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Assay of Des-Gamma-Carboxyprothrombin Using Staphylocoagulase. Application to the Diagnosis of Cellular Hepatocarcinoma

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(Received 18 March 1985; accepted 16 May 1985)

A new clotting method is described to assay des-gamma-carboxyprothrombin (DCP), using staphylocoagulase and adsorbed undiluted citrated plasma. The thrombin-coagulase formed was tested with a chromogenic substrate. The results were expressed in milliunits (m.u.). All 96 normal plasmas had less than 15 m.u. (mean 3.58 m.u.). Out of 56 non-hepatectomized cellular hepatocarcinomas, 40 had DCP levels between 20 and 420 m.u. (average between 40 and 60 m.u.); 71.4% of cellular hepatocarcinoma had an increased DCP and 90% were positive either in alpha-foetoprotein or in DCP. Ten cases of non-cellular hepatocarcinomas had normal DCP levels. We found no cases of cirrhosis or chronic hepatitis, whether active or persistent, with abnormal level of DCP. Out of 127 patients tested, no case was found with a high DCP and a low level of "total factor II", which could be interpreted as a vitamin K deficiency. Only one case of hepatocarcinoma had 25 m.u. of DCP and a low total factor II (20%) and 2 had less than 10% total factor II with no detectable DCP.

Keywords: des-gamma-carboxyprothrombin (DCP), hepatocarcinoma

Introduction

Liebman et al. have recently reported [3] that des-gamma-carboxyprothrombin (DCP) was increased in 67% of the cases of cellular hepatocarcinoma. Plasma prothrombin is synthetized in the hepatocyte, where it is modified in the presence of vitamin K by a post-translational gamma-carboxylation of 10 glutamic acid residues near its end-terminal part, this allows the molecule to become functional, i.e. to react with calcium and with phospho-lipids. The vitamin K related carboxylation also permits the adsorption of factor II (as well as of factors VII, IX and X) on different adsorbents, like barium sulphate or aluminium hydroxide [4, 6].

The American authors used a radio-immunological method to assay DCP, which needed, on the one hand, the preparation of a specific antibody reacting against "normal" prothrombin, and, on the other hand, the use of another antibody active against DCP. Such reagents are difficult to prepare in a reproducible way. If the RIA has the advantage of allowing DCP assay on serum, it has the disadvantage of being a long and complex technique, difficult to apply in many clinical laboratories.

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We have therefore developed a clotting method, based on the property of DCP to be less adsorbable than normal prothrombin, and on the fact demonstrated by us many years ago [5, 8, 9, 10], that DCP is easily converted into thrombin by staphylocoagulase (SC), a substance secreted by certain strains of staphylococci, first studied by Tager [11].

The results obtained by this new method in 56 cases of cellular hepatocarcinoma are presented here.

Material and Methods

A clotting assay of DCP will be described, as well as the assay for "Total factor II", both using staphylocoagulase.

1. DCP clotting assay

Plasma was obtained after centrifugation of blood collected on sodium citrate: 9 parts of blood for 1 part of 0.13 M citrate. The citrated plasma was first adsorbed on bentonite to remove the fibrinogen (to avoid any clot formation during the assay), and then on aluminium hydroxide to take out normal prothrombin, leaving the unadsorbed DCP, which was subsequently transformed into thrombin by SC. The thrombin activity formed was subsequently assayed by a chromogenic substrate.

(a) Fibrinogen adsorption with bentonite (according to previously published studies [4]). Bentonite from Prolabo (Paris) was used as a suspension made of 100 mg of powder for 1 ml of saline. To 0.5 ml of plasma, 0.05 ml of bentonite suspension was added; after 5 min of incubation at room temperature, the plasma was centrifuged for 15 min at 4500 *g*.

(b) The adsorbed plasma was then further adsorbed twice with a gel of aluminium hydroxide: Moist gel BDH, which is a suspension of 25% aluminium hydroxide in dist. water, (W/V). To 0.5 ml of plasma, 0.05 ml of gel were added. After 15 min of incubation at 37 °C, a first centrifugation was done for 15 min at 4500 *g*. Then, a second adsorption of the supernatant, identical to the first, was done, followed by an identical centrifugation. (Two consecutive adsorptions with 0.05 aluminium hydroxide were more efficient than a single one with 0.1 ml.)

(c) The residual DCP was activated into thrombin-coagulase by staphylocoagulase [11].

Staphylocoagulase from Diagnostica Stago (92600 Asnières, France), with an activity of 1000 CNTS units [7] was used. The complex which instantaneously forms with DCP, displays a thrombic activity, and does not require calcium nor any activator [8, 10].

In the cuvette of a Beckmann Spectrophotometer were successively introduced:

– 0.1 ml adsorbed undiluted plasma
– 0.1 ml SC

Then after 30 s, for the stabilization of the mixture, were added:

– 1.5 ml of Tris-Imidazol buffer, pH 8.5

– 0.1 ml of chromogenic substrate CBS 34 47, from Diagnostica Stago. This substrate is split by thrombin to liberate p-nitroaniline. The yellow colour is proportional to the amount of thrombin-coagulase formed, which reflects the content of DCP.

The kinetics of the colour increase was followed by measuring the change of optical density at 390 nm (which was preferred to 405 nm, since bilirubin at this wavelength was less able to interfere).

The measurement was done against a "blank", made of the same mixture, where SC was omitted and replaced by buffer, i.e. 0.1 ml adsorbed undiluted plasma + 0.1 ml Tris-imidazol buffer + 1.5 ml Tris-imidazol buffer + 0.1 ml CBS substrate. The use of such mixture as a blank avoided any interference due to the colour or turbidity of the plasma.

A positive control was made of a pool of plasmas obtained from subjects treated by antivitamin K. A negative control made of a pool of normal plasmas was also used daily. Both positive and negative controls were treated in the same way as the samples to be tested.

The increase of optical density (OD) was recorded (Vitatron), and the results were expressed in milliunits (m.u.) per ml; one m.u. being the increase of OD unit: 1000 during 1 min. It is possible either to record the OD curve to deduce the increase of OD per minute, or to measure the end-point at the third minute by stopping the reaction by adding acetic acid, then dividing by 3 the number obtained.

2. Measurement, using SC, of "Total factor II content"

This was not necessary to assay DCP, but we found it useful for better interpretation of the results.

The method has been previously described [5]; it is a fast and simple one-stage assay which was done by addition of SC on unadsorbed plasma, diluted 1 to 20 with NaCl-imidazol buffer, pH 7.35; it measures the content of both normally carboxylated prothrombin and decarboxylated prothrombin:

To 0.1 ml of unadsorbed plasma, diluted 1 to 20, were added:

0.1 ml of bovine fibrinogen (unsensitive to SC), in a solution containing 4 per 1000 of clottable fibrinogen.

0.2 ml of staphylocoagulase, in a dilution clotting normal plasma in this test in 40 s. A calibration curve gave a straight line, when the reverse of dilutions were plotted against the one stage clotting time.

Results

1. Normal plasma

Ninety-six healthy blood donors were tested. Their plasma content of DCP was found to be between 0 and 12 milliunits (m.u.) the mean being 3.58 m.u. This allowed us to fix the upper limit of normal at 15 m.u.

2. Antivitamin K (AVK) patients

Twelve subjects were found to have between 74 and 420 m.u. of DCP, with a rough correlation with Quick's time.

We used 3 successive pools of AVK patients: pools of about 50 non-selected patients gave 305 m.u. (pool 1), and 250 m.u. (pool 3); pool 2, consisting of patients selected for a Quick's ratio lower than 25 %, was found to contain 400 m.u. of DCP.

When diluting adsorbed AVK plasma with adsorbed normal plasma and plotting the DCP m.u. against the reverse of the dilutions, a straight line was obtained in semi-logarithmic coordinates (Fig. 1).

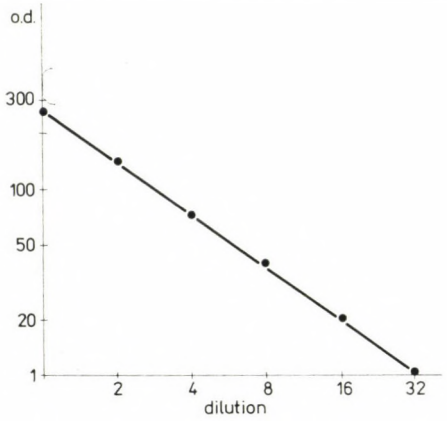

Fig. 1. Calibration curve by diluting AVK plasma with normal plasma (both adsorbed) Optical densities X 1000 against the reverse of dilution

3. Cellular hepatocarcinoma

The DCP clotting assay, as all clotting assays, requires the plasma to be tested on the day the blood is taken. It may also be done on fresh frozen plasma; but since it could not be done on serum, it was impossible to make a retrospective study, such as the one done by Liebman et al. Thus our study was entirely prospective, done with the collaboration of hepatology departments from several hospitals in Paris and the Paris area.

DCP level was measured in a total of 56 cases of non-hepatectomized hepatocarcinoma.

The diagnosis of hepatocarcinoma was confirmed by histology in 23 cases. In the other cases, diagnosis was based on one or several data: scanner, arteriography, echography, suggesting a tumoral process in patients with a severe alteration of the general status, or in the clinical context of evolutive cirrhosis. An increase over 80 ng of alpha-foetoprotein (AFP) was one of the symptoms in favour of hepatocarcinoma. Any cirrhosis, whether alcoholic or post-hepatitic, with a level of AFP higher than 300 ng, was also considered as highly suggestive of hepatocarcinoma.

Metastatic carcinoma of the liver will be considered later on, and has not been included in this series.

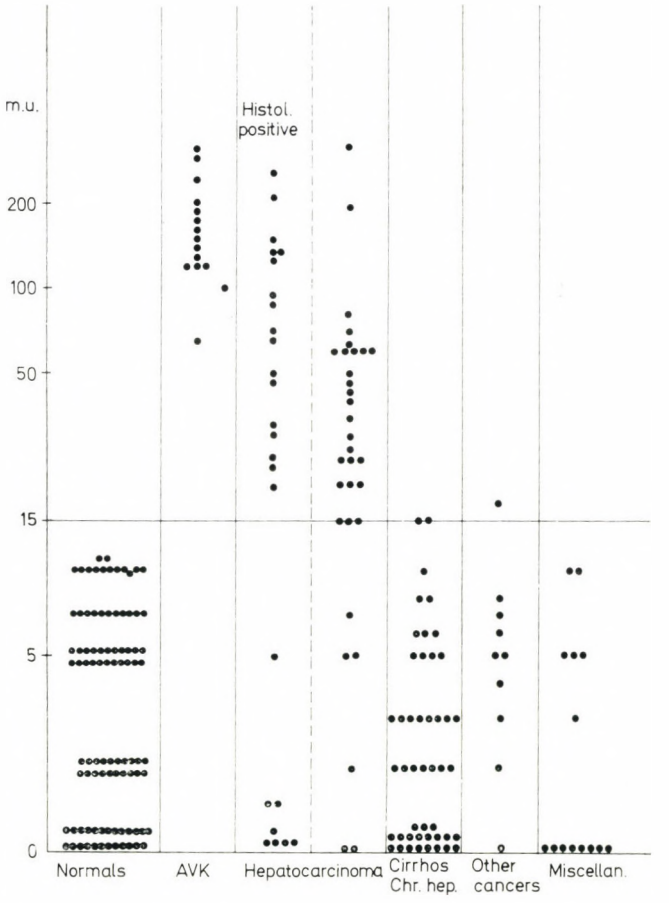

Fig. 2. DCP levels expressed in milliunits in normals and in patients. The horizontal line at 15 m.u. is the pathological threshold

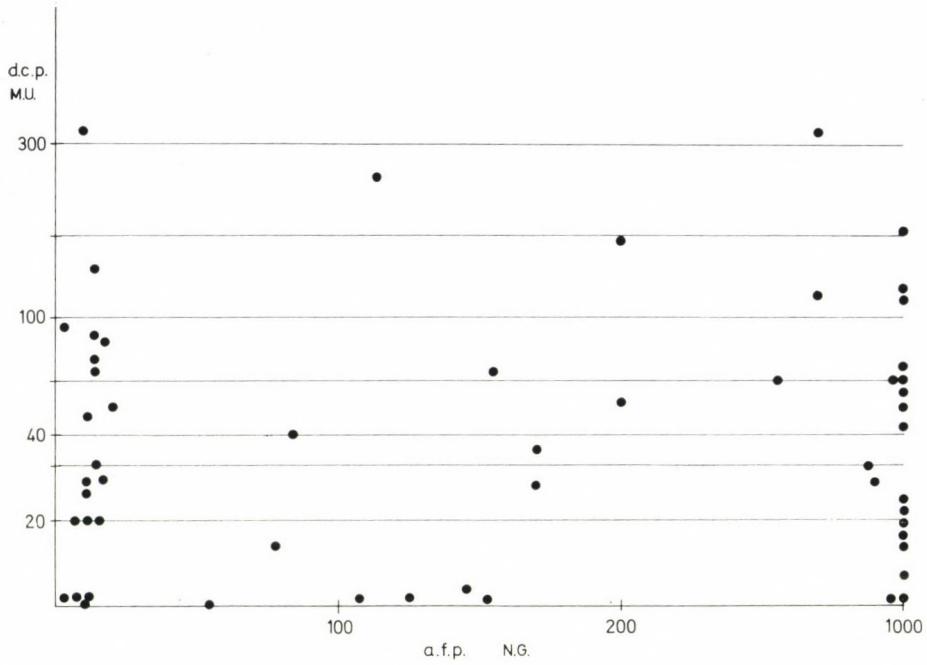

Fig. 3. Correlation between alpha-foetoprotein units expressed in ng and DCP expressed in m.u. in 54 cases of cellular hepatocarcinomas

Three hepatectomized cellular hepatocarcinomas were also excluded, since it is known from the American study that DCP returns to normal after surgery, as long as the disease is not recurring.

We found more than 20 m.u. in 40 out of 56 hepatocarcinomas (non-hepatectomized) (see Fig. 2); thus 71.4 per cent of the cases had an increase of DCP. The levels ranged from 20 m.u. to 420 m.u., with the mean between 40 to 60 m.u.

When the assays were repeated, the cases below 8 m.u. remained unchanged; among the high levels, two were found increased at a second or third assay, where two were found lower, although remaining in the pathological range. Thus, outside hepatectomy, the abnormal finding of increased DCP was quite reproducible. It remains to be seen if medical treatment (chemotherapy, hormonotherapy) may or may not modify the DCP level.

We did not find a significant statistical difference between the DCP content in hepatocarcinomas with or without histological confirmation (73.9 and 71.4 per cent, respectively), but, when positive, the mean appears somewhat higher in the group confirmed histologically (around 70 m.u. compared with 40 m.u.)

Comparison between DCP and AFP levels in cellular hepatocarcinomas. DCP was higher than 20 m.u. in 40 out of 56 (71.4%), AFP was higher than 80 ng in 32 out of 54 (59.2%) (we had no communication of the results in 2 cases).

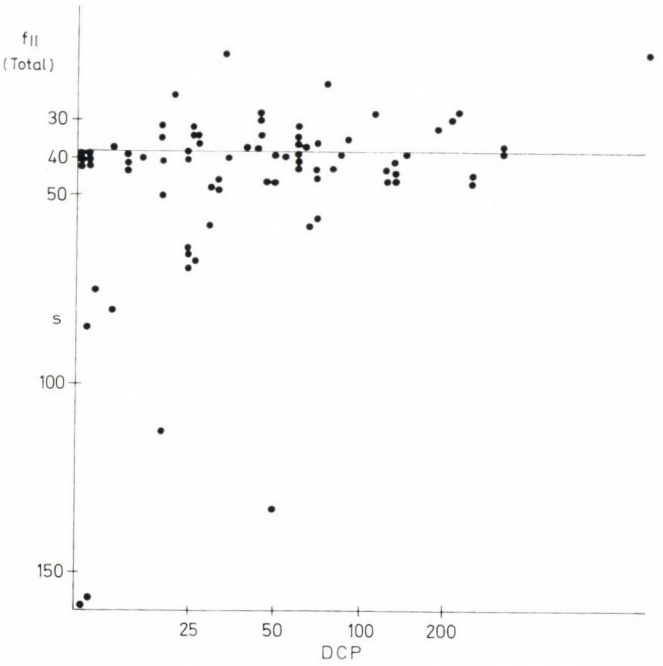

Fig. 4. Total factor II levels (time in s) in 56 cases of cellular hepatocarcinoma

Both markers were simultaneously positive in 23 out of 54 cases (42.6%ld
Both markers were simultaneously negative in 4 out of 52 – histologicale) confirmed – cases (7.7%). They were also both negative in a fifth case diagnosʌ. as hepatocarcinoma by the hepatologist although histology was lacking.

There was a low correlation between the DCP+ and the AFP+, as shown in Fig. 3. Thus the two markers appear to be complementary, as outlined by Liebman et al. [3]

In this series, one or the other marker was positive in 49 cases out of 54, that is to say in 90% of the cases.

Interpretation of the results in context of the "total factor II level". It can be seen in Fig. 4 that half of the hepatocarcinoma cases had a total factor II level normal or increased (clotting time equal to or shorter than 40 s). Among the cases with a decreased factor II, some had a moderately prolonged clotting time. Six patients had a time longer than 59 s (level of total factor II less than 35%) associated with a low or slightly increased content of DCP. This excludes a vitamin K deficiency and suggests that a very low level of prothrombin synthesis, related to heavy hepatocellular damage, may interfere with DCP production by the tumour and may explain the negative test in 2 of these cases having 20% and less than 5%

of total factor II, respectively. On the contrary, all the high levels of DCP, found in these hepatocarcinomas, were not associated with low levels of total factor II, excluding the interference of any vitamin K deficiency.

4. Cirrhosis and chronic hepatitis

Forty-five patients with evolutive cirrhosis (whether alcoholic or post-hepatitic), or with chronic hepatitis (whether persistent or progressive), were studied. The DCP content was below 16 m.u. in all cases. Only 2 cases had 15 m.u., most of the cases were under 6 m.u., 19 out of 45 having 40 DCP (see Fig. 2).

We were unable to classify only one case having a DCP level of 90 m.u.: a Sicilian man HBs positive, with a chronic active hepatitis, dialysed for a glomerulonephritis secondary to rheumatoid purpura, with abnormal lung pathology. This man was immuno-depressed, if there was no focal lesion in the liver suggesting a cellular hepatocarcinoma, such an evolution may not be excluded and only the evolution could tell; unfortunately this patient returned to his country and could not be followed up. Vitamin K deficiency could be excluded since the total factor II content was 130%.

In other words, there was only one case on 45, where DCP could have been false positive; this is to be compared with the results given by the alpha-foetoprotein, which was higher than 80 ng in 6 out of 35 cases here studied (17%).

5. Secondary metastatic carcinoma and primary non-hepatocellular carcinoma of the liver

DCP was found normal in 7 cases of metastatic carcinoma of the liver (see Fig. 2), whether the primary carcinoma was in the gastro-intestinal tract, or was of other origin (ENT or prostate). Three non-hepatocellular carcinomas of the liver also had normal DCP content: 1 was an endothelio-reticulosarcoma, and 2 were cholangiocarcinomas.

6. Various diseases

DCP was measured in 16 cases belonging to various pathology: haemochromatosis, thalassemic patients, acute hepatitis, angiocholitis, etc. and the level was always found to be in the normal range. One benign adenoma of the liver was also normal.

Discussion

1. Sensitivity and specificity of the clotting method described

The plasma being tested undiluted allows a sensitivity comparable to the radio-immuno-assay. Between 0 and 12 m.u. may be detected in normal subjects. The test does not require ionized calcium and excludes the interference of factors

VII, IX and X, which are partly removed during the adsorptions. No activators are required for the formation of the specific complex between prothrombin and staphylocoagulase; thus they cannot interfere, the same is true for the inhibitors (such as heparin or hirudin or antithrombin III) as we have previously demonstrated [5].

Aluminium hydroxide was preferred to other adsorbents such as barium citrate which give a turbidity. Serum cannot be used for the assay since it shows a great variability in prothrombin consumption, even when the blood is taken in the presence of thromboplastin, diluted 1 to 50.

Chromogenic assay was found more sensitive and more reproducible than clotting assay to evaluate the amount of thrombin-coagulase formed.

2. Interest of the DCP clotting assay for the diagnosis of cellular hepatocarcinoma

Our results agree with those of Liebman et al. [3]. On 76 cases of cellular hepatocarcinomas, they found 67% of cases with more than 300 ng (which they considered as the threshold of significance with their method). The clotting-chromogenic assay may even be more sensitive and more specific, since we found 71.4% positive, but no false positivity in evolutive cirrhosis by contrast with AFP which was higher than 80 ng in 6 cases (13.3) and with the RIA which was positive in some cases of cirrhosis [1].

Only one case out of our series of active chronic hepatitis was found DCP positive (90 m.u.), but this patient could not be followed up and classified.

Like Liebman et al., we found DCP and AFP poorly correlated, which makes the assay of both markers complementary. Ninety per cent of the cellular hepatocarcinomas were positive with one or the other marker (84% in the American series). DCP was more often positive than AFP and much more specific of hepatocellular carcinoma.

DCP assay was normal in 10 cases of liver cancer not concerning the hepatocyte, 7 being metastatic secondary carcinomas and 3 primary non-hepatocellular carcinomas of the liver (1 reticulosarcoma and 2 cholangio-carcinomas).

In none of the 127 patients studied here did we encounter a reduction of total factor II associated with an increased DCP, low enough to suggest a vitamin K deficiency. When total factor II was below 50% of the normal (one stage SC clotting time longer than 51 s), the DCP was always low. In only two cases in which DCP level was 30 m.u. (see Fig. 4). Total factor II was low (20 and 25 percent); Vitamin K was injected in one patient with hepatocarcinoma (20 mg IV on 2 successive days) and a new clotting assay done 4 days later was unchanged. Finally only 4 cases of cellular hepatocarcinoma had very low total factor II (one stage SC time longer than 56 s), expressing a very severe hepatic disturbance, in 2 of these cases DCP was very low, which may reflect the poor synthesis of prothrombin by the hepatocyte.

In three cellular hepatocarcinomas, DCP was found normal but the assay was done after hepatectomy; such cases should be followed to appreciate if DCP

will rise in case of relapse, as was observed by Friedman [3]. It remains also to be seen if chemotherapy or hormonotherapy may modify an elevated DCP content.

In conclusion, specific DCP clotting assay may be done in any well trained coagulation laboratory, with reproducible results. It brings new and valuable information for the diagnosis of cellular hepatocarcinoma. It should be done in all the cases of liver disease where the assay of alpha-foetoprotein is actually done, since the use of these two markers greatly improves the biological diagnosis of cellular hepatocarcinoma.

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PMN* Migration Measured by Bottom Filter Counts in Modified Boyden Chambers. Chemokinetic Deactivation and Vinblastine Inhibition of Chemotaxis

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Polymorphonuclear leukocyte (PMN) migration and chemotaxis were studied by a modified Boyden chamber technique in 100% autologous serum with casein as attractant. PMN chemotaxis was induced by increasing the top gradient concentration of casein to 20 mg/ml. Under non-gradient condition, casein 20 mg/ml inhibited PMN migration, which was thought to be due to chemotactic deactivation. Vinblastine 0.01 μg/ml partially inhibited PMN migration in a casein gradient with a top gradient concentration of 20 mg casein/ml. It is suggested that PMN migration in the casein gradient was composed of both vinblastine-sensitive chemotaxis and vinblastine-resistant chemotaxis.

Keywords: chemokinetic deactivation, vinblastine-resistant chemotaxis, vinblastine-sensitive chemotaxis

Introduction

Polymorphonuclear leukocyte (PMN) migration is a complex process which can be divided into non-stimulated random movement, stimulated random movement (chemokinesis) and chemotaxis. Chemotaxis can be further divided into a chemotaxis which is inhibited by microtubule antagonists (MAs) and an MA-resistant chemotaxis [1, 2]. It is essential to recognize that different assays of PMN migration measure different combinations of the partial processes of PMN migration. An old modification of the Boyden chamber technique appeared to measure almost exclusively the MA-sensitive chemotaxis (3–5].

By tradition, PMN migration is measured in a balanced salt solution, sometimes supplied with 10 or 20% serum. The dilution with serum represents an attempt to reduce the variable influence of motility enhancing factors and motility inhibiting factors found in serum. In contrast, the present study was designed to investigate the casein-induced PMN migration in 100% autologous serum by the modified Boyden chamber technique mentioned above.

* *Abbreviations:*
MA: microtubule antagonist
PMN: polymorphonuclear leukocyte

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Material and Methods

Pretreatment of blood samples

Venous blood from the antecubital fossa was obtained with Vacutainer® from 19 healthy donors. The blood was collected into heparinized glass tubes (16 IU heparin/ml blood) and then mixed with an equal volume of 2% dextran T 250 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.9% NaCl. The suspension was allowed to settle in plastic tubes for 30 min at room temperature. The leukocyte-rich supernatant was centrifuged at 150 *g* for 10 min and then washed in 0.9% NaCl. After another centrifugation at 150 *g* for 10 min, the cells were resuspended in the serum of the donor. The cell suspension was transferred to three plastic tubes, one without additives, one with casein (Merck, Darmstadt, FRG) to a final concentration of 20 mg/ml, and one with vinblastine (Eli Lilly, Indianapolis, Ind., USA) to a final concentration of 0.01 μg/ml. Prior to the experiment, vinblastine was dissolved in 0.9% NaCl and kept in refrigerator for, at most, 48 hours. Pre-incubation with or without vinblastine was performed at room temperature for 30 min.

The modified Boyden chamber technique

The apparatus consisted of two plexiglass disks, two silicone rubber rings, and two filters (Millipore Filter Corp., Bedford, Mass.). The apparatus was held together by two metal clips. A millipore filter of 3 μm pore size divided the apparatus into a top compartment and a bottom compartment. A millipore filter with pore size 0.45 μm was placed at the bottom of the lower compartment. Fluid could be injected into each of the two compartments by means of injection needles inserted through the silicon rubber rings [5–7].

Experimental design

The design of the experiment is shown in Table 1. Casein, 20 mg/ml, in donor's serum served as attractant. Hydrogen ion concentration was adjusted to pH 7.4 by means of tramoxan buffer (Addex-Tham®, Pharmacia Infusion Uppsala, Sweden). The attractant was injected into the bottom compartment and 1×10^6 cells of PMNs in autologous serum were injected into the top compartment. The chambers were then incubated for 3 hours at +37 °C. During incubation, some of the cells migrated through the intercompartment filter and settled on the bottom filter. After incubation, the bottom filters were stained with Mayer's hematoxylin-erythrosin and PMNs were counted along a diameter of the filter at $\times$ 320 magnification.

Table 1

The role of non-stimulated random movement (a), chemokinesis (b), control chemotaxis (c) and vinblastine-resistant chemotaxis (d). S: Autologous serum. C: Casein 20 mg/ml. V: Vinblastine 0.01 μg/ml.
$Q_1 - Q_3$: Interquartile range

		a	b	c	d
Upper compartment		S	SC	S	SV
Lower compartment		S	SC	SC	SC
Donor	1	3	—	99	55
	2	21	—	81	419
	3	20	0	86	22
	4	25	20	386	324
	5	95	9	651	38
	6	148	35	48	63
	7	119	92	409	326
	8	65	11	300	444
	9	229	58	344	830
	10	32	22	1989	275
	11	97	26	105	19
	12	19	36	275	130
	13	48	2	58	49
	14	156	22	549	420
	15	609	32	1601	225
	16	16	3	3	18
	17	59	2	81	8
	18	23	2	188	38
	19	28	4	1080	583
Median		48	20	275	130
$Q_1 - Q_3$		21-119	3–36	81–549	38–419
Range		5–609	0–92	3–1989	8–830
Significance	W		$2p < 0.001$	$2p < 0.001$	$2p < 0.044$
	KV			$P < 0.001$	

Statistics

The Wilcoxon matched-pairs signed-ranks test (W), two-tailed probabilities, and the Kruskal-Wallis one-way analysis of variance (KV) were calculated according to Siegel 1956 [6]. The confidence intervals of medians were calculated according to Wonnacott & Wonnacott 1977 [7].

