stimulation with PHA or with PHA/PMA. The induction of five genes was blocked by the immunosuppressive drug cyclosporin A (CSA), which appears to affect initial events in T cell activation. This expression in T lymphocytes, especially the sensitivity to CSA, indicates a common immunomodulatory role of these structural related proteins.

B. Sarkadi





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