Results

In order to overcome the inhibitory effect of serum on PMN migration, we had to increase the casein concentration to 20 mg/ml.

The results of the experiments are shown in Table 1. Two chambers measuring "chemokinesis" were withdrawn from analysis due to a mix-up of serum (Table 1b).

No chemokinesis could be demonstrated by the present experimental design (Table 1, b). On the contrary, PMN migration in casein without gradient was even less than in autologous serum (Table 1, a-b, W, $2p < 0.001$).

A significant PMN chemotaxis was induced by 20 mg/ml casein, as compared to the non-gradient serum control (Table 1, a-c, $2p < 0.001$). The dispersion of individual values was, however, wide and skew. In two preparations, from donors 6 and 16, no chemotaxis could be demonstrated.

Pre-incubation of PMNs with 0.01 μg/ml vinblastine produced a partial inhibition of PMN chemotaxis, which was significant statistically (W, $2p < 0.044$). The vinblastine-sensitive chemotaxis (Table 1, d) provided 53% of control chemotaxis (Table 1c).

After vinblastine treatment, a chemotactic response still remained (Table 1, d), which provided 47% of control chemotaxis (Table 1, c), and was significantly higher then non-stimulated random movement (W, $2p < 0.049$) and "chemokinesis" (Table 1, b-d, W, $2p < 0.001$).

Discussion

The main findings of the present study were as follows.

1. PMN chemotaxis was induced by casein in 100% autologous serum on increasing the top gradient casein concentration to 20 mg/ml (Table 1, c).
2. Under non-gradient conditions, 20 mg/ml of casein inhibited PMN migration (Table 1, b).
3. Vinblastine, 0.01 μg/ml, decreased PMN chemotaxis to 47% of the non-vinblastine value (Table 1, c-d).

It is an interesting fact that casein induced directional PMN migration in autologous serum in a gradient which approached 20 mg/ml cell suspension. This casein concentration corresponded to the mean casein concentration in human colostrum [8], a medium which was reported by other authors to contain actively migrating lymphocytes [9, 10].

In contrast to the casein gradient, a casein non-gradient concentration of 20 mg/ml significantly inhibited PMN migration (Table 1, b), both in comparison with non-gradient autologous serum (Table 1, a) and in comparison with a casein gradient in autologous serum (Table 1, c). This finding was in agreement with previous results by bottom counts in modified Boyden chambers; 80–95% of PMN chemotaxis was abolished by therapeutic concentrations of microtubule antagonists [3–5], leaving scarce room for MA-resistant modes of migration in the casein gradient.

It is reasonable to assume that the present PMNs in non-gradient casein were overburdened by a continuous abundance of attractant, analogous to the chemotactic "deactivation" or "desensitization" reported by previous authors using other

experimental systems [11]. Such a chemotactic deactivation is thought to be due to an increased adhesivity of the leukocytes [12–14]. In vitro deactivation was reported to have an in vivo correlation with severe infections [15, 16].

Vinblastine, 0.01 μg/ml, inhibited PMN chemotaxis to 53%, which was statistically significant. This finding was in agreement with numerous previous findings showing that microtubule antagonists in therapeutic concentrations inhibited leukocyte chemotaxis [1–5, 17–19]. It should, however, be emphasized that MA inhibition of PMN chemotaxis varied in different assay systems; 80–95% were measured in bottom counts in modified Boyden chambers [3–5], 30–40% by the leading front technique [1, 2], and as absent or slight by migration under agarose [20–22].

After chemotaxis inhibition by vinblastine (Table 1, d), there was still a PMN migration significantly higher than control migration in non-gradient serum (Table 1, a, 2 $p = 0.049$). There are at least two possible explanations of this observation.

A vinblastine concentration of 0.01 μg/ml could be as close to the threshold concentration of chemotaxis inhibition as to produce only a partial inhibition. In a previous study, however, vinblastine 0.01 μg/ml inhibited PMN chemotaxis to 87%, i.e. as much as did higher concentrations of vinblastine [4].

Another possible explanation of the partial vinblastine inhibition of PMN chemotaxis in the present study is that the experimental conditions used – autologous serum and a steep casein gradient – induced a MA-resistant PMN chemotaxis in addition to the MA-sensitive PMN chemotaxis seen in previous experiments with 20% serum and a less steep casein gradient [3–5].

It is conceivable that PMN migration and PMN chemotaxis are complex processes, composed of partial processes and triggered by a wide variety of attractants, such as serum factors, casein, factors derived from leukocytes or bacteria [1, 23, 24]. In a previous study, evidence was provided that microtubule antagonists inhibited the release of chemotactic factor from adherent and phagocyting leukocytes [25]. By inference, all other sorts of chemotaxis provide "MA-resistant chemotaxis".

The Boyden chamber technique of quantifying PMN migration is thought to be less precise and reproducible than the leading-front technique [23] and migration under agarose [26]. Nevertheless, since different methods appear to measure different combinations of the partial processes of PMN migration and PMN chemotaxis, it is suggested that modifications of the Boyden chamber technique are useful in the analysis of selected problems.

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Current Treatment of Patients with Inhibitors to Factor VIII

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The development of inhibitors against factor VIII or IX in hemophilic or non-hemophilic patients is a relatively rare condition, but basic and clinical studies of these inhibitors have been extremely fruitful and have led to the following: a better understanding of the structure–function relationships of the factor VIII and IX molecules; increased knowledge of the genetic defect in classic hemophilia and hemophilia B; newer insights into antibody formation against clotting factors; and an increased number of therapeutic options for the treatment of patients who have inhibitors to factor VIII or IX.

The prevalance of inhibitors to factor VIII:C in the hemophiliac population of the U.S. was obtained in the NHLBI multi-center trial using 1500 patients and is estimated to be 14.5% [1]. The vast majority of these inhibitors occurred in severely affected hemophiliacs less than twenty years of age with factor VIII:C levels of less than 0.03 u/ml. However, occasional older patients or those with F. VIII:C greater than 0.03 u/ml did develop inhibitors.

Keywords: factor VIII, factor IX, inhibitor

Characteristics of Inhibitors to Factor VIII:C

Early studies which supported the hypothesis that antifactor VIII:C inhibitors were antibodies included the demonstration that factor VIII inhibitors could be completely saturated by factor VIII [2]. Later it was shown that rabbit antihuman IgG neutralized antifactor VIII inhibitors, a clear indication that these inhibitors were antibodies [3]. These observations were followed by the classical studies of Shapiro who showed that factor VIII inhibitors in his patients were IgG immunoglobulin with both IgG heavy chains and Kappa light chains [4]. While other studies have also demonstrated heterogeneity of the antifactor VIII inhibitors the high preponderance of antibodies of the IgG4 heavy chain subclass and kappa light chain class is striking.

Factor VIII inhibitors are more likely to occur in severely affected patients, presumably because such patients lack the factor VIII antigen and transfused VIII is recognized by these hemophiliacs as foreign material. It has been shown that factor IX inhibitors have developed in patients with factor IX deficiency who lack the gene for factor IX protein, Gianelli [5] and Antonarakis [6] has found deletion of the gene in 1 of 4 inhibitor patients with severe factor VIII disease gene rear-

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rangement and recombination in two and a normal gene in the fourth patient. There have been no convincing reports of factor VIII inhibitors occurring in hemophiliacs who have not been exposed to exogenous factor VIII. Investigators at the Genentech Laboratories have not found a change in gene structure in 14 inhibitor patients examined. It is known, however, that mildly affected hemophiliac patients may develop factor VIII antibodies and these inhibitors may be different than those in severely affected patients since in most cases the inhibitors disappear and do not recur upon rechallenge with factor VIII. It may be that the antibodies occurring in mild hemophiliacs are autoantibodies.

Types of Inhibitors

Patients with inhibitors to factor VIII can be divided into two categories: low responders and high responders. Patients classified as low responders have maximal inhibitor titers of 2 to 5 Bethesda units/ml even when challenged with factor VIII. Low responders make up approximately 20% of the inhibitor population. These inhibitors may disappear and not return with factor challenge. Patients classified as high responders have an anamnestic response to factor VIII infusions, i.e. have a rise in titer of the antibody when the patient is rechallenged with factor. The inhibitor titers are usually in excess of 10 Bethesda units/ml. Such patients make up approximately 50-60% of inhibitor patients. The authors have observed that each patient appears to have a maximal titer of the inhibitor production that is not exceeded with each factor challenge. The implications are that high and low responder patients are on the same spectrum but low responder patients have a lower maximal threshold.

Inhibitors to Factor VIII: Von Willebrand's Disease

Inhibitors to von Willebrand factor have been described in patients with severe homozygous von Willebrand's disease. These inhibitors may be specific for the von Willebrand factor, the factor VIII:C, or F. VIII antigen, and unlike inhibitors to factor VIII:C, they are not time or temperature dependent [7].

Antibodies directed against both the moieties of FVIII–VWF complex have also been found in the acquired von Willebrand syndrome (AVWS) that develop in association with immunological and other underlying disorders.

Fifteen cases of VWD complicated by the development of antibodies have been reported. The prevalence of reported antibodies in severe VWD seems to be roughly similar to that of F. VIII antibodies in severe hemophilia A [8]. The small number of reported cases makes it difficult to thoroughly evaluate whether or not the development of antibodies increases mortality in severe VWD.

Therapeutic Alternatives in the Treatment of Inhibitor Patients

When a patient shows a lack of response to conventional F. VIII therapy, the presence of an inhibitor should be suspected, and the plasma analyzed for its

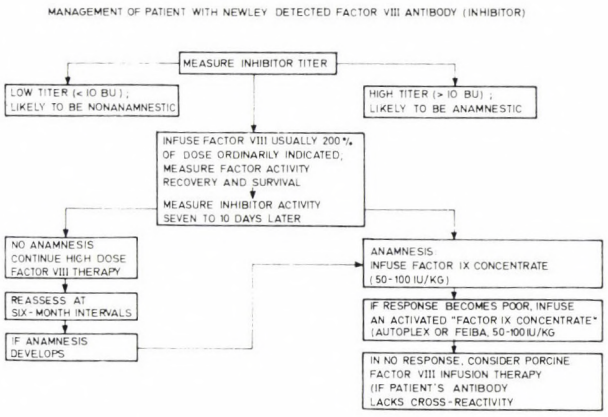

Fig. 1. Scheme from Aledort

presence. The assay recommended in the U.S. is the Bethesda assay [9] and the most commonly used assay in Europe is the New Oxford Unit. When the inhibitor is confirmed, it is prudent to follow the patient at 2-week intervals with repeat titer determination to ascertain whether the inhibitor may disappear. Additional F. VIII challenge dose should then be given and weekly titers done to determine whether an anamnestic type inhibitor is present (see scheme from Aledort, Fig. 1)[28].

There are two main approaches to the treatment of patients with factor VIII inhibitors. The first is to attempt to either temporarily remove or permanently eradicate the inhibitor, and the second is to try to achieve haemostasis when the necessity arises in the continued presence of the inhibitor using one of a variety of therapeutic approaches.

The inhibitor eradication type of treatment has been attempted using plasma exchange, affinity absorption of antibodies from plasma, immuno-suppressive drugs, intravenous infusions of gamma globulin concentrates and the production of immune tolerance using either high or low dosage human factor VIII. Although all of these methods have been reported to be successful in certain patients, none has been found to be universally acceptable. Each of the modalities of treatment will be discussed in detail.

Plasmapheresis

Removal of an antibody has been accomplished for many disorders using plasma exchange. The most efficient of these appears to be through the use of the IBM or Haemonetics machine to extract plasma from the whole blood through the machine. Fluid replacement and blood volume are maintained with saline. Nearly complete removal of the inhibitor titre can be accomplished when the procedure is repeated for 3 to 4 days, when four or more complete blood volumes are passed

through the machine. The measure however is temporary and return of the inhibitor can be anticipated [10].

Immunoabsorption

Antibodies against factor VIII and factor IX have been removed from the patient's plasma by extracorporeal adsorption to protein A, and in hemophilia B also by specific immunoadsorption in a continuous, extracorporeal whole blood system.

Since the antibodies formed in both hemophilia A and B, as well as in the acquired forms are immunoglobulins, they can be absorbed from plasma to protein A, in high titer.

To induce equilibrium between the intra and extravascular space, and allow for more complete removal of the inhibitor, it is sometimes useful to perform the adsorption in a few consecutive days or within short intervals. The technique can be used in small children and the elderly. However, as the extracorporeal volume during the procedure is about 600 ml, the patient's fluid balance needs to be carefully watched. When antibodies are removed by protein A adsorption, intravenous immunoglobulin is given after the procedure in an attempt to induce tolerance.

In one 10-year old severe hemophilia B patient, about 270 000 units of anti-factor IX were removed by passing this patient's blood volume four times through the Factor IX-Sepharose columns. After following this procedure for two consecutive days, his titer decreased from 125 BU/ml of plasma to 7.2 BU/ml and conventional substitution therapy could then be given [11]. This procedure has obvious merit and needs to be confirmed, but it also has a short term effect on the inhibitor titre.

Immunosuppressive Drugs

Various immunosuppressive drug regimens have been used for hemophilia inhibitors. Corticosteroid therapy alone has been ineffective for the patient with a congenital clotting factor deficency and an inhibitor. Despite animal studies documenting the effectiveness of both azathioprine and cyclophosphamide as immunosuppressive agents in hemophiliacs, therapeutic benefit has been largely restricted to cyclophosphamide. Cyclophosphamide was associated with the disappearance and the persistence of inhibitors when used as a single intravenous dose (6.25 mg/kg) or when combined with factor VIII as reported by Hultin et al. and in agreement with previously reported cases [12].

Similar regimens of combined cyclophosphamide and specific factor replacement have been claimed to be somewhat beneficial. Lusher and Evans treated two factor VIII deficient hemophilics with intravenous cyclophosphamide 10 mg/kg, followed immediately by factor VIII concentrate and an additional 3-day course of oral cyclophosphamide [13]. No subsequent rise in antibody titer was noted.

However, measurable factor VIII levels were not achieved in these patients. The authors noted that a delay in reappearance of the antibody for as long as 3 months could be achieved by this regimen, providing factor VIII doses were large enough to yield a level of 50% or more and neutralized the antibody. Therefore immunosuppression was effective in reducing antibody production, but the anamnestic response was not delayed. Dormandy had collected in the past the published and unpublished experience of many investigators with immunosuppressive therapy of 52 severely affected hemophiliacs with antibodies [14]. This review documents that some degree of therapeutic success was achieved on 40 occasions in 18 of 25 patients by using cyclophosphamide and factor VIII. Thus, immunosuppressive therapy may play a useful, though not completely defined, adjunctive role.

Production of Tolerance

Immune tolerance regimens are another type of treatment that has been used for inhibitor patients. Recent observations have suggested that when factor VIII concentrates are given for a prolonged period, the inhibitor titer falls, sometimes to undetectable levels. These observations have been shown by Rizza and Matthews, who recently reported their experience using factor VIII replacement therapy in conventional dosage for hemorrhagic episodes [15]. Twenty-four patients were observed over a number of years; the inhibitor titre disappeared or fell in approximately half their patients. No specific mechanism for the reduced inhibitor levels has been presented.

Recently, Brackmann and co-workers from the Bonn Hemophilia Center have reported the use of long-term twice daily high-dose factor VIII replacement together with concurrent administration of activated factor IX concentrate in a large number of patients with both high and low responding factor VIII inhibitors. Of 17 patients with high responding inhibitors, 15 have completed therapy and all but one of these patients has had complete disappearance of their inhibitor as judged by post infusion fall off and recovery of factor VIII. Eradication of the inhibitor occurred within an average of 25 months from the initiation of treatment [16]. However the cost of the large amounts of factor VIII is extremely high and this method therefore is currently not feasible in many countries. In addition the commitment of the patient to receive twice daily infusions cannot always be sustained.

White et al. reported a case in which a patient was treated with a continuous high-dose factor VIII replacement regimen, modified slightly from the Bonn protocol [17]. Although this patient's inhibitor did not completely disappear the patient achieved tolerance as evidenced by therapeutic levels of factor VIII after infusion and could safely undergo major surgical procedures. In contrast to the Bonn protocol, activated factor IX concentrates were not used which conceivably might have contributed to the failure of the inhibitor to disappear. Many of the patients reported by Brackmann and Gormsen remain responsive to factor VIII long after treatment was stopped, suggesting that tolerance is indeed produced and not merely temporary desensitization.

Gomperts et al. [18] reported a case in which high dose factor VIII concentrate was infused over 12 months in a child with hemophilia A and a high titer inhibitor. This regimen was successful in producing clinical tolerance to the factor VIII and was associated with a fall of inhibitor titer from 85 BU to <1 BU. Therefore several centers have shown that tolerance can be produced in these patients by repeated factor VIII infusions with immune suppression of antibody production.

Human Factor IX Complex Concentrates

The alternate form of treatment which may achieve hemostasis for the inhibitor patient is the use of Human Prothrombin Complex Concentrates (PCC). The rationale for use is based on the hypothesis that hemostasis is brought about through the activation of thrombin via the extrinsic clotting pathway. The mode of action of PCC which brings about hemostasis is unknown. Traces of activated VII, IX and X have been found in all of the PCC but these do not seem to be the active principle which activates the clotting system.

In 1969 Breen and Tullis reported that prothrombin-complex concentrates given to a patient with Hemophilia A produced shortening of the silicone clotting time and cessation of hemorrhage [19]. The potential therapeutic implications of this observation were noted in 1972 by Fekete et al., who reported beneficial clinical results with activated prothrombin-complex concentrates in patients with hemophilia A and factor VIII inhibitors [20].

Subsequently, other investigators began to use both prothrombin-complex concentrates commercially available in the United States, Konyne and Proplex, with seemingly good results, using the same dosage of 25–30 u/kg as was customary when using factor VIII. But by late 1976, some physicians believed that the regular commercially available factor IX products were no longer effective in the control of bleeding and others found that substantially increased dosage was necessary to achieve hemostasis. In a controlled study reported by Lusher et al. for the treatment of hemarthrosis, dosage levels approximating 75 factor IX u/kg of of body weight were found effective in 75% of cases, using the standard factor IX products [21]. Recently other PCC have been used and found effective for hemostasis in the treatment of hemarthrosis when used in similar high dosage. The repeated use of these products appeared safe when used alone. When antifiberinolytic agents have been used concomitantly; however deep vein thrombosis and pulmonary thrombosis, as well as myocardial infarctions have been reported [22].

Activated Human Factor IX Complex

Efforts have been subsequently made to produce activated prothrombin complex concentrates which would be safe and effective in the control of bleeding episodes in inhibitor patients. One such product, Feiba Immuno, was prepared from normal human plasma by Österreichisches Institut für Hämoderivate and contains an activity which appears to by-pass factor VIII in hemostasis. Another is

Autoplex, the activated product manufactured by Hyland Laboratories (Costa Mesa, California) whose active substance is different from that found in Feiba.

Feiba has been used extensively in Europe and the U.S. for treatment of bleeding episodes in patients with inhibitors who have few available therapy alternatives.

In one study in the U.S. a total of ninety-three percent of 165 bleeding episodes (hemarthrosis, hematoma, and mucous membrane bleeding) were controlled within 72 hours using 80 units per kg. In only seven percent or 12 episodes, was the product not effective, and few reactions were seen. Therefore, Feiba appeared to have a high degree of efficacy in the control of bleeding episodes in patients with factor VIII inhibitors. [26].

Anti-inhibitor coagulant complex, Autoplex has been used extensively in the United States and has been found useful for multiple bleeding episodes in hemophiliacs with inhibitors. When used for hemarthrosis at 50–80 u/kg Abilgaard et al. found 95% of the patients responding after 50 u or greater were used, and with 25% responding when 25–50 u were used [23]. Feiba and Autoplex are different in substance and appear to work in some patients better than others. Objective data for response however is difficult to find, and the majority of the data remains subjective in nature. However, these remain the standard most widely used short term therapies for the patient.

Porcine Derived Factor VIII

The use of a porcine-derived factor VIII concentrate also has application in the treatment of inhibitors. This form of treatment has the advantage of being readily monitored by measurement of the level of circulating factor VIII in the patient's plasma and thus dosage regimens can be more easily evaluated and likely clinical effect predicted. The current product, Hyate:C, manufactured by Speywood Laboratories, England, is a highly purified freeze dried concentrate of porcine antihemophilic factor (factor VIII:C). The dosage of Hyate:C required for control of bleeding varies for individual patients, and is calculated using the same criteria used for human F. VIII following a recovery and survival study. On rare occasions Hyate:C may give rise to acute infusion reactions, such as anaphylactic shock. Other reactions such as fever, chills, headaches, nausea, vomiting and skin rashes may also occur but are easily controlled with Benedryl and/or steroids. The only contraindication for its use is related to the cross-reactivity between the human and porcine F. VIII. Patients with greater than 50 B.U. inhibitor usually have a cross-reactivity of the human F. VIII with porcine F. VIII, which may render the porcine product inactive. Therefore, the patient's plasma must be tested with the porcine F. VIII to determine eligibility for use [24].

If a mean antibody cross-reactivity of 25–30% is assumed, it can thus be predicted that patients with an anti-human factor VIII titer of greater than 45–60 Bethesda units would be unlikely to benefit from the treatment with Hyate:C unless their antibody shows an unusually low affinity for porcine factor VIII [25].

Von Willebrand's Syndrome Inhibitors

Treatment of patients with severe VWD complicated by antibodies is aimed at the management of acute bleeding episodes. It is usually possible to achieve measurable F. VIII:C plasma levels with high dose cryoprecipitate or concentrates, although low titer inhibitors against the VWF:Ag and VWF:RICOF appear clinically significant. This approach can usually control soft tissue and post-operative bleeding. It is much more difficult to augment VWF:Ag and VWF:Ricof, to shorten the bleeding time and to stop mucosal bleeding. For mucous membrane bleeding, soft tissue and joint bleeding, Feiba at 90–100 u/kg has been useful in this type of patient [26].

Management

Low Responders

The following criteria are suggested for the management of the patient with a persistent low titre inhibitor to factor VIII. If the patient is actively bleeding or requires surgery and the inhibitor titer is less than 5 Bethesda units, factor VIII concentrate alone is usually sufficient to control any bleeding provided larger amounts of concentrate are used. The amount of factor to use cannot be calculated since the kinetics of action of all inhibitors is different. The optimal amount must be found by trial, but a useful rule of thumb is to give twice as much Factor VIII to a low titer inhibitor patient as one would to a noninhibitor patient.

High Responders

If the titer is greater than 5 Bethesda units, bypass of the inhibitor with either prothrombin complex concentrate or an activated prothrombin complex concentrate will usually be necessary. The cost differentials between the activated and non-activated products are such that one cannot be completely dogmatic about the approach to treatment. Suggestions for treatment are made with this problem of cost in mind.

Acute hemarthrosis in the high responder patient can be treated with non-activated PCC, 50–80 u/kg factor IX given at 12-hour intervals, with up to 3 doses. If bleeding is not controlled, then an activated product (APCC), Feiba or Autoplex at 100 u/kg should be used, using Feiba at 80–100 u/kg or Autoplex at 50–100 u/kg, depending on the magnitude of the bleeding episode. Many studies have shown APCC to be useful as the primary modality of treatment using the doses mentioned above.

Central Nervous System: Documented

It is difficult to know which head injury to treat and which to follow expectantly, particularly in a small child prone to repeated head injury. Eyster has shown

that the latent period between minor injury to onset of symptoms may be as long as two weeks [27]. The practice in our clinic is the following: the clinic is to be notified of all head injuries, and the magnitude of the injury discussed with the staff. For those on Home Care the child is to be infused at home with 100 u/kg of PCC immediately and brought into hospital for neurologic evaluation, if neurologic symptoms are present or the history is significant. A CAT Scan is done and if bleeding is found the patient is admitted for a minimum of 14 days of treatment. If no bleeding is found the child is treated daily for a total of three days with PCC at 100 u/kg. Confirmed central nervous system bleeding or other life threatening bleeding should be treated with porcine factor VIII when possible (if anti-porcine titer is less than 3 BU). If porcine factor VIII cannot be used then activated PCC (100–110 u/kg) should be given every 4–8 hours for at least 72 hours and continued daily for a minimum of 14 days depending on the magnitude of the bleeding. The protracted length of therapy has been necessary because of the high rate of recurrent bleeding if therapy is less. Life threatening bleeding refers to bleeding in the retropharynx, retroperitoneal and gastrointestinal tract. Large amounts of blood may be lost before being detected, and a small amount of blood in the retropharyngeal area may cause airway obstruction.

Emergency Surgery

Surgery can be carried out on inhibitor patients using the product most suited to the patient's current inhibitor titer. A low titer high responder can usually be treated with high dose F. VIII until the inhibitor titer returns at 5–7 postoperative days. At that time, treatment should be changed to activated PCC. For some simple procedures, physicians have turned to the APCC for hemostasis from the beginning of the procedure. If the titer is somewhat higher some physicians would prefer to use porcine factor VIII. Examples are available from multiple authors using all modalities.

Elective Surgery

In high responder inhibitor patients elective surgery is usually not recommended and is only recommended if the potential outcome is sufficient to justify the risks and expense. These must be thoroughly understood by the patient. Patients with high titer F. VIII inhibitors have been successfully taken through joint replacement procedures (hip and knee) at the Hospital for Special Surgery–Cornell University Medical Center (see Figs 2 and 3). In patients with titer levels of 10 Bethesda units or more therapeutic plasma exchange can be used for both elective and emergency procedures, using either extracorporeal adsorption of the plasma to sterile protein A-Sepharose® in columns, or plasmapheresis with normal plasma or concentrate given after the last pheresis. Human F. VIII can be used subsequently for hemostasis until the antibody returns. Continuous infusion of F. VIII has been quite useful for this purpose.

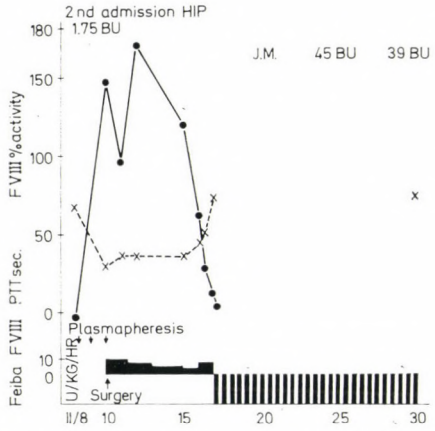

Fig. 2. Management of patient for elective surgery, use of plasmapheresis, continuous factor VIII infusion, and Feiba

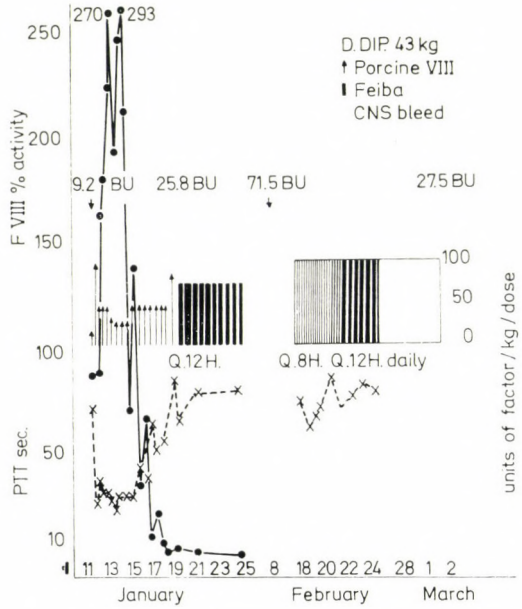

Fig. 3. Management of patient for central nervous system bleeding, use of porcine factor VIII (Hyate: C and Feiba)

When dental extractions are done in high responders, non-activated PCC can be used with EACA started 4 hours after the last dose of PCC. However, some oral surgeons have been able to use Avitine and EACA alone in many cases and PCC has not had to be administered.

Cost Analysis of Therapy

With the recent change in management of inhibitor patients a clearer definition of inhibitor responses reveal a cohort of patients who respond to factor VIII. Unactivated F. IX, Autoplex and Feiba, the activated F. IX products, and newly prepared porcine F. VIII have been effectively used to treat minor and major bleeding episodes in patients with high titre antibody.

A study was done to analyze hemophilia patients in the downstate area of New York, over the period 1981–1982, comparing patterns of replacement material for factor VIII and IX deficient patients with and without inhibitors.

The study showed that not only was factor utilization by the inhibitor patient substantially higher as compared to non-inhibitor patients in 1981, it increased further to double that of factor VIII patients in one year, e.g., more than twice as many u/kg/yr were used in 1982 as compared to 1981 (1427 versus 3050).

While the cost of therapy for factor VIII patients stabilized at $9000 per year, the costs of factor replacement for inhibitor patients rose to a high of $32860/pt/yr in 1982. The inhibitor patients are therefore a substantial drain on the blood supply as well as the limited health care resources of the world.

Despite tremendous advances in this field, many questions still remain unanswered, but the current foundation of basic and clinical knowledge about inhibitors offers opportunities for both effective treatment and further research.

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Ganglioside Composition in Common Acute Lymphoblastic Leukaemia

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Human leukaemic lymphocytes of the common acute lymphoblastic leukaemia (cALL) subtype were analysed for ganglioside composition by overpressured layer chromatography-densitometry. Two major ganglioside components, GM_3 (58.0–77.8% of the total sialic acid content) and GM_1 (20.7%–29.2%), and some minor compounds, GM_2 (trace amounts to 9.6%) and unidentified gangliosides with chromatographic mobility between GM_1 and GD_{1a} (trace to 12.6%), were isolated from these cells. The relative amount of GM_3 in cALL cells was found to be about double that in normal lymphocytes (65.7% vs. 37.7%), but lower than in chronic lymphocytic leukaemia (CLL) cells (65.7% vs. 81.5%). The cALL cells contained trace amounts of the ganglioside GD_3, a compound present in CLL cells (5.6%) but absent in normal lymphyoctes.

Keywords: cALL, ganglioside, GD_3, GM_3

Introduction

Gangliosides are sialic acid containing glycosphingolipids (GSL) located mainly on the surface of virtually all mammalian cells. Dramatic changes in GSL composition and metabolism have been observed in cells taken from a large variety of human cancer including leukaemia (reviewed in [6]). Changes in GSL composition of leukaemic cells are essentially quantitative rather than qualitative. A ganglioside (GD_3) which is not found in normal human leukocytes has, however, been demonstrated in acute myeloblastic leukaemia [12] and chronic lymphocytic leukaemia (CLL) cells [5].

By the overpressured layer chromatography method (OPLC) elaborated by us [9], CLL lymphocytes of B-cell origin were shown to be different from normal lymphocytes in their ganglioside profile, qualitatively by the presence of GD_3, and quantitatively by the predominance of less polar compounds on the malignant cells

Abbreviations: ALL, acute lymphoblastic leukaemia; cALL, common acute lymphoblastic leukaemia: cALLA, common acute lymphoblastic leukaemia antigen; CLL, chronic lymphocytic leukaemia; CyIg, intracytoplasmic immunoglobulin; E, sheep erythrocyte rosettes; EA, erythrocyte-antibody rosettes; EAC, erythrocyte-antibody-complement rosettes; GSL, glycosphingolipids; OPLC, overpressured layer chromatography; SmIg, surface membrane immunoglobulin; TLC, thin-layer chromatography; Abbreviations of the ganglio-series gangliosides such as GM_3, GM_2, GM_1, GD_3, GD_{1a}, GD_{1b}, GT_{1a}, GT_{1b} follow the system of Svennerholm [10].

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[1]. With respect to the distribution of ganglioside species in human acute lymphoblastic leukaemia (ALL) several inconsistencies could be found in previous reports [7, 11, 12].

With the recently developed OPLC, the present study has been focussed on elucidation of the composition of gangliosides extracted from ALL cells. To the best of our knowledge, this is the first report on ganglioside composition of ALL cells identified as common ALL (cALL) subtype by multimarker analysis.

Materials and Methods

Patients

The studies were performed on samples from three newly diagnosed, untreated patients admitted to the National Institute of Haematology and Blood Transfusion in Budapest in April and May 1983. Classification of the ALL cells was based on morphological and cytochemical examinations of peripheral blood smears, on characterization of the cell surface markers: surface membrane immunoglobulin (SmIg) and cytoplasmic immunoglobulin (CyIg), sheep erythrocyte rosettes (E), erythrocyte-antibody rosettes (EA) and erythrocyte-antibody-complement rosettes (EAC), and on immunofluorescent analysis employing a panel of monoclonal

Table 1

Clinical and laboratory characteristics of three patients with ALL[1] at initial presentation

No.	Initials	Age	Sex	WBC count in PB $\times 10^9$ l^{-1}	Percentage of lymphoblasts in PB	FAB[2] classification	Immunologic subtype	Survival time in months
1	L.G.	37	Male	120.0	100	L2	common ALL[3]	8
2	T.L.	29	Male	43.2	100	L1	common ALL	2
3	G.F.	16	Male	46.8	100	L2	common ALL	18

[1] Abbreviations: ALL, acute lymphoblastic leukaemia; PB, peripheral blood; WBC, white blood cell.
[2] L1 and L2 classification by the French-American-British scheme [2].
[3] By virtue of the expression of the common acute lymphoblastic leukaemia antigen, and by lacking the markers of T- and B-cells and intracytoplasmic μ heavy chains.

antibodies (J5, OKT-3, OKT-4, OKT-8, My7, B1, Mo1, Mo2). The pertinent clinical and laboratory characteristics of these patients at the time of initial presentation are listed in Table 1.

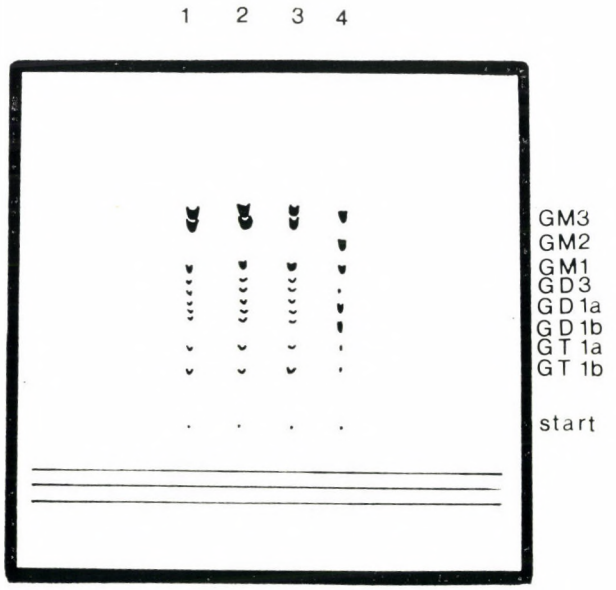
Isolation and purification of lymphocytes

Leukaemic blasts were collected by machine leucapheresis (Patient L. G.) with an IBM Blood Cell Separator (Armonk, New York) or obtained by phlebotomy of 100 ml of blood (Patients T. L. and G. F.). Contaminating red blood cells were eliminated by dextran gravity sedimentation and osmotic lysis, while platelets were removed by differential centrifugation as described in detail previously [1]. The purified cell preparations had a white cell/red cell ratio of 50 : 1 and white cell/platelet ratio of 5 : 1. Viability of the cells was greater than 90%.

Purified preparations of normal lymphocytes were obtained from healthy blood donors by the same procedures.

Isolation of gangliosides and ganglioside species

Techniques used for isolation, identification and quantitation of the gangliosides have been described previously [9]. Briefly, the total lipid extract obtained from the purified cell preparation was separated into lipid classes by ion-exchange chromatography on DEAE-Sephadex A-25 column and by reversed phase high-performance liquid chromatography. The interclass fractionation of sulphatides and gangliosides, and further intraclass separation of gangliosides were performed with OPLC on the same thin-layer chromatographic (TLC) plate with step gradient development. Gangliosides were visualized on the chromatograms by resorcinol-



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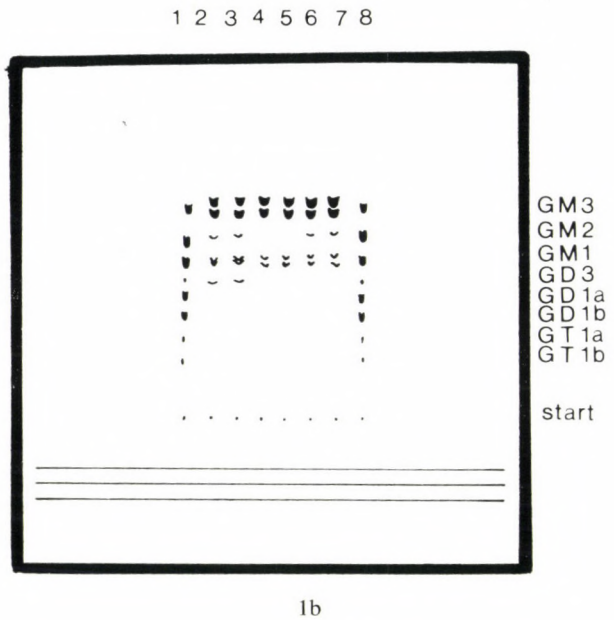

1b

Fig. 1. Separation of gangliosides from normal lymphocytes (a) and common acute lymphoblastic leukaemia cells (b) by overpressured layer chromatography. The plate (10× 10 cm Merck HPTLC Si 60, E. Merck, Darmstadt, F. R. G.) was developed with the solvent systems 1. chloroform : methanol = 7 : 3 (v/v), 2. chloroform : methanol : 0.25 KCl/H_2O = 55 : 36 : 9 (v/v/v), then stained with rescorcinol-HCl reagent at 100 °C for 15 min. All spots were purple. a — Samples 1, 2, 3 were normal lymphocytes; Sample 4 was ganglioside standard. b — Samples 1, 8 were ganglioside standards; Samples 2, 3 were cells from Patient T. L.; Samples 4, 5 were from Patient L. G.; Samples 6, 7 were from Patient G. F

HCl. Ganglioside species were identified by comparing the mobility of the resorcinol-positive bands with that of the standards. Ganglioside compositions were analysed by thin-layer chromatography-densitometry. All values were determined from at least three parallel chromatographic measurements.

Results

Figure 1 shows the chromatographic separation on silica gel thin layer plates, of gangliosides from human ALL cells and normal lymphocytes. A mixture of gangliosides GM_3, GM_2, GM_1, GD_3, GD_{1a}, GD_{1b}, GT_{1a} and GT_{1b} was used as standard.

The TLC patterns of the three ALL cell samples were essentially similar (Fig. 1b). Two sets of prominent doublets – cochromatographed with GM_3 and GM_1 – were detected in all samples examined. Additional faint bands located at

the positions of GM_2 (lanes 2 and 3, 6 and 7) and between GM_1 and GD_{1a} (lanes 2 and 3) were also visible. In contrast, normal lymphocytes had a more complex pattern, with at least 9 identifiable bands which migrated like GM_3, GM_2, GM_1, GD_{1a}, GD_{1b} and between GM_1 and GD_{1a} (Fig. 1a).

The amount of gangliosides present in the bands of TLC plates was quantitated by densitometric scanning and these data are summarized in Table 2. In ALL

Table 2

Ganglioside composition[1] of ALL[2] cells as compared to that of CLL and normal lymphocytes

Patients		Ganglioside content, % of the total					
		GM_3	GM_2	GM_1	GD_3	GD_1	GM_1–GD_1[3]
ALL cells	L.G.	77.8	trace	22.2	trace	—	—
	T.L.	58.0	8.7	20.7	—	—	12.6
	G.F.	61.2	9.6	29.2	trace	—	trace
	Mean	65.7	6.0	24.0	trace	—	trace
CLL cells[4]		81.5	trace	7.5	5.6	4.1	—
Normal lymphocytes		37.7	6.1	7.5	—	19.6	29.2

[1] Determined by scanning densitometry of the thin-layer chromatographic plates.
[2] Abbreviations: ALL, acute lymphoblastic leukaemia; CLL, chronic lymphocytic leukaemia. The nomenclature for gangliosides is based on the system of Svennerholm [10].
[3] Unidentified compounds with lower TLC mobility than GM_1 and faster than GD_1.
[4] Mean value from 6 CLL cell samples.

cells GM_3 was the most abundant component (range: 58.0%–77.8%, mean: 65.7%), and GM_1 the second most abundant component (range: 20.7%–29.2%, mean: 24%). Only trace amounts of GD_3 were detected in two samples (L. G. and G. F.), while a high proportion (12.6%) of gangliosides more polar than GM_1 was found in one sample (T. L.). In comparison with normal and CLL lymphocytes, cALL cells contained about double the amount of GM_3 than did normal lymphocytes (65.7% vs. 37.7%), but less than CLL cells (65.7% vs. 81.5%). The relative amount of GM_1 was strikingly higher in ALL cells than in normal and CLL lymphocytes (24.0% vs. 7.5% and 7.5%, respectively).

Discussion

With the development of new reagents to detect cell surface membrane structures, acute lymphoblastic leukaemia can now be divided into five major subgroups: "null" (undifferentiated or unclassified) ALL, common ALL, pre-B-ALL, B-ALL and T-ALL. Lymphoblasts from the majority (approximately 83%) of ALL

patients in all age groups are "non-B-non-T" cells [8]. They lack SmIg and CyIg, do not express the E-receptor and are not recognised by monoclonal anti-T antibodies. These malignant cells represent an expanded population arising from a clone of B-cell precursors, which originate in the bone marrow and are derived from the common lymphoid stem cell precursor. Two subgroups of "non-B-non-T" cell ALL have been identified. The "null" ALL does not express the common acute lymphoblastic leukaemia antigen (cALLA) defined by monoclonal antibodies (cALL/J5), while cALL expresses the classic cALL/J5 antigen. As far as we know, the present one is the first report on the ganglioside composition of ALL cells of the immunologic cALL subtype identified by multiple marker analysis. The cALL cells in this study had a ganglioside profile characterised by a high proportion of GM_1 (20.7%–29.2%), by the predominance of GM_3 (58.0%–77.8%), by some GM_2 (trace to 9.6%) and trace amounts of GD_3. In comparison with normal lymphocytes, the proportion of GM_3 and GM_1 was greatly increased, whereas that of more polar compunds were significantly decreased in all cALL cell samples examined.

There are several inconsistencies between our results and those in the previous reports, as well as among the findings of different authors. Besides the difference in methodology, cellular heterogeneity of the various ALL cell samples studied may be a reasonable explanation. Thus far the ganglioside composition of only one patient with null ALL has been reported, but the classification of the null-cell type was only based on the fact that the leukaemic lymphocytes neither formed rosettes nor had L3 morphology [12]. Three compounds were identified, i.e. GM_3 (81%), sialosylparagloboside (Spg, 11%) and GM_1 (5%). ALL cells with such markers can however, fall either in the cALL or in the "null"-ALL category. In adults, cALL and null-ALL occur at frequencies not far different from each other (50% vs. 35–40% of the total ALL cases, respectively) as reported by Bowman and Mauer [3]. In the first report on chemical analysis of gangliosides from ALL patients, whose cells were not immunologically subclassified, Tsuboyama et al. [11] showed the presence of GM_3 (83.4%), GM_2 (2.1%), GM_1 (9.8%) and unidentified compounds with TLC mobilities between GM_1 and GD_{1a} (4.8%). GM_1 at high proportions comparable to our findings (17%–25% vs. 20.7%–29.2%), GM_3 (73%–80%) and Spg (2%–4%) were demonstrated in T-ALL [12].

No definitive information is available on whether glycolipids would play a functional role in defining tumorigenicity and metastatic property. Differences in ganglioside composition have, however, been observed in a variety of transformed cells as well as in spontaneous malignancies. In general, high-tumorigenic cell variants or cells from highly malignant, highly metastatic neoplasms showed a low GM_3 content and a high level of GD or GT ganglioside (for review see [6]). Human melanoma is a typical example for this alteration and it is concluded that this pattern is characteristic of malignant vs. normal phenotypes of human melanocytes [4]. In contrast, ALL cells showed a drastic increase in the proportion of GM_3 in comparison with normal lymphocytes, as indicated by us and others [11, 12]. Interestingly, B-cell CLL lymphocytes expressed GM_3 and GD_3 at even much high-

er proportions than did the cells from these ALL patients (81.5% vs. 65.7%, and 5.6% vs. trace amounts, respectively). It is not known whether the increase in the level of GM_3 and GM_1 and the absence of higher compounds (GD, GT) in ALL cells was due to differences in the levels of enzymes that are responsible for the hydrolysis or synthesis of these gangliosides.

In conclusion, as in other types of human leukaemia. cALL cells showed drastic changes in the distribution of GM_3, GM_1 and gangliosides with more complex structures, as compared to their normal counterparts. Both the biological importance and the mechanism of these changes remain to be elucidated.

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New Congenital Deficiency of High Molecular Weight Kininogen and Prekallikrein (Fitzgerald Trait). Study of Response to DDAVP and Venous Occlusion

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The prolonged partial thromboplastin time observed in the plasma of a 36 year old asymptomatic man was related to the reduced prekallikrein activities (coagulant; antigenic; and amidolytic) and the absence of coagulant and immunologic activities of high molecular weight kininogen (HMWKg). The patient's plasma also exhibited impaired surface-mediated fibrinolysis and impaired generation of kallikrein. The coagulation defect was identified as the "Fitzgerald trait". The levels of CH50, C2, C4 and C-1 inactivator were normal.

Venous occlusion in the patient gave rise to a normal release of extrinsic plasminogen activator from the vascular endothelium. The administration of DDAVP led to a FVIII/VWF response which was similar to that obtained in healthy subjects. No alteration could be observed in the contact phase proteins after DDAVP administration.

Keywords: prolonged partial thromboplastin time, prekallikrein, HMWKg, "Fitzgerald trait", DDAVP, venous occlusion

Introduction

High molecular weight kininogen (HMWKg) is a non-enzymatic cofactor in the contact activation reaction, including the surface-dependent initiation of kinin liberation, intrinsic coagulation and the fibrinolytic pathyways [1]. HMWKg is a two-chain, disulfide-linked molecule containing a heavy chain of apparent MW 64 000 and a light chain of apparent MW 46 000 [2]. The light chain is that portion of the kininogen molecule which possesses HMWKg coagulant activity [3], and the major structural antigenic determinants that distinguish HMW or low molecular weight kininogen (LMWKg).

Congenital HMWKg deficiency, either isolated (Fitzgerald trait) or associated to LMWKg (Flaujeac and Williams traits) is very rare and only 7 families displaying such anomalies have been reported [4–9]. Recently it has been suggested that a defective response of Factor VIII/von Willebrand Factor and extrinsic plasminogen activator to infusion of 1-D-amino-8-D-arginine vasopressin (DDAVP) and venous occlusion, respectively, could exist in patients with congenital defects in some of the proteins involved in the contact system, in particular a congenital deficit of F. XII and prekallikrein [10, 11].

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In this report, we describe the coagulation and immunological studies carried out in an asymptomatic 36 year old man who exhibited striking laboratory anomalies in which the absence of HMWKg and reduced preakallikrein levels were detected. Moreover, it was possible to observe that the response of FVIII/VWF to DDAVP infusion and the release of extrinsic plasminogen activator after venous occlusion were not modified by the absence of HMWKg.

Material and Methods

The proband was a 36 years old male with no previous bleeding history. He was hospitalized at the University Hospital for extirpation of a pancreatic cyst. Routine coagulation studies before surgery revealed a very prolonged APTT (Table 1). The family history revealed no consanguinity.

Blood collection. Blood samples were collected by venipucture through a 19-gauge needle and immediately added to 0.13 M trisodium citrate at a ratio of 9:1. Platelet-poor plasma was obtained as previously described [12]. Pooled normal plasma was similarly prepared from 20 normal donors.

Coagulation assays. Factors II, V, VII, VIII:C, IX, X, XI, XII, HMWKg and prekallikrein were assayed in a one-stage test using as substrate platelet-poor plasma obtained from patients congenitally deficient in the respective factors (supplied by Behringwerke A. G., Marburg, FRG) as previously described [13]. Fletcher factor (plasma prekallikrein) deficient and HMWKg factor deficient plasmas, were obtained from George King Biomedical Inc. (Overland Park, KS, USA) Enzymatic activity of prekallikrein was measured using the synthetic chromogenic substrate S-2302 (Kabi Vitrum, Stockholm, Sweden) as described previously [14]. Fibrinolytic activity of euglobulin fractions (dilution 1:10; pH 5.9) was measured by the fibrin plate method, as described by Haverkate and Brakman [15]. Additionally, euglobulin fractions were applied to the plates with ε-1-inactivator (Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands) at a concentration equivalent to the normal plasma level in order to measure extrinsic activators only [16]. In this experiment the results are expressed in millimeters of diameter of the lysed area.

Immunological assays. Quantitative immunological determination of von Willebrand factor antigen (VWF:Ag), HMWKg and its light and heavy chains was carried out according to the method proposed by Laurell [17]. Antigenic activity of prekallikrein was investigated by radial immunodiffusion [18]. Rabbit antisera against prekallikrein, VWF:Ag and C-1-INA, were otained from Behringwerke (Marburg, FRG). Rabbit antisera against total kininogen, HMWKg and light and heavy chains of HMWKg were purchased from Miles Laboratories (Elkhazt, Indiana, USA).

Crossed immunoelectrophoresis of total kininogen was carried out in agarose (1 % w/v) in 0.028 M tris-barbital buffer at pH 8.6 as described by Weeke [19].

Generation of amidolytic activity of plasma kallikrein: This activity was

studied after incubation of plasma with the same volume of a solution of kaolin (10 mg/ml) [20].

Complement studies. Total plasma complement CH50 and antigenic determination of C-1-inactivator (C-1-INA) and C4 components were determined as previously reported [21].

Venous occlusion. This study was carried out after subjecting the forearm of the proband to venous stasis for a 20 min period, maintaining a constant pressure of 10 mm Hg above the diastolic pressure. Blood samples were obtained before the start and at the end of the study. The result obtained in our patient was compared with that recorded in 5 normal individuals under identical conditions.

DDAVP infusion. 0.4 μg/kg was added to 50 ml physiological saline and infused over 30 min. Blood samples were collected immediately before infusion, and 30 and 120 min after it had been started. The results obtained in the proband were compared with those found in five normal subjects after DDAVP infusion.

Results

The coagulation studies in our propositus are shown in Table 1. APTT were prolonged using 3 different activators, cellite, kaolin and ellagic acid, though this anomaly was corrected by the addition of normal plasma. In contrast, APTT was

Table 1

Coagulation studies

	Propositus	Normal
Platelet, 10^9/l	325	150–450
Bleeding time, min	5	<7
Prothrombin time, s	13	12–14
APTT		
Cellite, s	94	32–40
Kaolin, s	126	30–40
Ellagic acid, s	78	26–33
Inhibitor screen (PT, PTT)	negative	negative
Factors II, V, VII, VIII:C, IX, X, XI, and XII, %	>55	>50

only corrected after prolonged incubation with activators (data not shown). Direct measurement of HMWKg in the propositus by a coagulant assay revealed the absence of this activity. Moreover, a prekallikrein assay using Fletcher's trait plasma as substrate showed 20% activity, whereas with an amidolytic assay, activity was found to be 28%. When prekallikrein antigen concentration was measured by the radial immunodiffusion technique, its concentration was found to be 16%.

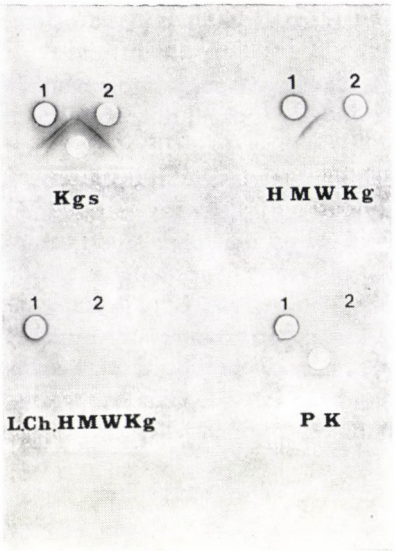

Fig. 1. Ouchterlony analysis of normal plasma (1) and proband's plasma (2) with antisera to total kininogen (Kgs), high molecular weight kininogen (HMWKg), light chain of HMWKg (L. CH. HMWKg) and prekallikrein (Pk)

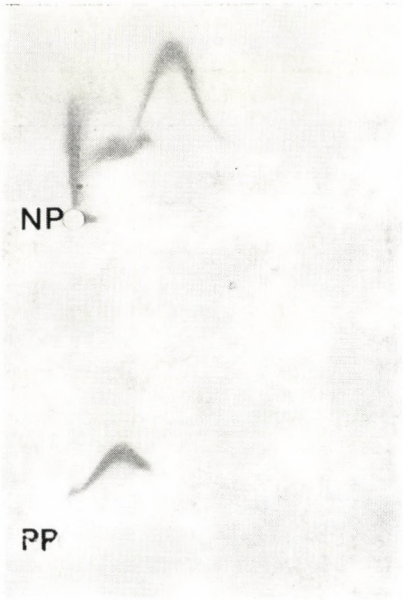

Fig. 2. Crossed-immunoelectrophoresis of normal plasma (NP) and patient's plasma (PP) with anti-total kininogen antisera

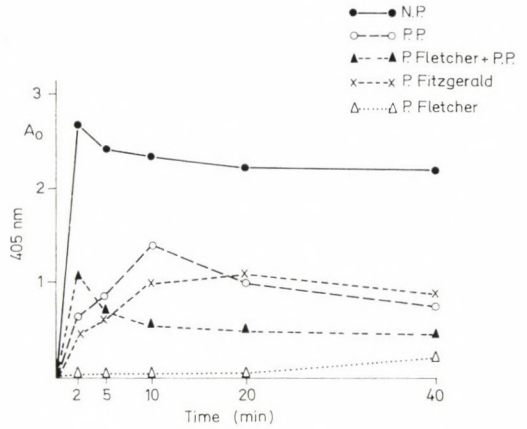

Fig. 3. Generation of plasma kallikrein amidolytic activity by exposure of plasma to kaolin. Normal plasma (●———●); Proband's plasma (○– – –○); Fletcher trait patient (△– – –△); Fitzgerald trait patient (×– – –×); 1 : 1 mixture of Fletcher trait and patient's plasma (▲– – –▲)

The proband's plasma was investigated by double diffusion in agarose against specific antisera (Fig. 1). Only one precipitation line could be detected when assayed against an antiserum against total kininogen, whereas normal plasma exhibited two precipitation lines corresponding to the HMWKg and LMWKg forms. With monospecific antisera against the HMWKg and the light and heavy chains of this protein, no precipitation could be observed (Fig. 1).

Cross immunoelectrophoresis of the patient's plasma, using an antiserum against total kininogen showed the absence of the more anodic precipitation arc, corresponding to HMWKg (Fig. 2).

The generation of plasma kallikrein amidolytic activity after exposure of the proband's plasma to kaolin was deficient and was not corrected by the addition of Fletcher's trait plasma (Fig. 3).

Table 2

Haemostatic changes after DDAVP (0.4 μg/kg) in five normal subjects (expressed as Mean$\pm$ $\pm$SEM) and a Fitzgerald trait patient

	VIII:C	VWF:Ag	FXI	FXII	PK	HMWKg
Fitzgerald trait patient						
Basal	140	109	87	75	28	<1
30 min after DDAVP	376	269	88	73	20	<1
Five healthy subjects						
Basal	98$\pm$8	87$\pm$4	86$\pm$4	100$\pm$5	96$\pm$3	98$\pm$5
30 min after DDAVP	402$\pm$33	259$\pm$8	82$\pm$4	102$\pm$5	98$\pm$4	97$\pm$5

DDAVP infusion ot the patient led to an increase (similar to that expected) in FVIII/VWF activities, whereas the proteins of the contact phase studied did not show any alterations (Table 2).

Table 3

Fibrinolytic activity, measured by fibrin plates, in plasma from Fitzgerald trait patient in basal conditions, after incubation of plasma with kaolin (4 mg/ml) at 37 °C for 30 min, and 20 min after venous occlusion. Results are expressed as diameter of lysis area (mm). PEF = Plasma euglobulin fractions; PEF+INACl = Plasma euglobulin fraction with Cl-inactivator

	Basal conditions		After incubation with kaolin	After venous occlusion	
	PEF	PEF+ +INHCl	PEF	PEF	PEF+INHCl
Fitzgerald trait patient	6.6	5.3	7.3	19.9	14.4
Five healthy subjects	14.2 (range 8.9–16.2)	8.8 (range 4–11)	20.6 (range 20.1–21.3)	20.7 (range 15–24.3)	14.9 (range 10–17.4)

Basal fibrinolytic activity as measured by fibrin plates was reduced. Moreover, kaolin-activated fibrinolytic activity was absent (Table 3). On the other hand, fibrinolytic activity generated after venous occlusion was normal, the response of the extrinsic activator of plasminogen being similar to what was expected (Table 3).

Discussion

The absence of haemorrhagic episodes in patients with a congenital deficiency in some proteins involved in the contact phase reactions is the reason why so few cases have been described up to the present. In our patient, who in spite of showing a prolonged activated partial thromboplastin time did not show any haemorrhagic episodes, it was possible to establish the origin of the anomaly by determining the absence of HMWKg by coagulant assays and immunochemical determinations. As expected, analysis of the patient's plasma revealed abnormalities in surface-mediated coagulation and fibrinolysis and a decrease in kallikrein generation.

Although thrombotic episodes have not yet been reported in patients with congenital HMWKg deficiency, the fact that several individuals with factor XII deficiency have experienced myocardial infarction and venous thrombosis [1] suggests that anomalies in the reactions dependent on the contact phase proteins do not preclude the risk of thrombosis. Likewise, it is not known whether the decrease in surface-activated fibrinolysis observed in vitro handicapped the ability of these individuals to lyse clots in vivo. Moreover, although several reports have demonstrated a depressed neutrophil mobilization in HMWKg congenital defi-

ciency patients [22], in our case, as in others described previously, no special incidence of infection was detected. Even though we are unaware of the physiological and pathological significance of the anomalies observed, they might have some role in the aspects just mentioned; further work is necessary to clarify this point.

Factor XI and prekallikrein both circulate in plasma in the form of a complex with HMWKg [23]. The absence of HMWKg would explain the reduced levels of prekallikrein found by us. On the other hand, factor XI clotting activity was found to be normal; however, since no monospecific serum against this protein was available, it was not possible to demonstrate reduced levels of F. XI antigenic activity in our patient, as reported by Bouma et al. [23] for the plasma of patients with HWMKg deficiency.

Recently, España et al. [24] have suggested an absence of extrinsic plasminogen activator release in a Fletcher trait patient after venous occlusion. These authors postulated the participation of prekallikrein in the release of circulating plasminogen activator in plasma from the vascular endothelium after venous occlusion. Since no reports have indicated whether the absence of HMWKg or low levels of circulating prekallikrein could alter the fibrinolytic response to venous occlusion, we were prompted to study this possibility in our patient. Our findings showed that the presence of HMWKg was not necessary for the generation of fibrinolytic activity after venous occlusion; moreover, in our case low levels of prekallikrein exerted no influence on the release of extrinsic plasminogen activator from the vascular endothelium.

It has been known for several years that the administration of DDAVP, a synthetic analogue of antidiuretic hormone, induced a rapid, marked and transient increase in FVIII/VWF [12]. Gamba et al. [10] suggested that the response of FVIII/VWF to DDAVP infusion could be mediated by contact phase activation, since in patients with factor XII deficiency they observed a lower increase in VIII:C after DDAVP than in the controls. In our patient, DDAVP gave rise to a response which may be considered normal for FVIII/VWF; furthermore, it was not possible to detect any change in the contact phase proteins, as we had found earlier for normal subjects [14].

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Plasma Exchange for Acute Cholestasis in Homozygous Sickle Cell Disease

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A child with sickle cell disease presented acute chest syndrome, acute cholestasis and a neurological syndrome. Bilirubin, LDH, alkaline phosphatase, urea and creatinins were markedly increased. Circulating immune complexes were slightly elevated. The patient improved dramatically after a plasma exchange.

Keywords: sickle cell anaemia, acute cholestasis, plasma exchange

Introduction

Acute and markedly increased jaundice associated with an elevation of conjugated bilirubin is a well recognized complication of homozygous sickle cell (SS) disease. The most common causes are serum hepatitis, extra-hepatic obstruction by gall stones, and acute cholestasis. Acute cholestasis is thought to result from acute sickling in the sinusoids; its course is variable, some cases being benign in spite of marked hyperbilirubinemia [1], and others leading to death with liver failure [2]. Factors determining this variable clinical picture are unknown. Circulating immunocomplexes have been found but their significance is not clear [3]. Partial exchange blood transfusion [4] and plasma exchange (Porfirio Hernández, personal communication) have both been associated with rapid resolution of symptoms in two patients with SS disease and acute cholestasis. In the following we report on a child with SS disease and a serious acute cholestasis with circulating immunocomplexes who improved dramatically after plasma exchange.

Case Report

The patient was a 9 year old, phenotypically Caucasian boy with SS disease and a foetal haemoglobin of 5.3% and glucose-6-phosphate dehydrogenase of 12.2 IU, electrophoresis B. The clinical picture was generally mild and he had received no blood transfusion before admission. On 13/3/82 he was admitted with bone pain, acute chest syndrome, marked jaundice and liver enlargement of 7 cm below the right costal margin. Consolidation of the right lung and a right pleural effusion were demonstrated radiologically. On admission, investigations indicated

hemoglobin 7.6 g/dl, reticulocytes 8.2%, total bilirubin 25 mg/dl, conjugated bilirubin 20 mg/dl.

After a few days of treatment with parenteral fluids and intravenous penicillin the clinical picture improved and on 17/3/82 the chest X-ray was normal. On 27/3/82 he presented once again high fever, increased jaundice, epigastric pain, signs of cardiac failure and generalized seizures that lasted 10 minutes. The cause of the seizures was not clear; there were no evident clinical signs of hepatic encephalopathy but the bilirubin, urea and creatinine levels were so high that they might have been toxic. The possibility of a vaso-occlusive crisis of the CNS could not be ruled out. Exchange transfusion was performed and he improved but 3 days later the seizures returned after which there were periods of lethargy and irritability. The spinal fluid and the fundi were normal, the EEG showed a slow inconstant rhythm (previously this had been normal). Two exchanges with fresh plasma as substitute were performed on 30/3/82 and 1/4/82; 600 ml of plasma was exchanged each time. That amount represented 60% of the total plasma volume. Immediately after the first plasma exchange the clinical state improved and a decrease of jaundice was noted; the neurological signs also disappeared. The child's recovery was complete.

Laboratory investigations before and after plasma exchange are summarised in Table 1. Coagulation studies performed on 30/3/82 indicated Factor II to be 1%

Table 1

Laboratory data

Data	30/3/82 Before plasma exchange	2/4/82 After plasma exchange
Bilirubin (mg/dl) (T/D)	60/45	5/3.6
SGPT* (IU/ml)	34	6
LDH+ (IU/ml)	660	250
Alkaline phosphatase (IU/ml)	340	179
Prothrombin time (s)	32	12
Urea (mg/dl)	540	15
Creatinine (mg/dl)	2.5	0.6
Immune complexes		
EAC rosette inhibition (%)	38 (N:0–25)	0
Polyethylene-glycol precipitation	0.085 (N:0.004–0.022)	0.010
Anticomplementary activity (uCH_{50})	1.05 (N:1.05–1.65)	1.65

* = Serum glutamic pyruvic transaminase
\+ = Lactic dehydrogenase

normal, Factor VII 1% normal and Factor X 13% of normal. The immunocomplexes were elevated by the technique of EAC rosette inhibition [3] and by polyethylene-glycol precipitation [5]. Anticomplementary activity assayed by the method of Johnson et al. [6] was at the lower limit of normal. Bacteriological cultures and tests for hepatitis B surface antigen were negative.

The presence of gall stones was ruled out by abdominal sonography. He was discharged and followed up at the outpatient clinic. A liver biopsy performed on 29/7/82 showed only minimal changes. On his last appointment, on 24/4/84 the patient was in good condition, with normal liver function tests and only slight jaundice.

Discussion

The patient had vaso-occlusive crisis in different organs but the most serious complication was the acute cholestasis that led to a hepato-renal syndrome. The exchange transfusion improved the clinical symptoms as reported previously [4] but only for a short period and in a few days the neurological signs reappeared. After plasma exchange there was a dramatic recuperation of the clinical condition as well as of the biochemical tests, as shown in Table 1. We do not have a definite explanation for the clinical improvement but perhaps the removal of bilirubin, urea, creatinine and other metabolites was important. Although the increase in the level of circulating immunocomplexes was modest it is possible that their removal had also contributed to the improvement of the patient.

We are indebted to Dr. G. Serjeant for review of the manuscript.

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Obituary

Professor Hermann Lehmann, emeritus professor of biochemistry in the University of Cambridge and a founder of molecular haematology, died on 13th July at the age of 75.

Hermann Lehmann was born in Halle in the province of Saxony, Germany, in 1910. He went to school in Dresden and later studied medicine in Freiburg and Heidelberg. His interest in biochemistry developed after he had taken his M. D. in Basle in 1934, and returned to Heidelberg to become an assistant to Professor Otto Meyerhof at the Kaiser Wilhelm Institute. His work there was notable for the demonstration of the reversibility of the phosphorylation of creatine and this led, in 1935, to an invitation to visit the Department of Biochemistry in Cambridge. He returned briefly to Heidelberg but seeing that the situation for him in Germany had worsened, he took up the offer of Professor F. G. Hopkins of Cambridge and emigrated to England in 1936.

His interest in haematology developed unexpectedly from work with Robin Hill in Cambridge on the iron content of chloroplasts. Because of this work he was recruited, when war broke out, to the army medical corps in India, where iron deficiency anaemia was a major problem. To meet this large-scale problem he introduced, with Heinz, the copper sulphate drop test for anaemia, a test that is still used for the rapid assessment of blood donors. In the case of the soldiers in India, if the drop of blood sank in the copper sulphate they were fit for active duty, if it floated they stayed back for supplementary iron therapy.

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This work on iron deficiency resulted in his being asked, after the war, to go to Uganda to investigate what was then called dimorphic anaemia. This dimorphic anaemia had a curious course – patients were hypochromic but on being given iron their blood picture changed to that of a macrocytic anaemia. Hermann Lehmann showed that the anaemia was due to a combination of hookworm infection and iron deficiency, and that the macrocytic appearance was really a reticulocyte response to iron therapy.

It was while he was in Uganda that he first became interested, because of sickle cell disease, in the haemoglobinopathies. From the viewpoint of the present time, the genetics of sickle cell anaemia seem simple and obvious. Reading Hermann Lehmann's notes of the time show why it was such a puzzle to the earlier investigators. Different surveys, even within the one area, had shown quite different rates of occurrence of sickle cell trait and of the disease, a discrepancy that seemed to contradict simple Mendelian inheritance. Hermann Lehmann explained this apparent discrepancy when he demonstrated that whereas homozygous sickle cell disease was seen in the government hospitals catering for the better-off African, it was not seen in rural districts where all homozygotes died in early infancy. He also showed the striking difference that could occur between tribal groups within the one country and, with others, he was able to relate the incidence of sickle cell trait to the severity of exposure to malaria.

Here we see the emergence of the central theme to all his work, namely his abiding interest in genetic diversity both in relation to anthropology and to evolution. His interest in anthropology is apparent in his book, with R. G. Huntsman, "Man's Haemoglobins"; it was also shown by his side-trip to the Andamese Islands where he demonstrated the origin of the islanders – work that gained him the Rivers medal of the Royal Anthropological Institute. His interest in evolution was evidenced in his later work, mostly after his retirement in 1978, that mapped the structural diversity and evolution of mammalian myoglobins.

The focus of Hermann Lehmann's interest in genetic diversity was of course in molecular disease. After his return to Britain and appointment to a Readership at St. Bartholomew's Hospital in 1951, he made the key observation that succinylcholine apnoea was related to a low, or nearly absent, concentration of pseudocholinesterase in the plasma. He later showed that this deficiency of pseudocholinesterase was genetically determined – an initial observation in the new science of pharmacogenetics.

It was at this time, in the early 1950's, that the techniques for the molecular analysis of haemoglobin were being established by Pauling, Itano, Ingram and others. Hermann Lehmann was one of the first to utilize these techniques in diagnostic medicine. At St. Bartholomew's he introduced the paper electrophoreses of haemoglobins as a screening, as well as diagnostic test. After his move to Cambridge in 1963 he established an Abnormal Haemoglobin Unit, that for the next decade was to provide the lead in the identification of the molecular abnormalities of human haemoglobin. Hundreds of variants were identified, many by his own laboratory, some by his students and trainees, and again many by people

who were inspired to enter the field by Hermann's ebullient enthusiasm. For him, the pursuit of a new variant had the excitement of the chase, he loved to lead the field and be the first to the catch. He conveyed this excitement to those who worked with him, and a district pathologist who diffidently entered his office with a new blood sample would leave proudly with the feeling that he, together with Watson and Crick, was moving forward the boundaries of life.

And it was true too. Hermann Lehmann foresaw the patterns that would emerge from the characterization of these variants and in particular from the study of the variants that result in malfunction and hence disease. He ignored the early criticism that the work was merely 'stamp-collecting' and had the satisfaction of seeing its use in the establishment of the universal nature of the genetic code, and in the prediction of the duplication of the α-globin gene as the explanation for the complex genetics of α-thalassaemia.

Together, the haemoglobin variants provide a comprehensive model of the genetic variation that will occur in the proteins of man. Many of the lessons have yet to be promulgated and accepted. For example, the unstable haemoglobin anaemias occur as a well-defined clinical syndrome but analysis of the variants shows an unexpected diversity of molecular lesions, most of which have arisen from recent, or de novo, mutations. Yet there is still an expectancy amongst the new generation of DNA geneticists that inherited diseases in general will be identifiable by standard restriction mapping, i.e. that each disease syndrome is due to one (or a few) founder lesions. Similarly, molecular biologists are now carrying out, en masse, site-specific mutagenesis to investigate questions of protein structure and function that were long ago answered by observations made with the abnormal haemoglobins.

The precise relationship between the molecular structure of haemoglobin and its normal and abnormal functions rests on the correlations made between the variant haemoglobins and the crystallographic structures obtained by M. F. Perutz and his colleagues. This partnership, of Perutz and Lehmann, provided a full description of the molecular pathology of haemoglobin – the correlation of dysfunction in disease with the aberrations in molecular structure. The combination of the studies converted haemoglobin from an inanimate model to a living molecule; for the first time it was possible to predict with confidence the changes that could occur within the molecule, with or without causing molecular disease.

Hermann Lehmann received many honours in his lifetime including honorary membership of the Haematology Societies of Britain, Costa Rica, Europe, Germany, Italy, Netherlands, Switzerland, Turkey, U.S.A., Venezuela and of the Pathology Society of Nigeria. Three British honours which particularly pleased him were his fellowship at Christ's College Cambride in 1965, his election to F.R.S. in 1972 and his appointment of C.B.E. in 1980.

To all who came in contact with him he will be remembered for his vitality and humanity. For those who were in direct competition with him the former might have initially been more obvious than the latter, but in the end it was always his humanity that won through. His life was full of quiet acts of kindness

and thoughtfulness; to struggling colleagues, to promising students, to the exile and to those in ill-health.

Hermann Lehmann married in 1942 Benigna Norman-Butler and it was a privilege for many haematological visitors to Cambridge to share the warm hospitality of their family home.

Abstracts

Anion transport in red blood cells and arginine-specific reagents. Interaction between the substrate-binding site and the binding site of arginine-specific reagents. L. Zaki and Th. Julien (Max-Planck Institut für Biophysik, Frankfurt am Main, 71 FRG). *Biochim. Biophys. Acta 818*, 325 (1985).

Phenylglyoxal is found to be a potent inhibitor of sulfate equilibrium exchange across the red blood cell membrane at both pH 7.4 and 8.0. The inactivation exhibits pseudo-first-order kinetics with a reaction order close to one at both pH 7.4 and 8. The rate constant of inactivation at 37 °C was found to be 0.12 min^{-1} at pH 7.4 and 0.19 min^{-1} at pH 8.0. Saturation kinetics are observed if the pseudo-first order rate constant of inhibition is measured as a function of phenylglyoxal concentration. Sulfate ions as well as chloride ions markedly decrease the rate of inactivation by phenylglyoxal at pH 7.4, suggesting that the modification occurs at or near to the binding site for chloride and sulfate. The decrease of the rate of inactivation produced at pH 8.0 by chloride ions is much higher than that produced by sulfate ions. Kinetic analysis of the protection experiments showed that the loaded transport site is unable to react with phenylglyoxal. From the data it is concluded that the modified amino acid(s) residues, presumably arginine, is (are) important for the binding of the substrate anion.

G. Gárdos

Aged human erythrocytes exhibit increased anion exchange. M. A. Zanner and W. R. Galey (Department of Physiology, University of New Mexico School of Medicine, Albuquerque, NM, USA). *Biochim. Biophys. Acta 818*, 310 (1985).

Young and old erythrocytes show different rate constants of anion exchange as measured by $^{35}SO_4^{2-}$ efflux at 37 °C. Results indicate that the rate constant for $^{35}SO_4^{2-}$ efflux ($SO_4^{2-} - Cl^-$ exchange) from old cells is approximately 20% greater than from young less dense cells. The cell water volume of older cells is also decreased. Based on these results and previously reported decreases of cell membrane area in aged cells we conclude that anion exchange ($^{35}SO_4^{2-}$) is increased in older, more dense human erythrocytes.

G. Gárdos

Stereospecific modulation of the calcium channel in human erythrocytes by cholesterol and its oxidized derivatives. L. Neyses, R. Locher, M. Stimpel, R. Streuli and W. Vetter (Department of Internal Medicine, University Hospital Zürich, Zürich, Switzerland). *Biochem. J. 227*, 105 (1985).

To study the effect of cholesterol and its pathophysiologically important oxidized derivatives (OSC) on the calcium entry channel, the human red blood cell was used as a model system. The calcium ejecting adenosinetriphosphatase (ATPase) was inhibited by vanadate. The cells were loaded with OSC at concentrations between 1.25×10^{-5} and 25×10^{-5} mol/l. 22-Hydroxycholesterol, cholestan-3β,5α,6β-triol, 5α-cholestan-3β-ol, 3β,5α-dihydroxycholestan-6-one and 3β-hydroxy-5α-cholestan-7-one stimulated $^{45}Ca^{2+}$ influx by up to almost 90%, whereas 25-hydroxycholesterol, 7β-hydroxycholesterol, 20α-hydroxycholesterol and 7-oxocholesterol in-

hibited influx by up to 75%. Both stimulation and inhibition were dependent on the amount of OSC incorporated into the membrane. More than 90% on the total modification of calcium influx by OSC was accounted for by an influence on the nitrendipine-inhibitable part of influx. Enrichment of cholesterol in the membrane greatly stimulated, and cholesterol depletion inhibited, Ca^{2+} influx. These results demonstrate that cholesterol and its oxidized derivatives are able to modulate the calcium channel in human red blood cells in a highly stereospecific manner.

Ágnes Enyedi

Role of phospholipids in the activation of the Ca^{2+}-dependent neutral proteinase of human erythrocytes. S. Pontremoli, E. Melloni, B. Sparatore, F. Salamino, M. Michetti, O. Sacco, and B. L. Horecker (Institute of Biological Chemistry, University of Genoa, Genoa, Italy; Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ, USA). *Biochem. Biophys. Res. Comm. 129*, 389 (1985).

Activation of the Ca^2-dependent neutral proteinase of human erythrocytes in the presence of Ca^{2+} and a digestible substrate is promoted by phospholipids such as phosphatidylcholine, phosphatidylinositol and phosphatidylserine. The presence of at least one unsaturated fatty acid chain is essential and metabolic derivatives such as dioleylglycerol, phosphorylserine and free fatty acids are ineffective. The most effective promoter was a freshly prepared mixture of phospholipids from human erythrocyte membranes. Activation involves conversion of the 80 kDa proenzyme (procalpain) subunit to the 75 kDa active proteinase and is irreversible. Phospholipids act by producing a large decrease in the concentration of Ca^{2+} required for the conversion of procalpain to active calpain.

Ágnes Enyedi

Peroxide-induced membrane damage in human erythrocytes. J. van der Zee, T. M. A. R. Dubbelman and J. van Steveninck (Sylvius Laboratories, Department of Medical Biochemistry, Leiden, The Netherlands). *Biochim. Biophys. Acta 118*, 38 (1985).

Erythrocytes exposed to H_2O_2 or t-butyl hydroperoxide (tBHP) exhibited lipid peroxidation and increased passive cation permeability. In the case of tBHP a virtually complete inhibition of both processes was caused by butylated hydroxytoluene (BHT), whereas pretreatment of the cells with CO increased both lipid peroxidation and K^+ leakage. In the experiments with H_2O_2, on the other hand, both BHT and CO strongly inhibited lipid peroxidation, without affecting the increased passive cation permeability. These observations indicate different mechanisms of oxidative damage, induced by H_2O_2 and tBHP, respectively. The SH-reagent diamide strongly inhibited H_2O_2-induced K^+ leakage, indicating the involvement of SH oxidation in this process. With tBHP, on the contrary, K^+ leakage was not significantly influenced by diamide. Thiourea inhibited tBHP-induced K^+ leakage, without affecting lipid peroxidation. Together with other experimental evidence this contradicts a rigorous interdependence of tBHP-induced lipid peroxidation and K^+ leakage.

G. Gárdos

Platelet activating factor-stimulated formation of inositol triphosphate in platelets and its regulation by various agents including Ca^{2+}, indomethacin, CV-3988, and forskolin. S. D. Shukla (Department of Pharmacology, University of Missouri Health Sciences Center, Columbia, Missouri). *Arch. Biochem. Biophys. 240*, 674 (1985).

When myo-2-[^{3}H]inositol-labeled rabbit platelets were stimulated with 1×10^{-9} M sn-3-AGEPC (platelet activating factor) for 5 s, the levels of [^{3}H]inositol monophosphate (IP), [^{3}H]inositol diphosphate (IP_2), and [^{3}H]inositol triphosphate (IP_3) increased about 1.5-, 3-, and 5-fold, respectively. Formation of these inositol polyphosphates was strikingly independent of extracellular Ca^{2+}. Inactive analogs of sn-3-AGEPC, i.e., lysoGEPS and stereoisomer sn-1-AGEPC, did not cause production of any inositol polyphosphate. Pretreatment of platelets

with indomethacin (5 μM) had little effect on this phenomenon. On the other hand, a platelet activating factor antagonist, CV-3988, blocked the AGEPC-stimulated production of radioactive IP, IP_2 and IP_3. Similarly forskolin, an activator of adenylate cyclase, at 5 μM or above completely abolished AGEPC-induced aggregation, [^{3}H]serotonin secretion, and formation of [^{3}H]inositol polyphosphates. In the light of the emerging role of AGEPC in inflammation, hypotension, and other cardiovascular processes, studies with platelets reported here indicate that forskolin could be a useful tool for manipulating AGEPC responses. It is further concluded that AGEPC-induced formation of inositol polyphosphate is an early response "specific" to AGEPC, mediated via extracellular Ca^{2+}-independent phosphoinositide phosphodiesterase, and could play a role in intracellular Ca^{2+} mobilization and platelet shape change.

G. Gárdos

Identification of Pl^{A1} alloantigen domain on a 66 kDa protein derived from glycoprotein IIIa of human platelets. E. Kornecki, S. Y. Chung, J. C. Holt, Cz. S. Cierniewski, G. P. Tuszynski and S. Nierwiarowski (Thrombosis Research Center, Temple University Health Sciences Center, Philadelphia, PA, and Department of Psychiatry, Neurosciences Research Unit, University of Vermont, Burlington, VT, USA). *Biochim. Biophys. Acta 818*, 285 (1985).

Incubation of platelets with chymotrypsin leads to the exposure of fibrinogen receptors and to the appearance of a 66 kDa membrane component on the surface of platelets. Both glycoprotein IIIa (GP IIIa) and a 66 kDa component were precipitated from detergent extracts of solubilized, surface radiolabeled chymotrypsin-treated platelets by human anti-Pl^{A1} antisera. Moreover, the presence of the Pl^{A1} antigen was identified on GP IIIa (but not on GP IIb) and on a 66 kDa protein by means of immunoblot procedures using platelet Triton X-114 extracts and these purified proteins. Anti-Pl^{A1} antiserum did not recognize GP IIIa on the surface of intact (untreated) platelets nor the 66 kDa protein on the surface of chymotrypsin-treated platelets of Pl^{A1}-negative individuals. The present data demonstrate directly that the 66 kDa protein is derived from GP IIIa and contains the Pl^{A1} alloantigen.

Ilma Szász

An adenosine triphosphate-dependent calcium uptake pump in human neutrophil lysosomes. M. S. Klempner (Division of Experimental Medicine, Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts). *J. Clin. Invest. 76*, 303 (1985).

Regulation of the cytosolic free calcium concentration is important to neutrophil function. In these studies, an ATP-dependent calcium uptake pump has been identified in human neutrophil lysosomes. This energy-dependent Ca^{2+} uptake pump has a high affinity for Ca^{2+} (Michaelis constant [K_m] Ca^{2+} = 107 nM) and a maximum velocity (V_{max}) of 5.3 pmol/mg of protein per min. ATP was the only nucleotide that supported Ca^{2+} uptake by lysosomes. The K_m for ATP was 177 μM. ATP-dependent Ca^{2+} uptake by neutrophil lysosomes was temperature- and pH-sensitive with optimal Ca^{2+} pump activity at 37 °C and pH 7.0–7.5. Mg^{2+} was also essential for ATP-dependent Ca^{2+} uptake by lysosomes. Azide and antimycin A had no effect on the energy dependent uptake of Ca^{2+} by neutrophil lysosomes. The chemotactic peptide formyl-methionyl-leucyl-phenylalanine inhibited ATP-dependent Ca^{2+} accumulation by isolated lysosomes. But oxycarbonyl-phenylalanine-leucine-phenylalanine, a competitive antagonist of the chemotactic peptide, blocked this inhibitory effect. These studies demonstrate the presence of an ATP-dependent Ca^{2+} uptake pump in human neutrophil lysosomes that functions at physiologic intracellular concentrations of Ca^{2+}, ATP, and H^+ and may be important to regulating neutrophil function by modulating cytosolic Ca^{2+}.

Ilma Szász

Inositol phosphate formation in fMet-Leu-Phe-stimulated human neutrophils does not require an increase in the cytosolic free Ca^{2+} concentration. F. Di Virgilio, L. M. Vincentini, S. Treves, G. Riz and T. Pozzan (C. N. R. Unit for the Study of the Physiology of Mitochondria and Institute of General Pathology, Padova, Italy). *Biochem. J. 229*, 361 (1985).

The accumulation of inositol phosphates in myo-[^{3}H]inositol-labelled human neutrophils stimulated with the chemotactic peptide fMet-Leu-Phe was measured. The challenge with the chemotactic peptide caused the generation of inositol monophosphate (InsP), inositol bisphosphate ($InsP_2$) and inositol trisphosphate ($InsP_3$). The formation of the three inositol phosphates followed a differential time course: $InsP_3$ accumulated very rapidly and transiently, whereas InsP increased steadily for more than 2 min. Inositol phosphate formation was only partially decreased by procedures which prevented the fMet-Leu-Phe-dependent increase of cytosolic free Ca^{2+} concentration.

Ilma Szász

Book Review

Blood relations, Blood groups and anthropology. By A. E. Mourant. Oxford University Press, London, 1983. 146 pages.

A. E. Mourant's book aroused interest not only in scientific circles, but also among the laymen, for men are attracted by the possibility of finding out more about their own biological being and that of their descendants, their people and of mankind in general. Blood groups is a notion that everyone has encountered one time or another. The question of how blood groups are inherited, what the knowledge of a person's blood group can tell of his origin, and how this knowledge can be used in studying the origins of people and ethnic groups, is raised. Another debated question, namely whether the blood group itself has any influence over a person's susceptibility to diseases, is also discussed. As the author himself states: "The objects of this book are to explain in as simple as possible, how blood-group anthropology works and how it is applied to particular populations."

The book, written in an easily understandable and clear language, yet not loosing its high standard, speaks to the masses. The thoughts and ideas present in it, however, can be of great value and use to the specialists too. One of these thoughts: "Most of the describable features are essentially hereditary in nature, but they can be affected by the history of the individual both before and after birth" is true for the population too, whose present is hardly or not at all understandable without the knowledge of their history. As the author states, one of the main aims of anthropologists is to "identify the historical and hereditary relations between populations." In the parts dealing with Africa, Asia, and Europe, we really do find that Prof. Mourant seeks the roots of the present genetical situation in the historical past. When discussing the European peoples, he pays special attention to the study of gipsies, and also to the uniqueness of the frequency of the Hungarian people's blood group.

In the last chapter, the author discusses the factors that bring about the changes in the gene frequency. In part, he illustrates the blood groups', plasma's, protein's, etc., connection with certain diseases or with the tendency to be susceptible to certain diseases.

The interesting topic, and its clear and simple exposition ensures that the reader will be introduced to the basics of population genetics on a high level.

Tamás Tauszik

VNU Science Press, Utrecht
Akadémiai Kiadó, Budapest

The VIIIth Congress of ESCO (European Sterility Congress Organisation)

will be held in Budapest, Hungary, between September 27–30, 1987.

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Time-Evolution of Erythrocyte Physico-Chemical Properties in Blood Stored at Different Temperatures

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Several interrelated biophysical and biochemical properties of erythrocytes and plasma from whole blood stored at 4 °C, 22 °C, and 37 °C with CPD preservative were determined over a 21-day period. Changes in red cell volume, deformability and osmotic fragility (all measured by resistive pulse spectroscopy) showed some surprising fine structure that correlated well with dynamic variations in extracellular species, especially sodium, potassium and lactate dehydrogenase. Several features of these data suggest the existence of three distinct stages of cell condition, apparently not recognized previously. Changes are interpreted primarily in terms of membrane behavior, and implications for storage monitoring are discussed.

Keywords: blood storage kinetics, deformability, fragility, plasma changes, resistive pulse spectroscopy, temperature-dependence, volume

Introduction

Whole blood in static storage is known to undergo continual physical and chemical changes over the 21-day period employed in blood banks. However, only limited aspects of these changes have been studied. Under typical conditions (4 °C), it is generally recognized [1, 2] that cells tend to swell, to gain Na^+, and to lose K^+. However, there is incomplete evidence as to how the time-evolution of chemical changes is correlated with erythrocyte mechanical and volumetric properties.

We present here such a study. Several physical properties of erythrocytes and chemical properties of the plasma taken from stored blood were tracked for 21 days. Cell volume, deformability, and osmotic fragility were monitored by Resistive Pulse Spectroscopy (RPS). Plasma changes of K^+, Na^+, lactate dehydrogenase (LDH), and glucose are also reported. Comparison of the evolution of these characteristics at 4 °C, 22 °C, and 37 °C leads to new information on cell deterioration mechanisms.

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Materials and Methods

Blood handling

Blood was donated at Alta Bates Hospital, Berkeley, by five healthy adults (4 males, 1 female) of ages 22–32 years. About 400 ml of blood was drawn into sterile Fenwal blood bags containing 63 ml of citrate-phosphate-dextrose (CPD) preservative [3]. To each bag was added 50,000 units of penicillin/streptomycin solution (Grand Island Biological Co.) to guard against both Gram positive and Gram negative bacterial contamination. After several gentle inversions to ensure homogenization, the contents of each bag were divided into five equal parts and stored separately in similar bags: three bags at 4 °C, one at 22 °C, and one at 37 °C.

The principal test-sequence involved tracking the properties of blood stored at 4 °C, 22 °C, or 37 °C during the entire storage period of 14 or 21 days, thus using three of the five portions of blood from a given donor. Before sampling, each blood was homogenized by 50 gentle inversions. In a parallel test-sequence, studies were made of cell responses to a change in storage temperature during the storage period (temperature-jump experiments), using the two remaining portions of blood from the same donor. Those blood bags were stored without sampling for 7 days at 4 °C and then, after an initial sampling, their temperatures were changed. Another sample was taken within a few hours at the new temperature, and thereafter the normal sampling sequence prevailed.

Chemical testing

An aliquot of 5–6 ml of blood from each bag underwent a battery of tests that included plasma analysis for K^+, Na^+, LDH and glucose, as well as numerous other components that showed less or no correlation with storage age and will not be reported here. In the temperature-jump experiments, only plasma K^+ and Na^+ were measured. The chemical analyses were performed in the Alta Bates Laboratory and in the Microanalytical Laboratory of the College of Chemistry at the University of California, Berkeley.

RPS testing

Erythrocyte volume, osmotic fragility, and deformability were evaluated by RPS at room temperature following procedures developed by Mel, Yee, and Akeson [4–6]. This technique is an outgrowth of the electronic particle counting/sizing ("Coulter") method. A cell traversing a small cylindrical orifice (48 μm diameter, 48 μm length), through which a known electrical current is flowing, gives rise to a measurable resistive pulse. Spectra containing the cumulative distributions of 5000–20,000 resistive pulses under various operating conditions yield the desired size and deformability information [5, 6]; any one spectrum is obtained in about 10 minutes.

For all RPS analyses, a two-stage dilution of the whole blood was employed. First, 50 μl of the blood was placed in 10 ml of 300 mOsm phosphate-buffered saline (PBS) at room temperature and given several minutes to equilibrate thermal-

ly. After a further dilution (200 μl of the PBS suspension added to another 10 ml PBS), cell volume was measured. The "slow flow" mode of RPS operation was used (average fluid speed through the orifice was 1.1 m/s) so that cells would not be deformed in passage. The resulting spectrum of pulses, which measure cell volume, was an essentially symmetrical distribution whose peak position is reported here as the population average, V. Cell deformability was assessed by repeating this procedure for "fast flow" (4.4 m/s), yielding a nonsymmetrical size distribution with a shoulder which is known to become more pronounced with cell deformability. The shape of this bimodal distribution is quantified by a bimodality index (BI) which, under appropriate specified conditions, is a measure of deformability. Under the present circumstances, this measure of deformability is believed to be dominated by membrane properties [5]. The computer-generated BI increases monotonically as the shoulder of the cell size distribution becomes more pronounced [4–8].

Osmotic fragility was measured in a time-dependent mode by an RPS procedure [5, 9] known as dynamic osmotic hemolysis (DOH). Here, 200 μl of cell suspension was added to 10 ml of 130 mOsm PBS, which led to rapid swelling and partial lysis. In the resulting mixture of ghosts and swollen cells, two RPS peaks emerge and it is known [5] that the smaller corresponds to the apparent ghost size. Following the procedure of Akeson and Mel [9], the mixture was monitored under fast-flow conditions for several minutes, while the distribution of "recovering" ghosts (small-volume peak) and nonlysed intact cells (large-volume peak) changed continuously. The relative areas under these two peaks represent the relative numbers of ghosts and swollen cells, and from these can be computed the time-evolution of the ghost fraction, GF. Although the ghost peak evolved towards and merged with the swollen-cell peak, there was sufficient resolution to track the former during a 1–4 minute period while a limiting value GF_∞ was being attained. Both the kinetics of ghost formation and GF_∞ were evaluated.

Results

We present first a typical set of biophysical results (volume, V; bimodality index, BI; ghost fraction, GF) obtained by RPS. These are followed by the chemical data on plasma composition. Experience with 21-day storage periods showed that all significant trends had already been established by the 14th day, so we limit discussion to samples monitored for 14 days. Only a selection of sample results are presented, for compactness, but bloods from all donors behaved similarly.

Because RPS measurements were made at room temperature, data on blood stored at 4 °C and 37 °C could have been slightly affected by cellular response to immersion in 22 °C fluid just prior to testing. Thus, only the data on blood stored at 22 °C can be taken as totally independent of such thermal shock. However, previous experience in this laboratory has shown that such shock is not likely to alter the chemical balance on such a short time scale, so there should be no signifi-

cant effects on V, BI, and GF of the sort that would ultimately result, on a long-time scale, from such balance changes (see the second test-sequence data described below). Data on V and BI are normalized to values recorded for control samples (fresh blood contributed at 37°C, on the same day) designated V_c and BI_c.

Cell volume

Figure 1 shows that storage at 4 °C produced little change in V; only an 8% linear increase occurred between days 3 and 14. Storage at 22 °C was nearly as

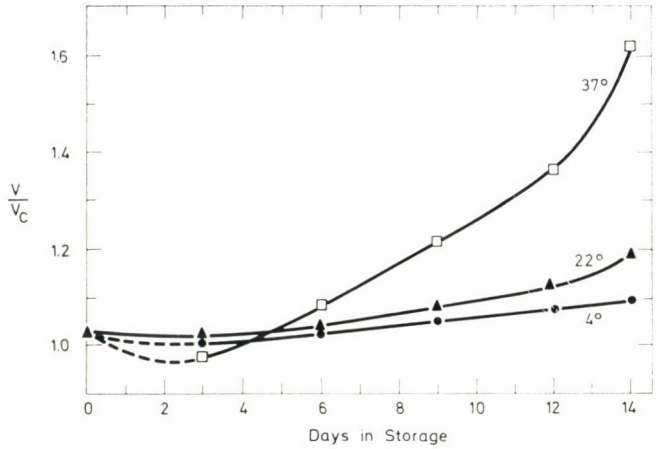

Fig. 1. Average cell volume (V) changes during blood storage at three temperatures. Ordinate is normalized to cell volume for a fresh control sample (V_c). Lines shown for 4 °C and 37 °C curves in the 0–3 day period are probable but not certain, since the 0-day point is at 22 ° for all samples. Because of this uncertainty, these lines are drawn as dashed; dashed lines are similarly used in Figs 2–8. Data shown for Donor Y

effective in preserving volume through the 6th day, though significantly more swelling occurred thereafter. At 37 °C this tendency for volume increase at longer times was greatly exaggerated, with 66% swelling recorded between days 3 and 14.

Cell deformability

Figure 2 displays the corresponding time-evolution of BI. Almost no changes were detectable for blood stored at 4 °C, while at 22 °C and 37 °C the BI dropped steadily. The speed of this drop was fastest at 37 °C, with the BI reaching almost zero by the 9th day. At 22 °C the rate of drop was intermediate, being roughly half that at 37 °C through the 9th day.

Osmotic fragility

Examples of several DOH curves are given in Fig. 3. The lysis subsequent to t = 0 (the point of initial cell exposure to osmotic stress) occurred very rapidly, with the major rise of GF usually completed within 25–50 s. The behavior of GF_∞

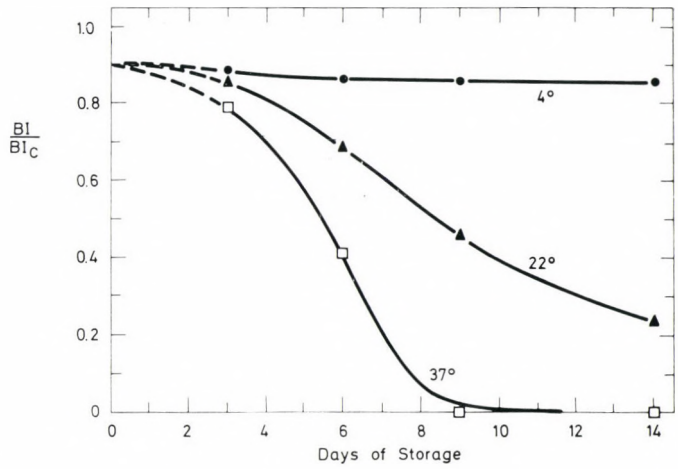

Fig. 2. Bimodality index (BI) changes during blood storage at three temperatures. Ordinate is normalized to the fresh control-sample value (BI_c). Donor Y

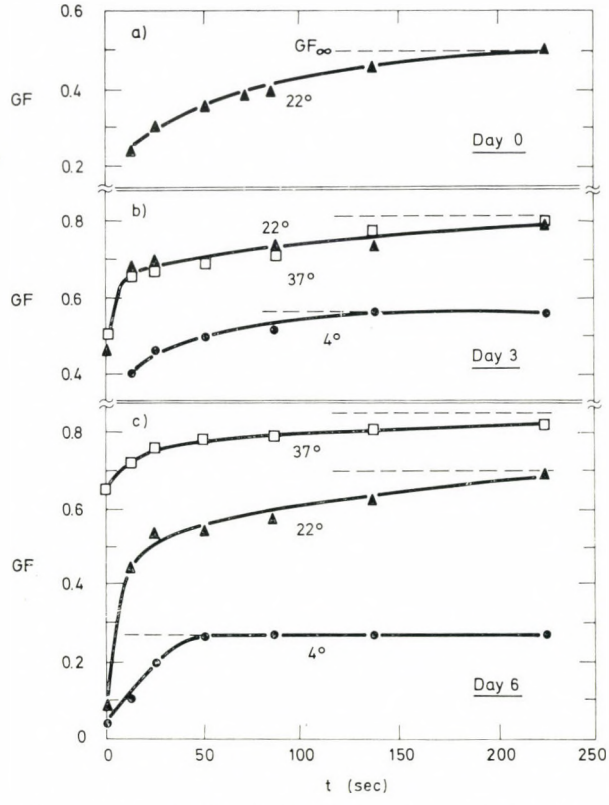

Fig. 3. Behavior of ghost fraction (GF) in DOH experiments during four minutes of RPS testing. (a) Blood within a few hours of donation, tested at room temperature. The plateau value $GF\infty$ is also plotted in Fig. 4. (b) Same blood, aged at three temperatures for 3 days. (c) Same blood, aged for 6 days. Donor Z

with storage time at different temperatures is shown in Fig. 4, for one donor. There is a surprising amount of structure in these curves. In the short term, fragility increased at all temperatures, with 22 °C being essentially as traumatic as 37 °C, and 4 °C far less so. In the long term, the 37 °C curve remained high while the other curves exhibited a maximum with a subsequent drop to a minimum, then another rise.

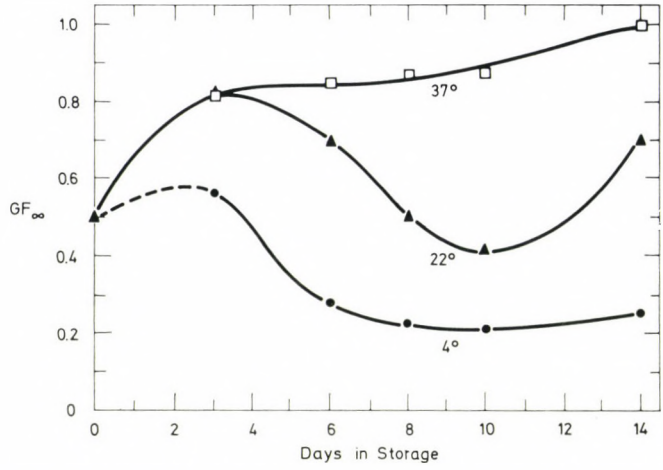

Fig. 4. Plateau values of the ghost fraction in DOH experiments (GF_∞) during blood aging at three temperatures. The GF_∞ is a measure of osmotic fragility and other cell properties (see text). Donor Z

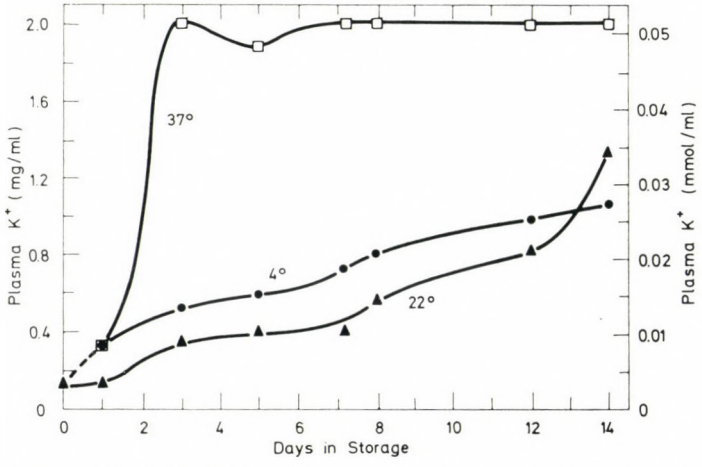

Fig. 5. Plasma potassium (K^+) changes during blood storage at three temperatures. Donor X

Potassium

Figure 5 shows the extent of K^+ leakage from cells into plasma during storage, a process also seen to be highly temperature-dependent. For the storage-aging at 4 °C, substantial leakage has already occurred by day 1; by the 3rd day the leakage rate slows and – if time-smoothed through 14 days – remains nearly constant. However, details within the data again reveal considerable structure during the 3–14 day period. Storage at 37 °C leads to a similar day 1 leakage, but between days 1 and 3 the leakage increases considerably; the high plasma level reached by the 3rd day remains almost unchanged thereafter. Results at 22 °C are not intermediate; plasma K^+ levels remain lower than those for both other temperatures, at all times prior to day 13 (and virtually no release occurs prior to day 1). However, between day 1 and day 12 structural features in this curve resemble those in the 4 °C curve. Additional data (not shown) indicated that K^+ leakage at 4 °C and 22 °C was continuing at 21 days.

Sodium

Blood stored with CPD contains excess Na^+, since CPD is sodium-rich [3] (hypertonic, at 460 mOsm). This starting point – 4.24 mgNa^+/ml of plasma – therefore constitutes a nonphysiological condition, but one typical of storage environments.

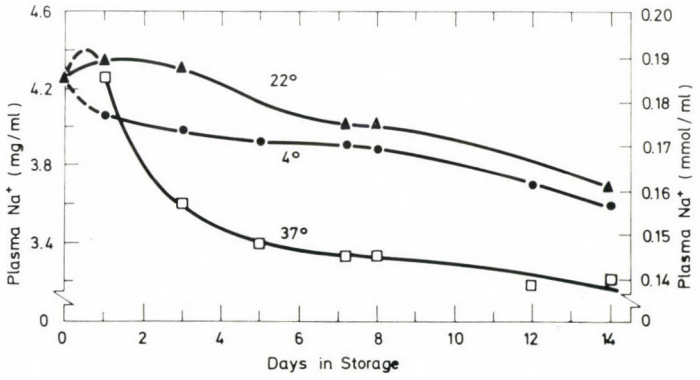

Fig. 6. Plasma sodium (Na^+) changes during blood storage at three temperatures. The speculative dashed-line maximum in the 37°C curve during the first day is guided by the broader maximum in the 22 °C curve; a similar maximum has been reported near day 1 by Wallas [10]. Donor X

Figure 6 shows the kinetics of plasma Na^+ change at different temperatures. Storage at 4 °C gives the simplest behavior, with plasma Na^+ decreasing monotonically into a slight plateau, then slowly declining again after day 7. At 37 °C plasma Na^+ at day 1 is the same as the initial value. Thereafter, the plasma level drops rapidly toward a low apparent plateau (about day 7) before beginning another slow decline. Cells stored at 22 °C exhibit an anomalous behavior in Na^+gain,

as they did for K^+ loss. At all times after day 1, the Na^+ level remains higher than that for either 4 °C or 37 °C. Just as for K^+, however, the most striking change in plasma Na^+ occurs for cells stored at 37 °C.

LDH

The LDH found in human erythrocytes occurs as five different isozymes, all having the same molecular weight and catalytic activity; the assay monitored

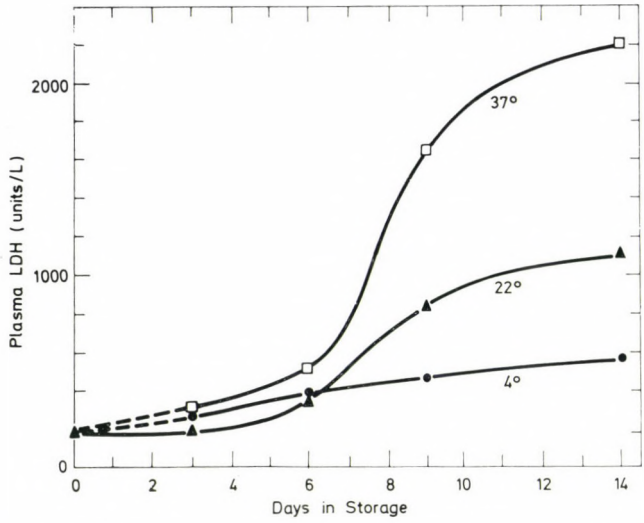

Fig. 7. Plasma lactate dehydrogenase (LDH) changes during blood storage at three temperatures. Donor Y

the collective activity of all isozymes in the plasma. As seen in Fig. 7, release of LDH is small for the first 6 days and of comparable magnitude for all three temperatures. For 22 °C and 37 °C, the release accelerates beyond day 6 and has a generally sigmoidal character. In contrast to the Na^+ and K^+ results, the LDH release is a monotonically increasing function of temperature beyond day 6.

Glucose

As in the case of Na^+, plasma glucose is artificially elevated due to its presence in CPD [3]. Figure 8 shows that the uptake of glucose is slight at 4 °C but much more pronounced at the two higher temperatures—the latter cases leading to long-term glucose levels that are identical. It is notable that the curves for all temperatures appear to exhibit slight inflections after day 7.

Temperature-jump experiments

These data are displayed in Figs 9 (for K^+) and 10 (for Na^+). There, the time scale is expanded by a factor of two to assist inspection of relatively rapid

changes occurring shortly after the jump was imposed. For comparison purposes, the corresponding data on isothermal storage at the three temperatures are included as dashed lines (taken from Figs 5 and 6). Results of such experiments have apparently not been reported heretofore, and the blood evolution during the first few days after the jump is far more complex than could have been predicted.

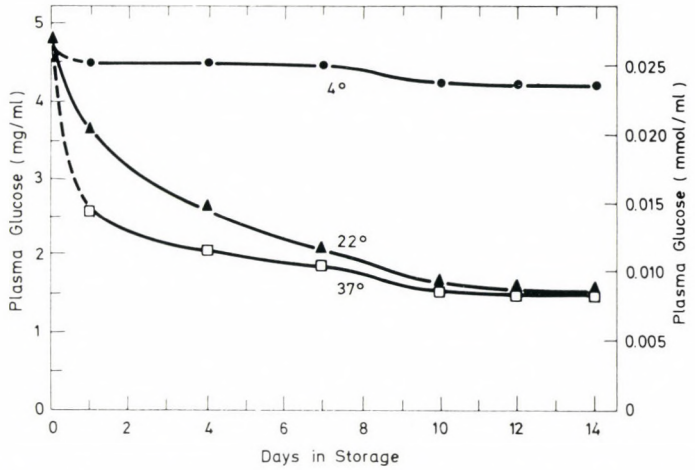

Fig. 8. Plasma glucose changes during blood storage at three temperatures. Donor Z

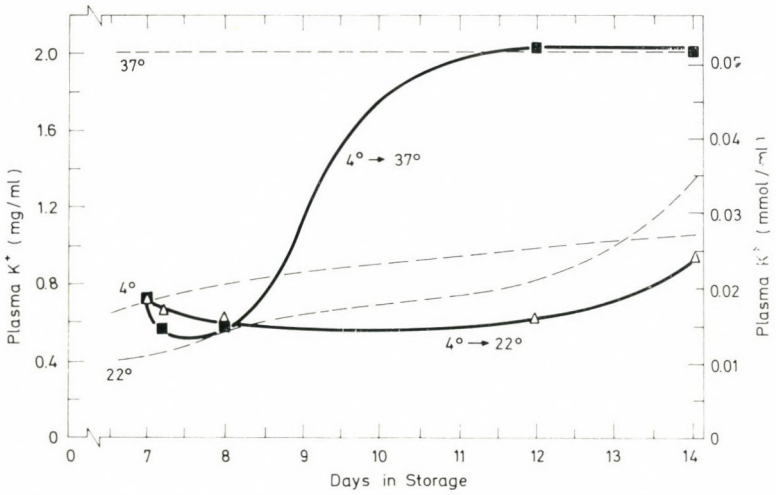

Fig. 9. Response of plasma K^+ to two different temperature jumps. Both jumps (labelled on figure) were initiated on day 7 of storage, and the new temperature was maintained through day 14. Dashed lines represent the curves of uniform-temperature storage, from Fig. 5. The time scale is expanded (relative to Fig. 5) for clarity of the display. Donor X

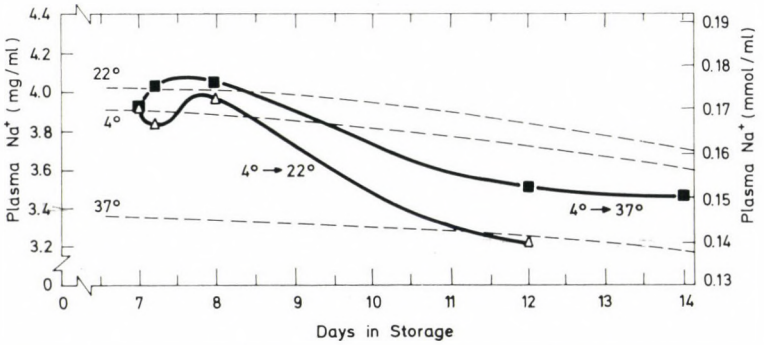

Fig. 10. Response of plasma Na^+ to two different temperature jumps. See caption of Fig. 9. Donor X

Discussion

The changes in red cells reported here fall into two general categories: chemical, and physical/mechanical. Chemical results, in the form of time-dependent changes of intra- or extra-cellular constituents, are for the most part to be ascribed to "membrane leakage", although metabolism or adsorption may play a significant role in some instances. The physical/mechanical results, though reported in terms of changes in three separate properties (cell volume, deformability, and fragility), are actually closely related to each other as well as to the chemical changes. In this discussion we will focus particularly on the experimental and theoretical linkages between the physical and chemical properties in order to arrive at a global picture, including new insights on the time- and temperature-dependent changes that occur during storage of whole blood.

Volume changes and membrane leakage

Since water is by far the principal volume-building constituent of the red cell (roughly 2 : 1 relative to hemoglobin, the second most plentiful component), increases and decreases in intact-cell volume reflect directly – and virtually exclusively – gains and losses in cytoplasmic water. Furthermore, red cells maintain rapid osmotic equilibrium with their external milieu, so changes in cell water content reflect cellular gains or losses of osmotically active solutes. Given the normally high intracellular concentration of K^+ ($\sim$3.6 mg/ml), and low intracellular concentration of Na^+ ($\sim$0.2 mg/ml), a cell shrinkage – at least of fresh red cells – usually therefore indicates a net loss of K^+, and a swelling a net gain of Na^+. (This is moderated to some extent by the presence and movement of other small molecules, such as glucose.) The fact that an interrelationship exists between volume regulation and sodium and potassium flux across the red cell membrane has been known for some time [11–13].

Our interpretation of the relationships between "leakage" (Figs. 5–8) and volume changes (Fig. 1) will emphasize certain qualitative and semiquantitative

linkages. To attempt a complete quantitative reconciliation of the results would require additional and more detailed data than we have available. The main point is made most simply for the 4 °C data, where the slight swelling of the cells observed over the 14-day period (Fig. 1) is seen to be quite consistent with the leakage results: sufficient Na^+ enters the cells from the plasma (Fig. 6) to balance approximately the slightly lesser K^+ loss (Fig. 5) and therefore to lead to a small net gain in osmotically active solute, causing a small net gain, also, in water and in volume. (In this case the disappearance of plasma glucose, Fig. 8, is negligibly small for purposes of this semiquantitative argument.)

For the 37 °C results the explanation must be a bit more complex, for at first glance the gain of Na^+ by the cells would seem to be somewhat less than the loss of K^+ – yet, the cells swell considerably. However, if we include the glucose results as well, there is sufficient net uptake of total solute species (and one that increases over time) to generate an increasing swelling of the cells. This general argument is further enhanced to the extent that glucose is being metabolized at 37 °C and produces additional, osmotically-active products inside the cell.

The 22 °C volume results, with swelling intermediate between that for 4 °C and 37 °C, are again consistent with the net change in plasma solute species (losses of Na^+ and glucose; gain in K^+), despite the apparent anomalies in Na^+ and K^+ transport viewed separately at 22 °C.

The time and temperature dependence of LDH movement (Fig. 7), which is not conceptually encumbered with complications of pumps or metabolism, is generally consistent with a simple progressive loss of ability of the cell in vitro to maintain a classical permeability barrier, and all the more so for higher temperatures. Whether the LDH that escapes originates within the membrane or within the cytoplasm is irrelevant to the argument, although its large molecular size favors the former. (If so, one could speculate further about the LDH release representing also a progressive mechanical weakening of the membrane.) The LDH loss from cells makes no significant osmotic contribution to cell volume change, since its molar concentration is vanishingly small.

This rather simple release-behavior for LDH, which parallels volume changes (Fig. 1) remarkably closely, is to be contrasted with the more complex temperature-dependence of Na^+ and K^+ kinetics, both of which show anomalous behavior at 22 °C. We will not attempt a detailed explanation of the latter; changes in the physical state of the membrane (i.e., phase behavior of lipids) and contributions from energy metabolism are probably involved.

Energy metabolism is clearly relevant to cell volume changes (more precisely, to non-changes). It is not widely appreciated that normal red cell volume must be maintained by establishment of an intricate nonequilibrium steady-state condition. The well-known 3 : 2 Na^+(out)/K^+(in) pump, when considered along with the normal constancy of intracellular Na^+ and K^+ concentrations, requires that: (1) a constant in-leak of Na^+ and out-leak of K^+ must occur, exactly balancing their respective pump rates; (2) a net in-leak of solute and water must normally balance, exactly, the net out-pumping of solute (with passively following water);

and (3) if the pump runs out of energy or otherwise is shut off, the continuing net leak inward will lead naturally to a passive increase in cell volume. These tendencies are seen in the present data – e.g., the rapid 37 °C glucose consumption (Fig. 8) at short times suggests strong pump activity, but this subsides after one day and the lower activity thereafter permits the rapid swelling seen in Fig. 1. At 4 °C and 22 °C all cell mechanisms are slowed relative to 37 °C; volume and glucose results for these cases are ordered as expected for temperature-dependent chemical kinetics.

Deformability and volume changes

Whatever the detailed molecular/membrane mechanism(s) underlying the overall volume changes, the relationships between changes in volume (Fig. 1) and deformability (Fig. 2) should be simple and straightforward, to a first approximation. Two fundamental physical properties of the red cell are: (a) the essential incompressibility of its aqueous cytoplasm, and (b) the normal inextensibility of its membrane surface area [14, 15]. (The minor deviations of these statements from absolute precision are not significant for the following argument.) The general geometrical concept of "excess surface" or "relative sphericity" [15] is also relevant. Thus, a *sphere* (with relative sphericity = 1) containing an incompressible liquid enclosed by a flexible but inextensible membrane has no degrees of freedom to permit its changing shape under stress – i.e., it is not deformable. On the other hand, a normal red cell [15] has relative sphericity = 0.69; there is considerable excess membrane surface beyond that required to enclose a spherical volume of about 90 μm^3, so the biconcave cell can easily be deformed into other shapes.

This simple, *geometrical* argument is fundamental, although there are many kinds of contributions to red cell deformability. Thus, the more a normal cell would swell and become sphere-like, the less mechanical deformability it is able to manifest. (From a practical point of view, a reduced deformability causes the cell greater difficulty in negotiating restricted passages of the microcirculation encountered in normal blood flow [16].) Comparisons of the curves in Figs 1 and 2 indicate that these ideas are, indeed, reflected in the present data. The slight increase in cell volume during storage at 4 °C is accompanied by a very slight decrease in deformability; at 37 °C, where apparent volume increases by 60% over 14 days, deformability decreases to a negligibly small value. In general, a substantial reduction in deformability is expected if storage times are sufficiently long, even at 4 °C Haradin et al. [17] have reported such a result, using the filterability test for deformability, for eight-week storage samples. LaCelle, in his earlier work [18], using the micropipette method, obtained a similar result. Decreased deformability has also been invoked [19, 20] as a factor – perhaps even the dominant factor – in the natural aging of the red cell progressing through its physiological life span.

Systematic monitoring of cell deformability in stored blood may eventually provide a major improvement in the characterization of blood/cell condition. Furthermore, because of its experimental convenience and simplicity, the BI should be a particularly useful means for accomplishing this.

Fragility

It is useful to recall that the data on cell physical properties, applicable to different storage temperatures and durations, were all derived from RPS measurements made at room temperature. As previously mentioned, the rapid change of cell temperature at the beginning of a test should not significantly influence the measured cell volume, as this is determined by the cell condition as it was prior to measurement and is thus essentially independent of the test temperature. For fragility and deformability, however, numerical results are sensitive to the temperature of measurement; we must thus separate this measurement phenomenon from that portion of the data which reflects the intrinsic condition of the cells at their different storage temperatures. This is accomplished by always using the same test temperature (here, 22 °C).

For example, it is well known that fragility of red cells measured at low temperature (e.g., 4 °C) is greatly increased [21], probably because the lipid-bilayer membrane is less fluid and, in a sense, more brittle at low temperature. If the membrane is also less permeable on a short time scale, this could be a factor contributing to fragility because osmotic stress should not, then, be rapidly relieved in a fragility measurement. However, such adverse membrane properties at low temperature should not be allowed to obscure the fact that low-temperature storage best preserves cell condition.

The room-temperature measurements of fragility and deformability are successful in manifesting this fact. This was seen earlier for deformability in Fig. 2, and for fragility it is evident in Fig. 4. Storage at the highest temperature (37 °C) produces the most fragile cells, storage at 4 °C the least fragile, and storage at 22 °C yields cells with generally intermediate fragility.

The monotonic rise of fragility with storage time at the highest temperature is reasonable and consistent with general cell degradation to be expected under such conditions. However, with respect to the "fine structure" of the 22 °C and 4 °C curves of Fig. 4, we have no ready explanation. Evidently the cell at 4 °C is able to reduce its fragility and hold it low during the 14-day storage interval. The 22 °C data are, in a sense, a combination of the 4 °C and 37 °C results: after an initial rise (as for 37 °C) the cell is able to reduce its fragility (as for 4 °C), but exhausts this ability after about 8–10 days. Thereupon its fragility increases, approaching that for 37 ° at long storage times. Our speculation is that for both the 22 °C and 37 °C storage, the basis for this behavior should lie in metabolic depletion. (Again, for long-term low-temperature liquid storage, fragility should eventually be expected to increase.)

General time correlations

Previous sections of this Discussion have analyzed and interpreted features of the measurements singly or in pairwise comparisons of two types of experiments. It is worthwhile at this point to take a brief overall look in the form of broader comparisons of the results considered all together.

Many of the figures, examined closely, suggest that at least slight transitions in behavior are occurring in a mid-range period of about 6 to 8 days' storage time.

Even if the significance of these, considered individually, could be doubted in some cases, their coexistence at this particular time period is unlikely to be a coincidence. For example, for the fragility data (Fig. 4), we see a tendency towards stabilization following earlier-time changes: for 4 °C a "recovery" from higher fragility, for 37 °C a leveling-off of the previous high rate of increase of fragility. All of the chemical data (K^+, Na^+, LDH, glucose; Figs 5–8) show, for all temperatures, structural changes in their curves which suggest a transition in the degree of cellular control in this time frame: better control prior to this time, some loss of control at later times. The volume data, as well (Fig. 1), show divergence for the three temperatures which becomes significant after this time. The corresponding changes in deformability (Fig. 2) seem to lead the others by a day or two; this could result either from the more sensitive nature of the BI measure of this property, or perhaps from the sensitivity of the property of deformability itself, serving to amplify changes in other, related cellular properties. This further supports the idea that the BI may prove to be a particularly valuable early indicator of change occurring during storage.

Other transition periods also seem to exist. In an earlier time frame, at about 3 days, there is evidence for entering a new phase following an initial transient phase: for volume, a change from slight shrinkage to swelling; for fragility, a change from an increasing to a decreasing BI (or, at 37 °C, to a reduced rate of increase). For the K^+, Na^+, and glucose data there appears to be something of a respite (or in some cases a recovery) from earlier rapid changes. It is as if cell metabolic processes at 3 days are still sufficiently effective to exert the extra effort necessary to combat ongoing degradation occurring during storage.

A final correlation of changes is seen in the late-time data, at about 12–14 days. Loss of volume control is progressing most rapidly, and fragility is once again increasing. The K^+ loss data show a significant change only at 22 °C, but this is the temperature for which the biggest change would be expected; at 37 °C the maximum changes have already occurred, and at 4 °C the cells are much better protected. The Na^+ changes in this late time frame, though less dramatic, are not inconsistent with this interpretation.

To summarize: we observe an early, rapid transient change in cell properties and speculate that at 3 days the cell energy stores are still sufficient for the cell to be able to work with some effectiveness toward recovery. By mid-storage times the cell has been able to re-establish a measure of homeostatic control. Then the control period comes to an end sometime near 7 days, and by 12–14 days enhanced cell deterioration has occurred. This may have reached a limit or be approaching one at 37 °C; at 4 °C the cell is far better preserved, although degradation has occurred and is continuing slowly.

Two final points are worth making. First, the inferences about "fine-structure" changes during the 14-day storage period are reasonable to make, given the time-coincidence of changes in a considerable body of data of different kinds (obtained in three different cooperating laboratories). Second, such inferences and correlations are made all the more credible by the fact that the data shown are

samples drawn from a number of different donors, hence are not idiosyncratic for any single individual. This coincident fine-structure in cell changes during storage has apparently not been reported before, probably because previous workers have not tracked so many properties as closely as was done here.

Temperature-jump responses

Figures 9 and 10 reveal that cell responses to temperature changes occur over vastly longer periods of time than needed merely for thermal equilibration. This much is expected, but the complex behavior seen in Figs 9 and 10 and the duration of the time scales needed to accomplish the full response could not have been predicted.

The change from 4 °C to 37 °C produced results simplest to understand. Both the initial K^+ and Na^+ responses (3 data points, days 7–8) indicate that energy stores have been sufficiently preserved during the earlier 4 °C storage for the cells to reassert a measure of homeostatic, ionic-gradient control: slightly "pumping up" cellular K^+ (i.e. lowering plasma K^+), and lowering cellular Na^+. Within 7 more days (i.e. by day 14), this ability has largely been exhausted: plasma K^+ is now up to its maximum value and Na^+ is again entering the cell from the plasma with kinetics comparable to the isothermal data at 37 °C. The fact that plasma Na^+ at day 14 is higher than the isothermal 37 °C data reveals that cell condition has benefited from its history of 7-day storage at 4 °C. For K^+, however, the degradation process has already run its course by day 14. These differences in K^+ and Na^+ evolution, seen in both the isothermal and temperature-jump experiments, can be viewed as reflections of fundamental differences in cell control mechanisms for these two components.

The 4 °C → 22 °C curves in Figs 9 and 10 are generally consistent with those for 4 °C → 37 °C and with the previous discussion, though they also contain some puzzling features. Consistency is found, for example, in the plasma K^+ drop immediately after the temperature-jump, and in its eventual increase, with kinetics very close to those shown for isothermal storage (at 22 °C). However, the Na^+ data contain puzzles: the curious "inversion flux" between days 7 and 8, and the fact that plasma Na^+ is always lower after the jump to 22 °C than after the jump to 37 °C. This latter suggests that the jump to 22 °C results ultimately in more cell degradation than at 37 °C. However, this may just be a continuing manifestation of the complex, earlier, inversion-flux response to the jump. We have no explanation for these 22 °C-phenomena. They join the class of anomalous cell/membrane responses detected in the vicinity of room temperature: for example, Figs 5 and 6 where the 22 °C-curves lie outside the bounds of the 4 °C- and 37 °C-curves, and other observations by Mel and Reed [8], Mohandas et al. [22], and Dellinger [23].

Conclusion

The simultaneous tracking of many physical properties of erythrocytes and chemical species in plasma has led to a new definition of details in cell evolution during long-term storage at 4 °C, 22 °C and 37 °C. Many features of these individual property changes were peculiar and not predictable according to common understanding, but most were shown to be consistent with an overall pattern of osmotic volume regulation, geometrical constraints on the cell, loss of membrane LDH, and changes in cell metabolism. Previously unrecognized stages in cell evolution were observed, with transitions at approximately 3 days and 7 days (varying somewhat with temperature). Certain behavior at 22 °C appeared anomalous, in the sense of not being intermediate between 4 °C and 37 °C behavior, possibly related to other anomalies reported in the literature for 22 °C. A new type of experiment involving a sudden change of storage temperature demonstrated that cell mechanisms respond in a complex kinetic fashion.

The role played in these tests by the antibiotic is not entirely known but is believed to be minimal. Antibiotic concentration in these blood samples was only slightly larger than the physiological levels attained in normal medical treatment. While some cell membrane alterations might be expected, such as could be reflected in deformability measurements, another study in this laboratory [24] with a different antibiotic (amikacin) showed that the BI was affected by less than 3% at a storage age of 12 days when comparable dosages were involved. In any event, comparison of the results at different temperatures and the major evolutionary trends should be largely independent of the antibiotic.

Acknowledgement

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Transfusion of Rh-Incompatible Blood Components to Cancer Patients

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To study the effects of Rh_0D incompatible transfusion of platelets and/or granulocyte concentrates, we analyzed the transfusion history of 118 oncology patients. Patients received a mean 36.2 units of random platelets, a mean 6.2 concentrates of single donor platelets, and a mean 8.4 concentrates of granulocytes. Patients were not treated with anti-Rh_0D specific immunoglobulin following the incompatible transfusions. The patients were followed for a mean 9.4 months to detect the appearance of Rh_0D antibodies. Of the 118 patients, 3 were lost to follow-up. Of the remaining 115, 2 cases (1.74%) developed anti-Rh_0D following administration of the incompatible transfusions.

Keywords: cancer patients, granulocyte transfusion, platelet transfusion, random donor platelets, Rh_0D incompatible, single donor platelets

Due to aggressive therapeutic regimens, oncology patients require substantial support with platelet and granulocyte concentrates that very often are Rh_0D incompatible and unavoidably contaminated with RBC. To study the effects of Rh_0D incompatible transfusion, we analyzed the transfusion history of 118 patients: 50 acute leukemias, 8 chronic leukemias, 14 lymphomas, 45 other malignant tumors and 1 Glanzmann's disease. The mean age was 45.7 years and 64 patients were male.

Treatment consisted of various chemotherapeutic protocols with multiple drug combinations. All patients were routinely blood grouped and Rh_0D typed as well as screened for "irregular" antibodies. They were repeatedly transfused with Rh_0D positive platelet and/or granulocyte concentrates, receiving a mean 36.2 units of random platelets (range 2–321), a mean 6.2 concentrates of single donor platelets (range 1–28), and a mean 8.4 concentrates of granulocytes (range 1–24).

Patients were not treated with Rh_0D specific immunoglobulin following the incompatible transfusions. The patients were followed for a mean 9.4 months (range 0.07–84 months) to detect the appearance of Rh_0D antibodies. Of the 118 patients studied, 3 were lost to follow-up. Of the remainder 115, we found 2 cases (1.74%) that developed anti-Rh_0D following the administration of the incompatible transfusions. Thus, our studies show that cancer patients treated with modern chemotherapeutic regimens have a very low capacity to develop anti-Rh_0D when compared with the normal population.

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Introduction

Modern chemotherapeutic regimens administered to cancer patients may produce temporary immunomyelosuppression characterized mainly by pronounced thrombocytopenia and neutropenia [1]. During that time the patients are supported with transfusions of platelets, granulocytes, and red cell concentrates. However, some of the patients are Rh_0D negative, and compatible blood components such as platelet and granulocyte concentrates are not always readily available. Thus, constraints in the supply of Rh_0D-negative components make it necessary that Rh_0D-positive blood components be transfused to Rh_0D-negative patients. In spite of all technical safeguards adopted, Rh_0D platelet and granulocyte concentrates are unavoidably contaminated with variable numbers of red blood cells capable of alloimmunizing an Rh_0D-negative recipient.

Woodrow and Donohue reported that as little as 0.1 ml of Rh_0D-positive red blood cells can immunize an Rh_0D-negative individual [2]. It has also been documented that when Rh_0D-negative normal volunteers were given Rh_0D-positive blood, only 60–80% became alloimmunized by the Rh_0D antigen [3].

Lichtiger et al. reported in a study conducted on cancer patients, the total absence of alloimmunization to the transfusion of Rh_0D-positive platelet concentrates contaminated with red blood cells [4]. In a prior study, Goldfinger and McGinnis found that 7.8% of Rh_0D-negative immunosuppressed patients became alloimmunized after receiving multiple transfusions of Rh_0D-positive platelet concentrates [5].

In this study, we report the experience encountered at The University of Texas M. D. Anderson Hospital and Tumor Institute (UTMDAH) where 118 severely immunomyelosuppressed cancer patients were repeatedly transfused with Rh_0D-incompatible platelet and granulocyte concentrates and only two cases developed Rh_0D antibodies.

Materials and Methods

The clinical histories of 118 consecutive Rh_0D-negative cancer patients at UTMDAH who received transfusion of platelets, granulocytes, and blood were analyzed (Table 1). The patients studied ranged in age between 5 and 77 years (mean age 45.7 years) and 64 patients were male. The patients included in the analysis represented a variety of neoplastic diseases (Table 1) and were treated with various chemotherapeutic protocols with multiple drug combinations that included: cisplatin, vinblastine, methotrexate, chlorambucil, doxorubicin, prednisone, allopurinol, vincristine, cyclophosphamide, and hydroxyurea, among others. Of the 118 patients studied, three were lost to follow-up, 104 are dead, and 11 are still alive. In no case was Rh_0D-specific immunoglobulin given after incompatible Rh_0D transfusions were administered.

All patients were routinely tested for blood group and Rh_0D-type according to methods routinely employed at the UTMDAH Blood Bank and in compliance with the Standards of the American Association of Blood Banks (AABB) [6]. In addition, all patients were screened for irregular antibodies using a potentiating medium (albumin or low ionic strength solution) at room temperature at 37 °C. Antihuman globulin testing was also performed. If the screening was positive, the patient's serum was tested against a panel of red blood cells of known antigens to determine the specificity of the antibody according to methods already published [7]. Platelet concentrates transfused to these patients were derived either from random units of blood or from single donors undergoing apheresis with Haemonetics-30 (H-30) or IBM 2997. Granulocyte concentrates were obtained from relatives of the patient, using the H-30 or IBM 2997. Hydroxyethyl starch was used and donors were premedicated with corticosteroids [8].

Donor testing

All donors were blood grouped, Rh_0D typed, and screened for irregular antibodies according to methods currently in use at our institution and in compliance with recommendations of the Standards of the AABB [6].

Contamination of platelet and granulocyte concentrates with Rh_0D-positive red blood cells

The actual contamination of platelet and granulocyte concentrates was obtained from the routine quality control determination performed on each apheresis concentrate (Table 2). In reference to the random units of platelets, the procedure and calculation have been reported previously [4] (Table 2).

Patients received a mean of 36.2 units of random donor platelets (range 2–321), a mean of 6.2 concentrates of single donor platelets (range 1–28), and a mean of 8.4 concentrates of granulocytes (range 1 – 24). Patients were followed for a mean 9.4 months (range 0.07 – 84 months) to detect the presence and/or development of Rh_0D and other blood group related antibodies (Table 3).

Results

The 118 patients included in this study received massive hemotherapeutic support throughout the evolution of their neoplastic process and treatment. Many of the components transfused were Rh_0D-positive due to logistical constraints in obtaining and transfusing Rh_0D-compatible components. During the course of treatment, three patients were lost to follow-up. Of the remaining 115 patients, only two were found to have developed Rh_0D antibodies at 4 and 48 months after having been transfused with Rh_0D-incompatible components. Of the remainder that remain alive, antibody screening remains negative.

Table 1

Clinical summary

Description	No. of Patients	Sex	Age Mean (Range)	Component transfused mean (range)			Follow up No. of Months Mean (Range)	Dead or Alive	Development of Anti-Rh_0D
				Random Platelets No. of Units Mean (Range)	Single Donor Platelets No. of Units Concentrates Mean (Range)	Granulocyte Concentrates No. of Units Mean (Range)			
AML	39	20 M 19 F	49.4 (18–72)	44.9 (4–163) 35 Patients	6.3 (1–28) 23 Patients	10.3 (2–24) 21 Patients	11.08 (0.1–60)	33 Dead 6 Alive	2 Patients 1–Developed Anti D+C 4 Mos. after 1st Transfusion. 40 Y/O Female. B Neg. 2–Developed Anti D and Anti E 48 Mos. after 1st Transfusion. 36 Y/O Female. O Neg.
AMML	6	3 M 3 F	48.6 (21–75)	91 (12–321) 6 Patients	3.75 (2–6) 4 Patients	5 (4–6) 2 Patients	8.72 (0.3–26)	6 Dead	No evidence of Anti-Rh_0D
ALL	5	4 M 1 F	16.6 (6–19)	46.4 (2–122) 5 Patients	—	5 2 Patients	15.8 (1–40)	4 Dead 1 Alive	No evidence of Anti-Rh_0D
CLL	1	M	54	146	—	—	10	1 Dead	No evidence of Anti-Rh_0D
CML	7	3 F 4 M	44 (26–70)	102.4 (12–294) 7 Patients	19 1 Patient	3 (2–4) 2 Patients	20.66 (0.3–48)	7 Dead	No evidence of Anti-Rh_0D

Lymphomas: 9 Lymphomas 4 Hodgkin's 1 M Myeloma	14	11 M 3 F	51.2 (25–77)	28.8 (4–103) 13 Patients	2.5 (1–4) 2 Patients	5 1 Patient	2.3 (0.13–9)	12 Dead	No evidence of Anti-Rh_0D 2 Patients Lost to Follow Up
Ca of Breast	12	12 F	51.9 (33–73)	11.08 (4–54) 12 Patients	2.5 (1–4) 2 Patients	0	8.88 (0.07–36)	12 Dead	No evidence of Anti-Rh_0D
Ca of Lung	13	9 M 4 F	51.9 (24–69)	14 (2–40) 13 Patients	—	—	5.90 (0.3–14)	13 Dead	No evidence of Anti-Rh_0D
Glanzmann's disease	1	M	10	49	—	—	84	Alive	No evidence of Anti-Rh_0D
Other solid tumors	20	12 M 8 F	47 (5–75)	11.75 (4–40) 20 Patients	—	2.5 (2–3) 2 Patients	6.26 (0.03–60)	16 Dead 3 Alive	No evidence of Anti-Rh_0D 1 Patient Lost to Follow Up

Table 2

Contamination of blood components with Rh_0D-positive red blood cells

Random donor platelets mean (range)	Single donor platelets mean (range)	Granulocyte concentrates mean (range)
0.522 ml (0.21–0.74)	1.5 ml (0.3–4.2)	20 ml (8–31)

(Values expressed in ml's of packed red blood cells)

Table 3

Length of follow-up and number of patients

1 Month or less	2 Months or less	3 Months or less	4 Months or less	5 Months or less	6 Months — 84 Months
33 Patients	12 Patients	9 Patients	3 Patients	6 Patients	52 Patients

Discussion

In the current treatment of cancer, hemotherapeutic support plays a pivotal role. However, it has been documented that cancer patients who are subject to chemotherapy with multidrug combinations become immunomyelosuppressed and require massive hemotherapeutic support that includes transfusion of platelet and granulocyte concentrates [9, 1]. Usually the tranfusion of blood components are ABO and Rh_0D compatible. At times this cannot be done due to the fact that Rh_0D-negative platelets and granulocytes are not always available.

Platelets do not seem to carry Rh_0D antigens [10]. Likewise, Rh_0D antigens could not be found on granulocytes [11]. Although platelets and granulocytes cannot be implicated in Rh_0D alloimmunization, the red blood cell contamination in each unit of concentrate carries the definite potential for alloimmunization to occur with the consequent Rh_0D antibody development. Previous reports have found that as little as 0.1 ml of Rh_0D-positive blood can immunize an Rh_0D-negative recipient [2].

The volume of red blood cells contaminating the various concentrates transfused to the patients in our study easily surpassed 0.1 ml (Table 2). In spite of the continuous infusion of Rh_0D-positive components, we were only able to

find two patients out of 115 (1.74%) who developed anti-Rh_0D at 4 and 48 months after first being exposed to Rh_0D-positive platelet and granulocyte concentrates. It is interesting to note that they also developed antibodies to rh'C in one case and to rh"E in the other (Table 1).

Goldfinger and McGinnis [5] found that 7.8% of 102 patients with impaired immune status became alloimmunized to Rh_0D-incompatible platelet transfusion. Lichtiger et al. reported a study of 30 oncology patients who failed to reveal any alloimmunization to Rh_0D-platelet transfusion. Pfisterer et al. [12] demonstrated that patients with Rh_0D antibodies did not show shortened survival of Rh_0D-positive platelets labeled with ^{51}Cr. It is conceivable that aggressive chemotherapeutic regimens render cancer patients less responsive to repeated stimulation with Rh_0D-incompatible blood components. Two separate studies have pointed out that a high percentage of patients with neoplastic diseases failed to develop lymphocytotoxic antibodies to HLA antigens in spite of repeated stimulation [13, 14].

It could be speculated that in those cases in which patients have received ABO and Rh_0D-incompatible platelets the ABO incompatibility had a protective effect as it is postulated in the hemolytic disease of the newborn [7]. On the other hand, Shulman [15] and Duquesnoy et al. [16] could not demonstrate any significant effect of ABO incompatibility on the survival of platelets. However, in our present and previous studies the ABO incompatible transfusions were not the majority, as most platelet and granulocyte concentrates infused were actually ABO compatible [4].

The results of this study would indicate that transfusion of Rh_0D-incompatible platelet and granulocyte concentrates into cancer patients does not produce alloimmunization with the frequency encountered and reported in the normal population. Obviously, these patients do not seem to become immunized to that particular antigen and can safely tolerate numerous transfusions containing large numbers of Rh_0D-incompatible red blood cells, if compatible blood components cannot be found. Although we are not postulating indiscriminate transfusions of Rh_0D-incompatible components, these should not be withheld when needed.

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Effect of Dipyridamole on Adenosine Incorporation into Hypoxanthine Nucleotides of Fresh Human Red Cells

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Fresh human red cells were incubated for 2 hours in a medium containing adenosine, pyruvate and inorganic phosphate (APP medium), or in APP medium supplemented with 10^{-4} M dipyridamole (APPD medium). No measurable amount of ITP was found in fresh red cells, and the average IMP content in these cells was 0.18 ± 0.09 μmol/g Hb. After 2 hours incubation in APP medium, the IMP content increased almost 8.5-fold to 1.52 ± 0.78 μmol/g Hb. Under these conditions the ITP level also increased to 1.40 ± 0.84 μmol/g Hb. After 2 hours incubation of red cells in APPD medium, the average IMP content increased to 5.30 ± 2.33 μmol/g Hb, about 3.5 times that found in APP medium. At the same time ITP content was about 53.6% lower, that is 0.65 μmol/g Hb. In red cells incubated in APPD medium, penetration of 8-^{14}C-adenosine decreased by 50%, and incorporation of this nucleotide into the pool of all free nucleotides also decreased by 18.2% as compared to red cells incubated in APP medium. It is concluded that IMP is probably formed directly from AMP gained by the phosphorylation of adenosine during its penetration.

Keywords: human red cells, adenosine, hypoxanthine nucleotides, dipyridamole

Introduction

Adenosine is taken up rapidly by erythrocytes [1] because their membrane is highly permeable to this nucleotide [2]. According to Roos and Pfleger [3] the mechanism of adenosine uptake possesses two components. One component is saturable and kinetically resembles an enzymatic reaction. It is described by a Michaelis-Menten type equation and is inhibited by dipyrimadole, a well-known inhibitor of nucleotide uptake into a variety of cells [4–10]. A second component corresponds to diffusion and is not saturable, cannot be inhibited, and appears to represent diffusion across the lipophilic parts of the membrane [3].

It is not known whether the enzyme-like component consists of active transport, passive carrier-mediated transfer, or uptake induced by intracellular phosphorylation. All these possibilities may correspond to Michaelis-Menten kinetics and be inhibited by dipyridamole. Rozenberg and Walker [11] found that dipyridamole is a powerful inhibitor of ^{14}C-adenosine phosphorylation by platelets.

The aim of the present study was to examine the effect of dipyridamole on the incorporation of 8-^{14}C-adenosine into hypoxanthine nucleotides (IMP and

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ITP) of fresh human red cells incubated for two hours at 37 °C in a medium containing adenosine, pyruvate, inorganic phosphate and NaCl (APP medium).

Material and Methods

Fresh human blood was collected from healthy donors into heparinized glass bottles. The blood was centrifuged at 4 °C, the plasma and buffy coat removed, and the red cells washed three times with an excess of chilled 0.9% NaCl solution. Incubation of erythrocytes was performed at 37 °C in (1) APP medium, i.e. a solution containing adenosine (10 mM), pyruvate (10 mM), inorganic phosphate (50 mM) and NaCl (75 mM); or (2) APPD medium, i.e. APP medium containing 1×10^{-4} M dipyridamole. The pH of the two media was adjusted to 7.35 and added to the washed erythrocytes to obtain a haematocrit of about 20%. Before erythrocytes were added to 10 ml of APP or APPD media, 8-^{14}C-adenosine (specific activity 49 MCi/mmol) was introduced (1 μCi/ml erythrocytes). Suspensions of erythrocytes were placed in water bath at 37 °C under continuous agitation. Prior to the addition of the incubation media, aliquots of erythrocytes were removed and soluble compounds were extracted with perchloric acid [12]. After two hours incubation, samples of erythrocytes were removed and rapidly chilled in an ice-bath. The medium was removed by centrifugation, and the cells were washed three times with an excess of cold 0.9% NaCl solution. Acid soluble phosphate compounds were extracted in the same manner [12]. Separation of the phosphate compounds was achieved by means of column ion-exchange chromatography with Dowex-1, formate form [13, 14]. Hypoxanthine and adenine nucleotides in eluates were determined spectrophotometrically [15]. Identification of IMP and ITP was performed as described previously [14, 15]. The results obtained were expressed in μmol nucleotide/g Hb. Isotope counts were affected in APP and APPD solution before and after incubation of red cells, in erythrocytic acid soluble extracts and in the fractions obtained as a result of chromatographic separation of phosphate compounds. The counts were carried out in Wallace 81 000 scintillation counter using Brey solution. In each case the count lasted 5 minutes, and results were expressed in the number of decays per min (DMP).

Results

No measurable amount of ITP was present in fresh red cells. The average IMP content in these cells was 0.18 $\pm$ 0.09 μmol/g Hb. After two hours incubation in APP solution, the IMP content increased almost 8.5-fold to 1.52 $\pm$ 0.78 μmol/g Hb. Under these conditions ITP synthesis took place; the mean ITP level reached the value of 1.40 $\pm$ 0.84 μmol/g Hb (mean $\pm$ S.D. of 17 experiments). After two hours incubation of red cells in APPD medium, the average IMP content was 5.30 $\pm$ 2.33 μmol/g Hb and the ITP content 0.65 $\pm$ 0.26 μmol/g Hb

(mean ± S.D. of 15 experiments). Results are presented in Fig. 1. In 10 experiments, 8-^{14}C-adenosine was added to the APP and APPD media before the addition of red cells. The counts of labelled adenosine in erythrocytes (expressed in DPM/ml cells) after incubation in APP solution was 493.357 ± 82.212 and after incubation in APP medium it was only 226.439 ± 14.984. The percentage of labelled nucleotide penetration into red cells with APP medium was 21.7%, and with APPD medium only 10.8%. Comparing the counts of acid-soluble extracts with the total counts of isotope penetration into red cells, it was found that 47.3% of 8-^{14}C-adenosine had been incorporated into nucleotides after 2 hours of red

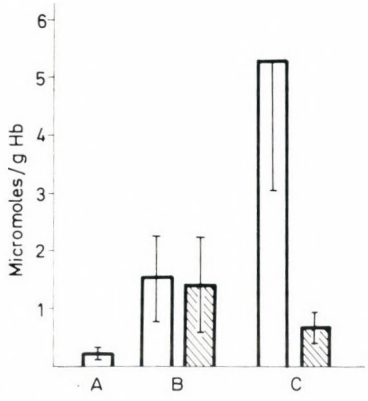

Fig. 1. Hypoxanthine nucleotide content of fresh human red cells before and after 2 hours incubation in APP and APPD medium. A— before incubation; B — after 2 hours incubation in APP medium; C — after 2 hours incubation in APPD medium; Open columns — IMP; hatched columns — ITP. Note that before incubation the red cells contained no ITP

cell incubation in APP medium and 29.1% after incubation in APPD medium. Taking for 100% the sum of nucleotide counts of erythrocytes incubated in APP medium, hypoxanthine nucleotides were found to contain 5.3% of 8-^{14}C-adenosine of which 1.3% was in ITP and 4.0% in IMP. Following incubation of erythrocytes in APPD medium (assuming that the sum of nucleotide counts reached 100%), hypoxanthine nucleotides were found to contain 13.4% of the isotope of which 12.9% was in IMP and only 0.5% in ITP.

Thus following 2 hours incubation of fresh erythrocytes in APPD medium, there was a marked decrease of 8-^{14}C-adenosine penetration into red cells and its incorporation into nucleotides with the exception of hypoxanthine nucleotides or, more precisely, of IMP. The synthesis of adenine nucleotides in red cells incubated in both media also differed significantly (detailed results will be published elsewhere).

Discussion

Under physiological conditions no free adenine or adenosine occurs in erythrocytes [16]. Purine bases and their nucleotides easily penetrate the red cell membrane [2]. However, in view of the fact that adenosine passes across the cell membrane quickly, its intracellular concentration is regulated by the enzymes which metabolize it [17, 18]. Inside the red cell, adenosine undergoes two processes, i.e. phosphorylation which leads to the formation of AMP and later to ADP and ATP, and deamination to inosine [10]. There is general agreement that dipyridamole inhibits adenosine uptake by erythrocytes [3, 10, 19] and platelets [4].

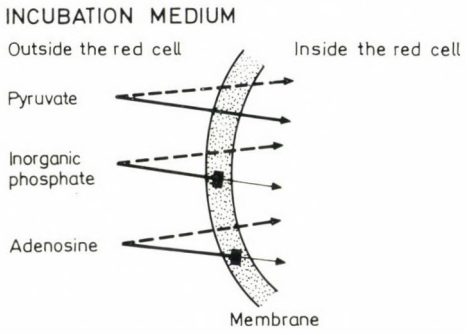

Fig. 2. Graphic illustration of the dipyridamole action. (– – – –) APP medium; (—) APPD medium; —■— inhibitory effect of dipyridamole

We have shown that dipyridamole inhibited the penetration of 8-^{14}C-adenosine into red cells incubated in APP solution. During 2 hours incubation in APP medium, 21.7% of the isotope, while in the presence of dipyridamole two times less (10.8%) penetrated the red cells. Bretschneider et al. [5] showed that in the case of blood platelets the inhibition of 8-^{14}C-adenosine is even higher and reaches ca 80%. The inhibitory effect of dipyridamole on adenosine penetration of erythrocytes, as shown in Fig. 2, reduced the synthesis of phosphate compounds [20–23]. In this study, in the course of APPD incubation of fresh erythrocytes we could confirm the inhibition of phosphate compounds synthesis and the reduced incorporation of 8-^{14}C-adenosine into nucleotides. We adopted the convention that the quantity of 8-^{14}C-adenosine taken up by the erythrocytes constitutes 100%; if this quantity was incorporated by the nucleotides of APP incubated cells, only 29.1% was taken up by APPD incubated cells. The reduced level of synthesis did not occur in the case of IMP; its quantity in APPD incubated erythrocytes was 3.5 times that in APP incubated was, amounting to 1.52 and 5.30 μmol/g Hb, respectively. At the same time the level of ITP synthesis in APPD incubated red cells was markedly lower than after incubation in APP medium; ITP concentration in APP medium was 1.40 μmol/g Hb, while in APPD

medium it was a mere 0.65 μmol/g Hb. This finding agrees with that of Zachara [20, 21] who incubated in APP medium erythrocytes of blood preserved in ACD medium for 11–15 days at 4 °C; in erythrocytes incubated for 2 hours in APPD medium ITP synthesis was somewhat smaller than in those incubated in APP medium, being 35.5% while the level of IMP synthesis was identical, i.e. there is a 3.5-fold increase in comparison with that in APP incubated cells. Zachara suggested [22] that the main cause of the lower rate of synthesis of phosphate compounds in erythrocytes incubated in APPD medium was an insufficiency of P_i inside the red cells (Fig. 2), because dipyrimadole considerably reduced the penetration of P_i across the red cell membranes [22, 24–26]. What remains unexplained, however, is the considerable increase of IMP after 2 hours incubation of erythrocytes in APPD medium. In view of the inhibitory effect of dipyridamole on adenosine deamination [27, 28] one would expect a direct phosphorylation of that nucleotide to adenine nucleotides. The adenosine which had penetrated the membrane might undergo phosphorylation to AMP, especially as dipyridamole present in the cell does not inhibit the activity of adenosine kinase [2]. The arising AMP might undergo deamination to IMP, as AMP deamination proceeds with considerable activity in the red cells and is not inhibited by dipyridamole [29]. Thus, such a mechanism might be responsible for the increased levels of IMP and APPD incubated erythrocytes.

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Prognostic Factors in Dysmyelopoietic Syndromes

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Dysmyelopoietic syndromes are characterized by cytopenia in peripheral blood and marrow maturation abnormalities. The condition has been studied in 33 patients with regard to the initial haematologic picture. A certain number of variables of prognostic value was observed: excess of marrow blasts, pancytopenia, and decreased growth of bone marrow cells in agar culture. The length of survival and the percentage of leukaemic transformation were different in patients with refractory anaemia and refractory anaemia with an excess of blasts.

Keywords: dysmyelopoietic syndromes, excess of blasts, in vitro growth, leukaemic transformation, pancytopenia.

Introduction

Dysmyelopoietic syndromes (DMPS) [1] correspond to several morphological entities which are characterized by the presence of one or several cytopenias and marrow maturation abnormalities. The bone marrow is usually cellular despite the associated cytopenias in peripheral blood. Aberrant cellular development is usually associated with abnormal morphologic characteristics involving the nucleus and cytoplasm. The occurrence of acut leukaemia [AL] is not obligatory and the time of appearance varies considerably. In the absence of transformation into AL, evolution can be prolonged, with death occurring due to some intercurrent disease or infectious or haemorrhagic complications of the cytopenia. According to the importance of the different anomalies of the blood and bone marrow, DMPS has been described as idiopathic sideroblastic anaemia [2, 3], refractory anaemia with an excess of blasts [4], oligoblastic leukaemia [5], subacute leukaemia [6], chronic myelomonocytic leukaemia [7, 8], or simply preleukaemic syndromes [9, 10]. New diagnostic criteria for the diagnosis of the various types of DMPS were proposed by the FAB Coooperative Group. Five types were described: (1) refractory anaemia (RA); (2) RA with ring sideroblasts; (3) RA with excess of blasts (RAEB); (4) chronic myelomonocytic leukaemia (CMML); and (5) RAEB in transformation. One of the main distinguishing features of these conditions is the proportion of blast cells in peripheral blood and bone marrow [1].

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This report presents a retrospective study of 33 patients with DMPS, based on parameters from an initial set of results. Statistical study of these parameters revealed a number of variables of prognostic value.

Materials and Methods

Patients

The diagnosis of DMPS was made in 33 patients between 1978 and 1984. DMPS was considered in patients with cytopenia and marrow maturation abnormalities, but they suffered neither from vitamin deficiency, nor metabolic problems. 19 patients had pancytopenia, 14 had no pancytopenia. Table 1 gives the repartition of these 33 patients into different types of DMPS.

Methods

Study of granulopoiesis was carried out in agar culture using placental conditioned medium as a colony stimulating factor [11, 12]. The number of GM-CFC was scored on day 10 in 23 patients. The growth of bone marrow cells was examined in Dexter-type long-term bone marrow culture from 21 patients [13]. The number of non-adherent cells and granulocyte-macrophage progenitors (GM-CFC) was determined weekly. Statistical analysis was carried out according to Fischer [14].

Results

Age, haemoglobin level, number of platelets and polymorphonuclear neutrophils separately did not influence survival. The percentage of blasts observed in bone marrow smears and the presence of pancytopenia appeared to be strongly correlated with the length of survival ($p < 0.05$) (Figs 1, 2). It seemed that patients with RAEB have a less favourable prognosis than patients with RA ($p < 0.05$) (Fig. 3). The rate of leukaemic transformation was higher in patients with RAEB

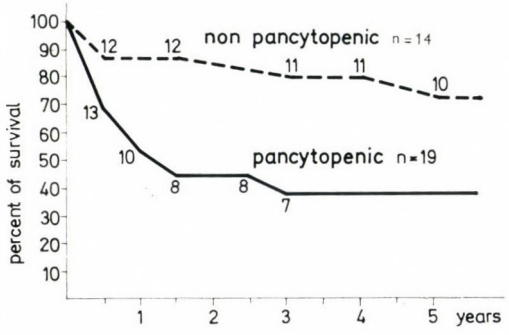

Fig. 1. Role of pancytopenia in survival of 33 DMPS patients

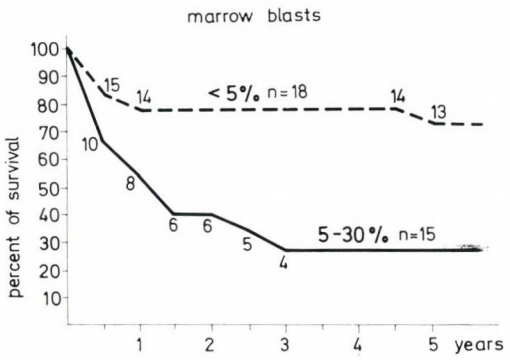

Fig. 2. Role of marrow blast number in survival of 33 DMPS patients

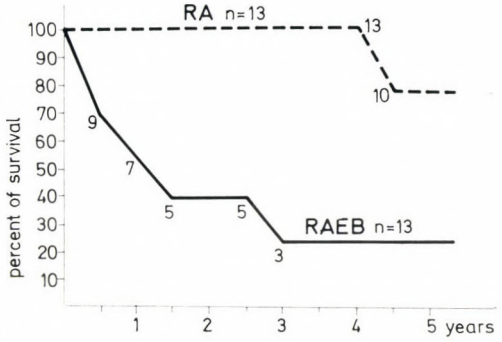

Fig. 3. Survival of patients with RA and RAEB

(Table 1). Leukaemic transformation was observed in 13 patients; out of these, 11 patients had earlier pancytopenia. Three patients died because of haemorrhagic complications, all of them had pancytopenia.

Table 1

Patients with DMPS: Diagnostic subdivisions

Patients		Colonies + clusters	Clusters	No growth
Pancytopenic patients	n = 12	5	5	2
Non-pancytopenic patients	n = 11	10	1	
Patients who died (because of leukaemic transformation or haemorrhagic complications)	n = 7		6	1
Survival patients	n = 16	15		1

Table 2

Growth type of bone marrow cells in agar culture from 23 patients

Patients		Colonies + clusters	Clusters	No growth
Pancytopenic patients	n = 11	1	5	5
Non-pancytopenic patients	n = 10	8	2	
Patients who died (because of leukaemic transformation or haemorrhagic complications)	n = 6		4	2
Survival patients	n = 15	9	3	3

Except for two cases, the number of GM-CFC was lower than in haematologically normal patients, or in vitro growth was not observed. Normal colonies and clusters grew in 10 cases from non-pancytopenic patients and in 15 cases from surviving patients (Table 2). The presence of clusters without colonies was an unfavourable sign.

The decrease of the number of non-adherent cells in long term cultures was similar in pancytopenic and non-pancytopenic patients. There was no difference in the decline of cell number in Dexter-type cultures from patients with or without leukaemic transformation. Colonies were not observed in agar cultures made from long-term cultures of patients with leukaemic transformation, and sometimes there was no growth in short-term agar cultures during long-term cultivation (Table 3).

Table 3

In vitro growth type of bone marrow cells in long-term cultures

Diagnosis	No. of patients	Pancytopenia		Non-pancytopenia		Death from		Alive
		with leukaemic transformation	without	with leukaemic transformation	without	acute leukaemia	haemorrhagic or infectious complications	
RA	13	—	6	2	5	2	1	10
RA with ring sideroblasts	4	—	—	1	3	1	—	3
RA with excess of blasts	13	8	2	—	3	8	2	3
CMML	1	1	—	—	—	1	—	—
RAEB in transformation	2	1	1	—	—	1	—	1
All patients	33	10	9	3	11	13	3	17

Discussion

The individualization of DMPS as a specific entity has been described recently. Over the past few years numerous articles have drawn attention to the clinical, biologic and morphologic symptoms, and have proposed several subclassifications of DMPS [1, 15]. The results indicate that DMPS includes diseases that are not rare and are characterized by fairly typical anomalies [1]. The diagnosis is not synonymous to preleukaemia, but there is an increased risk of transformation into AL. DMPS is a serious illness whether or not AL develops. The overall mortality rate is 72%, AL occurs in 32% of the cases [16]. Varela et al. [17] have follow-up data on 60 cases of DMPS; median survival for patients with RA was 47 months, for those with RAEB, 14 months. The proportion of cases in which AL occurred was significantly higher in the RAEB group. Juneja et al. [18] reported the data of 118 DMPS and found the highest transformation rate and shortest median survival in RAEB. Vallespi et al. [19] described statistically significant differences in survival among the five groups, and RAEB evolved into AL more often than the others. Our data regarding leukaemic transformation and survival are similar. As the data of Coiffier et al. [16] our study did not reveal any prognostic value for low granulocyte count, platelet number and haemoglobin level. The excess marrow blasts and the presence of pancytopenia appear to be the most important prognostic factors [16].

The number of GM-CFC was markedly decreased in all patients but two, in agreement with the findings of a number of authors [20, 21, 22, 23, 24]. According to Coiffier et al. [16] the excess of clusters is a characteristic feature of evolution into AL. Marrow cultures are likely to be of importance in assessing residual haemopoietic function in such cases, and may predict the progression into AL.

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The Importance of Aspartate Aminotransferase for Platelet Aggregation

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In bovine platelets aspartate aminotransferase has a high activity. The enzyme in vitro is inhibited in a dose dependent manner by aminooxyacetate ($IC_{50} = 10^{-4}M$), hydroxylamine ($IC_{50} = 10^{-4}M$), and cycloserine ($IC_{50} = 5 \cdot 10^{-3}$). The inhibitory effect of all the three compounds is strongest at low substrate (aspartate) concentration.

Blocking of aspartate aminotransferase activity by these compounds in intact platelets is accompanied by the inhibition of ADP and collagen-induced aggregation. Among the three compounds the strongest inhibitor of platelet aggregation was hydroxylamine, which was also the most effective inhibitor of aspartate aminotransferase.

Other metabolic blockers, i.e. dinitrophenol (DNP), rotenone and antimycin also inhibited the aggregation of platelets, and a synergism has been demonstrated between DNP, rotenone and antimycin A action on platelet aggregation and blockade of aspartate aminotransferase activity.

The results are interpreted to mean that transamination is of importance in the energy production in the activated platelet, probably through its participation in reducing equivalents transport from the cytosol to the mitosol via the malate: oxaloacetate: aspartate shuttle.

Keywords: aminooxyacetate, aspartate aminotransferase, cycloserine, hydroxylamine, platelet aggregation

Introduction

Aspartate aminotransferase (EC 2.6.1.1.) catalyzes the reversible transfer of the amino group of aspartic acid to the α-ketoglutaric acid, forming glutamic and oxaloacetic acids [1]. The enzyme occurs widely and plays an important role in cellular amino acid metabolism [1, 2]. Another important function of aspartate aminotransferase is its participation in the transfer of reducing equivalents from the cytosol to mitochondria [3].

The aspartate : oxaloacetate : malate shuttle (Scheme 1) proposed for the mitochondrial oxidation of cytosolic NADH includes a double transamination step, and two forms (mitochondrial and cytosolic) of aspartate aminotransferase are involved in this cycle [2, 3].

No data are available on the operation of this shuttle in blood platelets, but high concentration of aspartate and glutamate [4] together with the high activity

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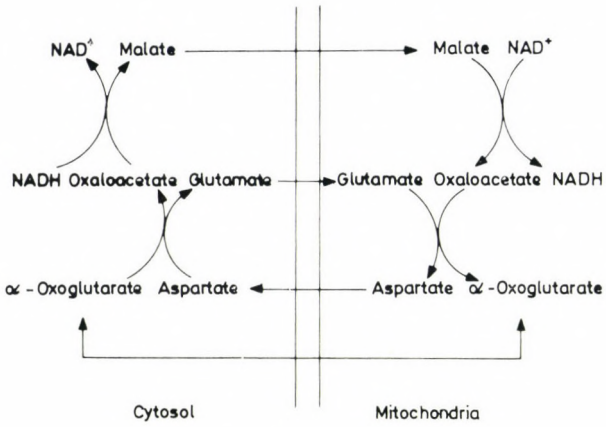
of aspartate aminotransferase and malate dehydrogenase [4] found in platelet homogenates suggest the occurrence of this cycle in these cells.

The present study was aimed at clarifying the question whether the reducing equivalent transport from the cytosol to mitochondria was of any importance for platelet aggregation.



Scheme 1

The results of experiments in which specific aminotransferase inhibitors were used suggest the existence of the malate aspartate cycle in bovine platelets and indicate that the closing of this shuttle is accompanied by the inhibition of platelet aggregation.

Materials and Methods

Chemicals

Collagen was the product of Hormonchemie, Munich, FRG., ATP, amino-oxyacetate (AOA), D-cycloserine (CS), 2,4-dinitrophenol (DNP), pyruvate (sodium salt), NADH, L-aspartic acid, α-ketoglutaric acid, N-2-hydroxylethypiperazine-N'-2-ethanesulfonic acid (HEPES), rotenone and antimycin A were purchased from Sigma Chemical Co. Aspartate aminotransferase, malate dehydrogenase, ADP (disodium salt) and lactate dehydrogenase were obtained from Boehringer, Mannheim, FRG. Other reagents were analytical grade products.

Preparation of platelet-rich plasma

Nine volumes of fresh bovine blood were collected in one volume of 3.8% (W/V) sodium citrate. Platelet rich plasma (PRP) was obtained by centrifugation of the blood in plastic tubes for 15 min at 300 *g* at room temperature.

Assay of aggregation

Platelet aggregation was followed turbidimetrically [5] by recording the light transmission through a stirred platelet suspension in a plastic cuvette of an aggregometer (Elvi Logos, Milan, Italy) at 37 °C. 1.3 ml PRP was pre-incubated in an aggregometer under stirring. After 2 min 1–10 μl of the inducer were added. The inducers were used at their threshold concentration required for inducing maximum aggregation in the control. The concentrations were between 6–10 μM for ADP and 8–15 μM for collagen. In order to test the inhibition of aggregation, 1–15 μl of a solution of the tested substances was added to the stirred PRP in the aggregometer 2 minutes before addition of the inducer. In control assays, 1–15 μl of saline or ethanol was added instead of the inhibitor solution. The amounts of ethanol (1–15 μl) added with rotenone, antimycin A and DNP did not per se affect the aggregation of platelets. Aminooxyacetate, cycloserine, hydroxylamine and ADP were dissolved in saline and the pH of the solutions was adjusted to 7.4 by 0.2 M NaOH.

Rotenone, antimycin A and DNP were used as ethanolic solutions. All experiments were performed at least in triplicate, using 3–5 different platelet preparations.

Isolation of platelet concentrate

Platelets were isolated from fresh bovine blood that had been collected into ACD solution (0.075 M citric acid + 0.085 M sodium citrate + 2% glucose) at a ratio of 9 : 1. The platelet rich plasma was separated by centrifugation at 300 *g* at 20 °C for 55 min. The platelets were pelleted by centrifugation at 1200 *g* for 15 min. The resulting platelet pellet was resuspended in a small volume of plasma and centrifuged at 100 *g* for 10 min. This step was repeated if necessary until contaminating erythrocytes and leukocytes had been removed. Finally, the platelets were collected by centrifugation at 1200 *g* for 20 min and washed twice in medium consisting of 0.14 M NaCl, 5 mM EDTA and 5 mM HEPES pH 7.4. The resulting platelet pellet obtained from 5 liters of blood was suspended in 20 ml of this medium and will be referred to as platelet concentrate.

Measurement of enzyme activities

Enzyme activities were measured in the high speed supernatant fraction obtained after centrifugation (150 000 *g* for 40 min) of ultrasonically disrupted (6 bursts each at 20 kHz for 15 s with 30 s intervals at 0 °C) platelet concentrate. Dithiotreithol and Triton X-100 were added to the final concentration of 1 mM and 0.5% W/V respectively prior to sonication.

Aspartate aminotransferase, alanine aminotransferase, malate dehydrogenase, lactate dehydrogenase and α-glycerophosphate dehydrogenase (cytosolic) were followed spectrophotometrically according to Bergmeyer [6].

The concentration of protein in the supernatant was determined by the biuret micromethod according to Zamenhof [7].

The concentration of platelets in platelet concentrates was determined by phase contrast microscopy [8].

Results

As can be seen from Table 1, the bovine platelets contained all enzymes which enable the operation of the malate: oxaloacetate: aspartate cycle, i.e. malate dehydrogenase and aspartate aminotransferase.

Table 1

Activities of some enzymes in bovine blood platelets

	Initial velocity (μmol of substrate $\cdot min^{-1} \cdot 10^{-11}$ cells)
1. Lactate dehydrogenase	50.2 ± 2.5
2. Malate dehydrogenase	49.2 ± 2.2
3. Aspartate aminotransferase	6.9 ± 1.6
4. Alanine aminotransferase	0.7 ± 0.12
5. Glycerol-3-phosphate dehydrogenase (cytosolic)	traces

Enzyme activities were measured in samples obtained from the same blood platelet preparation.

For further details see Materials and Methods.

Values are the means $\pm$ SD for results obtained with the five platelet preparations.

As is shown in Table 2, the latter enzyme was inhibited by aminooxyacetate, hydroxylamine and cycloserine. The inhibitory effect of the three compounds was much stronger at lower (20 mM) than at high (250 mM) substrate (aspartate) concentration, and was dose dependent. Cycloserine seemed to be a weak inhibitor since it blocked an enzyme at a concentration higher than 20 mM, whereas the aminooxy compounds (AOA and HA) exerted their inhibitory effect at a concentration below 1 mM.

The ADP-induced aggregation curves are shown in Fig. 1. It can be seen that the aggregation of platelets pretreated with aminotransferase blockers was inhibited in a dose dependent manner. The strongest inhibitory effect was observed after preincubation of platelet rich plasma with hydroxylamine, which inhibited nearly totally the ADP-induced platelet aggregation at the concentration of 1.5 mM (Fig. 1b). AOA seemed to be a less potent inhibitor than hydroxylamine (Fig. 1b), whereas cycloserine (Fig. 1a) was relatively weak since it only worked at concentrations above 10 mM.

Changes in the optical density of platelet-rich plasma following the addition of collagen are seen in Fig. 2. Collagen-induced platelet aggregation was affected

Table 2

Effect of hydroxylamine, aminooxyacetate and cycloserine on aspartate aminotransferase activity

High substrate concentration		Low substrate concentration	
Inhibitor concentration (mM)	Initial rate (nmol min^{-1} mg^{-1})	Inhibitor concentration	Initial rate (nmol min^{-1} mg^{-1})
control	14.0	control	14.0
0.5 HA	10.0	0.05 HA	9.0
1.0 HA	7.0	0.10 HA	6.0
2.0 HA	3.0	0.20 HA	2.0
4.0 HA	1.5	0.25 HA	1.0
8.0 HA	0.0	0.30 HA	0.0
0.3 AOA	8.0	0.05 AOA	9.0
0.6 AOA	4.0	0.10 AOA	6.0
0.9 AOA	3.2	0.20 AOA	3.0
2.0 AOA	2.0	0.30 AOA	2.0
2.5 AOA	0.0	0.50 AOA	0.0
5.0 CS	11.0	5.0 CS	8.5
10.0 CS	8.5	10.0 CS	5.5
15.0 CS	6.0	15.0 CS	4.0
20.0 CS	4.0	20.0 CS	2.0

Each cuvette contained a total volume of 2.5 ml : 200 mM triethanolamine HCl buffer pH 7.4, 0.2 mM NADH, malate dehydrogenase 0.7 U/ml, L-aspartate (20 mM low and 250 mM high substrate concentration) as shown, high speed supernatant fraction from the platelets (2 mg protein) and the inhibitors as shown. The reaction was carried out at 25 °C for 2–3 min, and started with α-ketoglutarate (final concentration 10 mM). The inhibitors were added to the measuring system containing enzyme, 2 minutes before the starting of the reaction. The extinction changes at 340 nm were recorded. Aliquots of the same supernatant fraction were used throughout the experiment. The results (one of five) of one representative experiment are presented.

HA = hydroxylamine, AOA × aminooxyacetate, CS = cycloserine.

by aminooxyacetate (Fig. 2b), hydroxylamine (Fig. 2a) and cycloserine (Fig. 2c). The inhibitory effect of all three compounds was dose dependent, and also in this case the strongest inhibitor was hydroxylamine, which totally inhibited the aggregation of platelets at the concentration of 0.5 mM (Fig. 2a), and the weakest one was cycloserine, which for a 100% inhibitory effect needed a concentration of more than 14 mM (Fig. 2c).

Figure 3a shows that dinitrophenol also inhibited ADP-induced platelet aggregation; as is seen, 3.6 mM DNP completely abolished this process.

Figure 3b shows the potentiation by aminooxyacetate of the inhibitory effect of DNP. In this experiment the platelets, prior to the addition of ADP, were incubated with concentrations of aminooxyacetate and DNP, which by themselves inhibited platelet aggregation only partially.

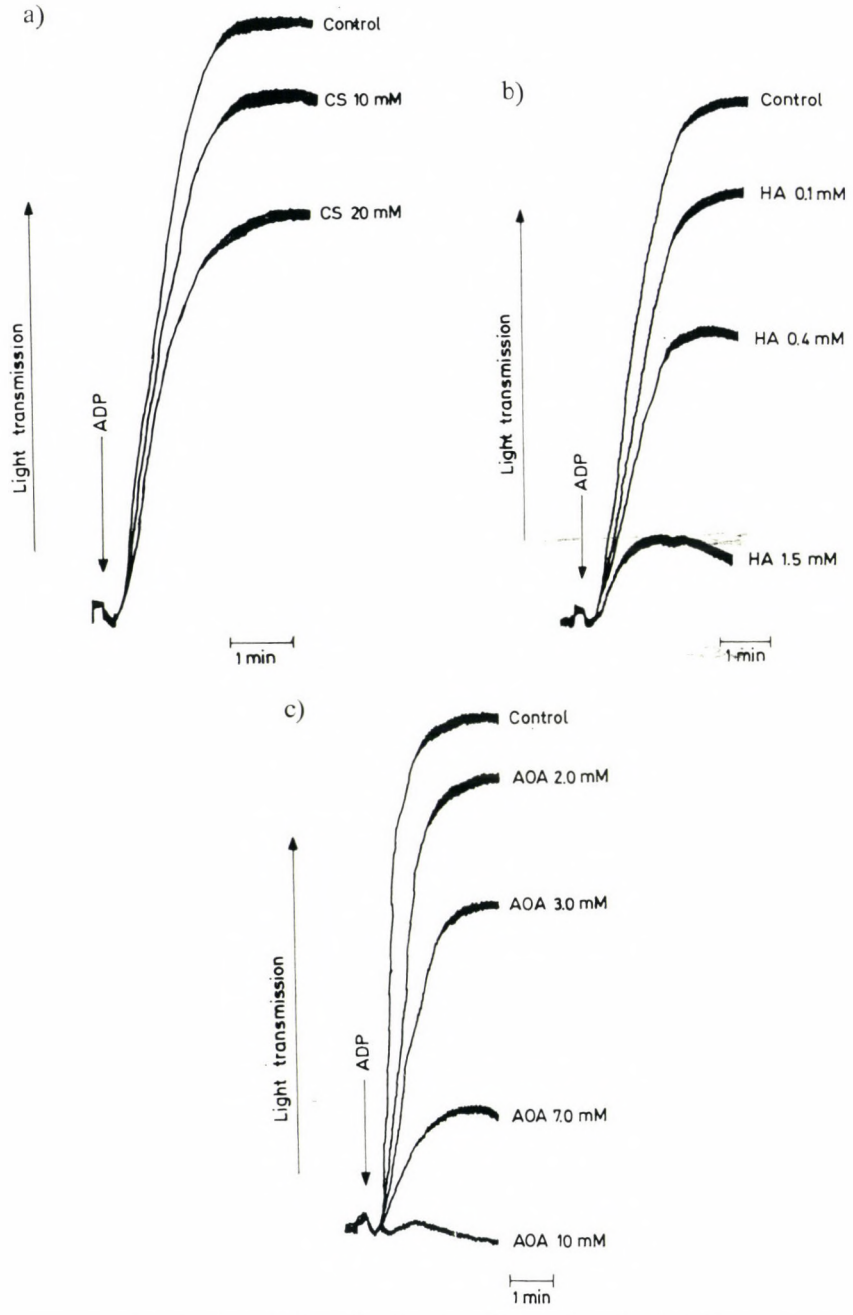


Fig. 1a, b, c. Effect of cycloserine (CS), hydroxylamine (HA) and aminooxyacetate (AOA) on bovine platelet aggregation induced by ADP. For experimental conditions see Materials and Methods

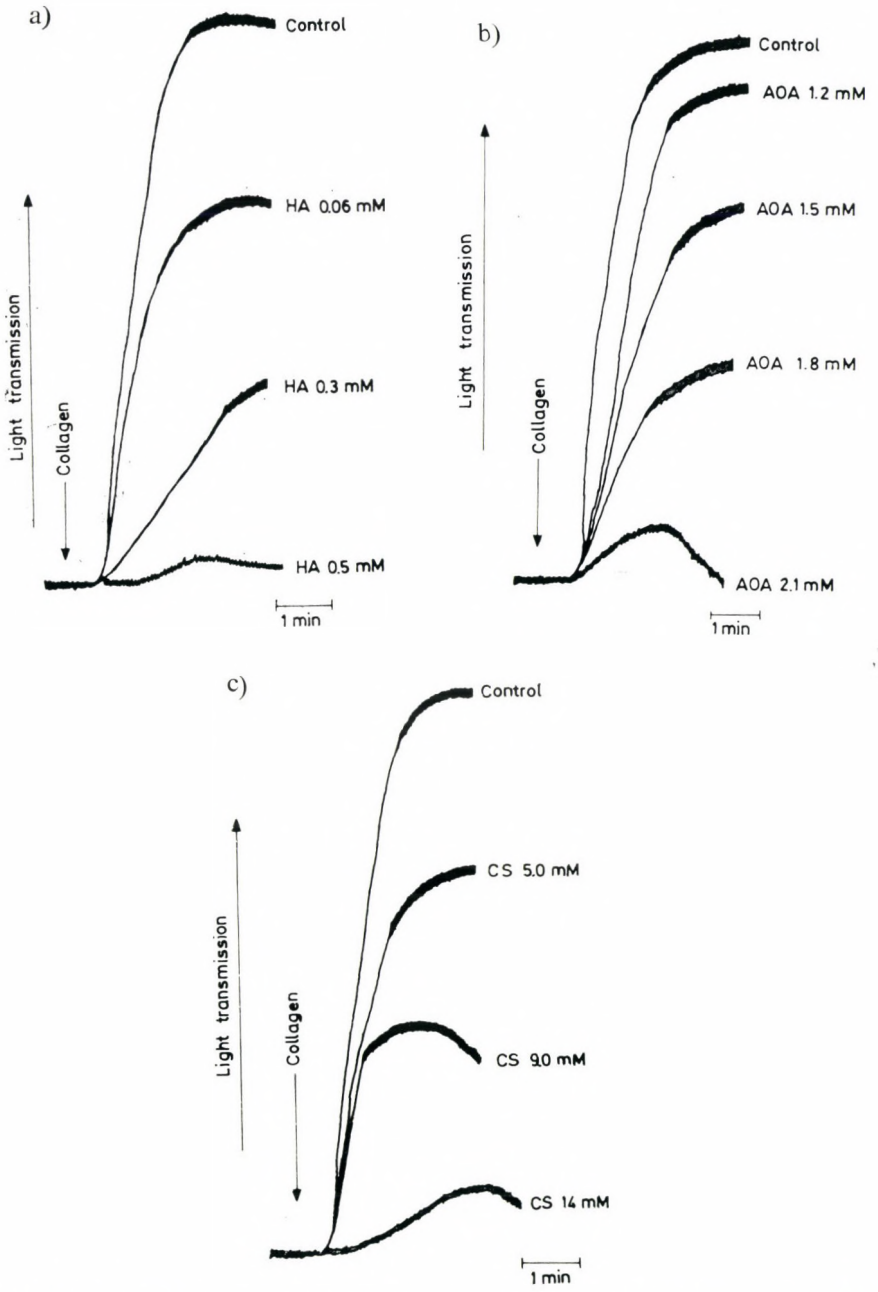


Fig. 2 a, b, c. Effect of hydroxylamine (HA), aminooxyacetate (AOA) and cycloserine (CS) on collagen-induced platelet aggregation. For experimental conditions see Materials and Methods

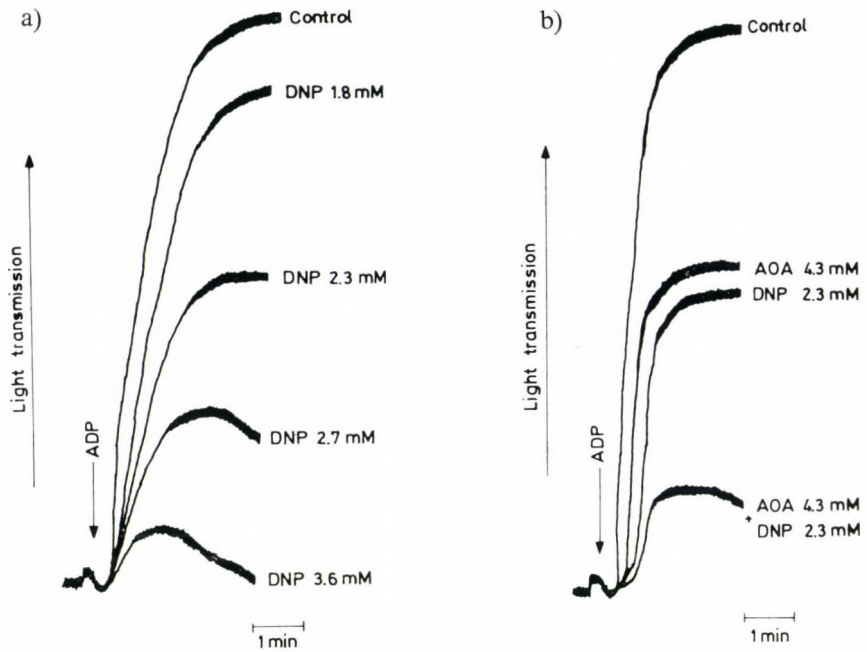


Fig. 3a. Effect of dinitrophenol (DNP) on ADP-induced platelet aggregation. DNP was added 2 min before the addition of ADP

Fig. 3b. Effect of subthreshold concentrations of aminooxyacetate (AOA) and dinitrophenol (DNP) on ADP-induced platelet aggregation. AOA and DNP were added 2 min before ADP at a concentration, which per se inhibited platelet aggregation by about 50%

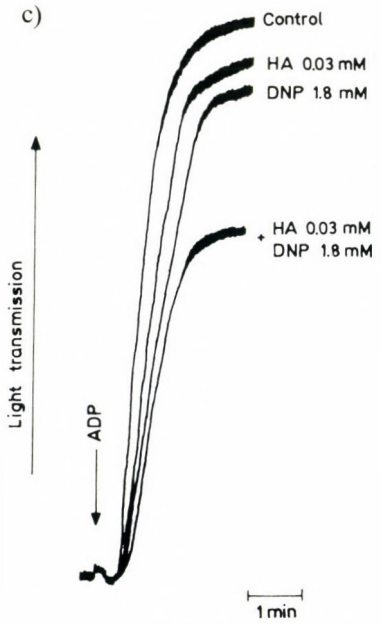


Fig. 3c. Effect of subthreshold concentrations of hydroxylamine (HA) and dinitrophenol (DNP) on ADP-induced platelet aggregation. HA and DNP were added 2 min before ADP at concentrations, which had a slight inhibitory effect

Figure 3c shows the results of similar experiments with hydroxylamine and DNP. In this case, too, a potentiation by hydroxylamine was observed.

As can be seen from Fig. 4, antimycin and aminooxyacetate added to PRP at concentrations which per se abolished the ADP-induced aggregation only slightly, inhibited the process strongly when added together.

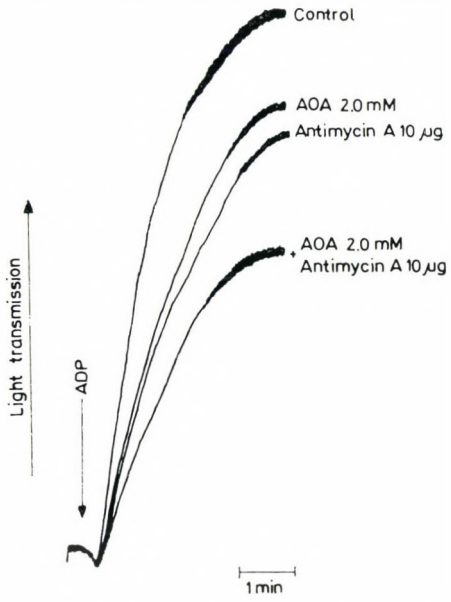


Fig. 4. Effect of aminooxyacetate (AOA) and antimycin A on ADP-induced platelet aggregation. AOA and antimycin A were added 2 min before ADP to the final concentration of 2 mM and 7 µg/ml, respectively

As shown in Fig. 5, similar results, i.e. a synergism in the inhibitory action on ADP-induced platelet aggregation were obtained after the addition of small concentrations of rotenone and aminooxyacetate.

Still, in contrast to DNP, neither rotenone nor antimycine A added alone to PRP at much higher concentrations were able to completely inhibit bovine platelet aggregation.

Discussion

Aminooxy compounds (H_2N-O-R) and cycloserine (L-4-isoxazolidinone) are known inhibitors of several aminotransferases, including aspartate aminotransferase [9, 10, 11]. The high affinity of aminooxyacetate ($H_2N-O-CH_2CO_2^-$) and hydroxylamine to phosphopyridoxal enzymes is well established [11], and more recently their interaction with the pyridoxal phosphate prosthetic group of pig heart aspartate aminotransferase has been reported [12].

All the three inhibitors were used as a probe for aminotransferase catalysed carbon, and reducing equivalent transfer across the renal mitochondrial membrane [13], isolated hepatocytes [14, 15, 16, 17], and tumour cells [18].

Taking all this in account and the fact that all three compounds blocked aspartate aminotransferase in bovine platelet homogenates (Table 2), it is justified

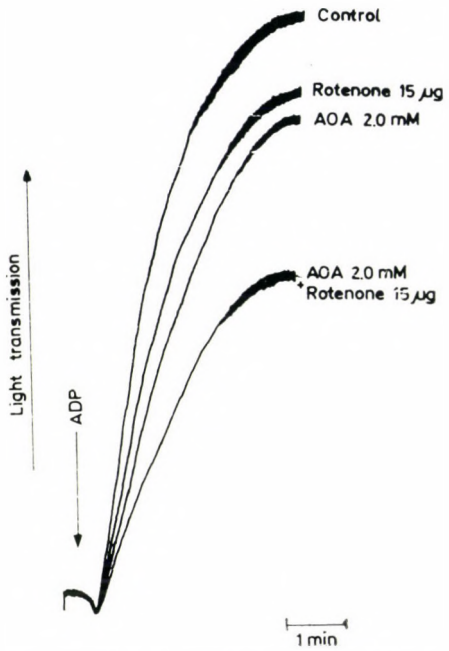


Fig. 5. Effect of aminooxyacetate (AOA) and rotenone on ADP-induced platelet aggregation. AOA and rotenone were added 2 min before ADP to the final concentration of 2 mM and 10 µg/ml, respectively

to assume that in blood platelets aminooxyacetate, hydroxylamine and cycloserine inhibit aspartate aminotransferase and thereby the whole glutamate oxaloacetate cycle. The possible consequences of alanine aminotransferase inhibition seems to be negligible due to very low activity of the enzyme in platelets (Table 1).

An inhibition of the glutamate aspartate shuttle in platelets must be accompanied by a nearly complete inhibition of energy production by mitochondria, because with the shuttle closed, glycolytically derived NADH can be reoxidized only by lactate dehydrogenase, and because such oxidation consumes pyruvate, which cannot serve as a substrate for ATP production in mitochondria.

An alternative scheme for the oxidation of cytosolic NADH, i.e. the α-glycerophosphate cycle [20] is questionable in platelets due to the very low activity of α-glycerophosphate dehydrogenase in the cytosol of human [4], porcine (our observation) and bovine platelets (Table 1).

Resting and activated platelets consume large amounts of metabolically derived ATP [22]. In the resting platelets, metabolic energy is required to maintain them in a responsive state, while in the activated state ATP may be used for several purposes in the basic platelet reaction including the induction-transmission steps, shape change, aggregation and secretion [19, 21].

In the resting platelets the vast majority (about 80%) of total energy production can be attributed to oxidative ATP production [19, 22].

Although the inhibition of oxidative phosphorylation in resting platelets stimulates glycolytic ATP production (Pasteur effect), such a compensation might not be sufficient in stimulated platelets [19], as is the case in rabbit [23] and bovine platelets (Fig. 3–5). Blockage of the glutamate aspartate shuttle by aminotransferase inhibitors is accompanied by the inhibition of platelet aggregation induced by ADP (Fig. 1), collagen (Fig. 2) and thrombin (not shown here). Hydroxylamine and aminooxyacetate are (as shown in Table 2) blocking transamination and are also potent inhibitors of platelet aggregation (Fig. 1).

Arrest of mitochondrial ATP production by antimycin A (Fig. 4), rotenone (Fig. 5) or DNP (Fig. 3a) inhibited or abolished the platelet aggregation induced by ADP. This was observed also with rabbit platelets treated with antimycin A, oligomycin and dinitrophenol (23). The inhibitory effect of oxidative phosphorylation blockers in bovine platelets is potentiated by the simultaneous partial blockage of the aspartate aminotransferase reaction (Fig. 3b, 4, 5).

This synergism suggests that in bovine platelets, too, the aminotransferase reactions are needed for energy production in these cells and that the glutamate aspartate shuttle plays an important role in the metabolic ATP supply for the basic platelet reaction.

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The Effect of Platelet Homogenate Fractions on Megakaryocytopoiesis in the Mouse

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Platelet homogenate suppresses megakaryocytopoiesis more effectively than intact platelets do. In mouse experiments mitochondrial, lysosomal, and microsome fractions separated by ultracentrifugation from platelet homogenate did not influence megakaryocytopoiesis, while the cytosol fraction inhibited it similarly as platelet homogenate. Besides thrombopoietin, the substance released from the platelets also takes part in the regulation of thrombocytopoiesis and this substance passes into the cytosol fraction on separation by ultracentrifugation.

Keywords: CFLP-mice, megakaryocytopoiesis, platelet homogenate, regenerating bone marrow

Introduction

The megakaryocytopoiesis-inhibiting effect of platelets and platelet extracts was proven in several experiments [1, 2, 3, 4, 5, 8, 14, 15, 16, 18]. Earlier we have shown that the suppressive effect of platelet homogenates is stronger than the megakaryocytopoiesis-inhibiting effect of intact platelets [12, 19]. It was thought that a substance released from the platelets themselves might be responsible for the inhibitory effect [13].

The experiments to be reported had the aim to establish to which cell constituent the substance was bound in the platelets. For this purpose the homogenate was fractionated by ultra-centrifugation and the effect of the fractions was examined on megakaryocyte-platelet production.

Material and Methods

The experiments were made on 2–3 months old mice of the CFLP strain. The animals were given 8 Gy whole-body irradiation by means of a THX-250 (Medicor) apparatus (5 min 28 s irradiation time, 230 kV, 15 mA, 1 mm Cu filter, 40 cm focal distance).

For the preparation of platelet suspension, blood was taken from the inferior vena cava of ether-anaesthetized animals. 5% EDTA Na_2 (pH 6.7) was

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used as anticoagulant. The blood was centrifuged at 1000 *g* for 20 min, then the supernatant (the platelet-rich plasma) was again centrifuged at 2500 *g* for 20 min. Then the platelets were washed twice with EDTA, occasional red blood cells were removed by addition ammonium oxalate. Finally, the residue was suspended in 0.9% NaCl solution. From the platelet suspension obtained in this way, a platelet homogenate was prepared using a hand-operated Potter homogenizer.

Fractionation of the platelet homogenate was done by means of a Janetzky VAC 601 ultracentrifuge. By centrifugation at 10 000 *g* for 10 min, we obtained the mitochondria, at 24 000 *g* for 30 min the lysosomes, and at 105 000 *g* for 60 min the microsome fractions. The last supernatant yielded the cytosol fraction containing the cell substances. Dilution of the various fractions of the platelet homogenate to the original volume was done with 0.9% saline.

Schedule of treatment

In previous experiments [12, 19] we found that the platelet homogenate given on days 8, 9, 10 following irradiation inhibited megakaryocytopoiesis in the regenerating bone marrow. In the present experiment we carried out the treatment with platelet homogenate or its fractions on days 8, 9, 10 after irradiation. Each mouse was given the homogenate from $1–2.5 \times 10^8$ platelets on each occasion, or the corresponding fractions i. v. in 0.2 ml doses. The animals were divided into 6 groups. The control animals were treated only by irradiation. The second group was given full platelet homogenate used for fractionation, while the remaining four groups received the mitochondrial, lysosomal, microsomal and cytosol fractions. The peripheral platelet count determined on the 14th day after irradiation was used for assessment of the effect, as suggested by Goodman et al. [7]. The rate of ^{75}Se-selenomethionine incorporation into the platelets measured according to Penington [17]. The platelet count was determined with a phase contrast microscope according to Fischer and Germer [6]. The results comprise the total values of the two series of experiments. Calculation of significance was done by Student's *t* test.

Results

The platelet count of untreated mice was $15.45 \pm 2.25 \times 10^{11}$/l. On the 8th day after irradiation with 8 Gy the platelet count was $7.70 \pm 1.83 \times 10^{11}$/l. If the animals were given platelet homogenate on days 8, 9 and 10, the platelet count on the 14th day was $7.37 \pm 1.37 \times 10^{11}$/l. The platelet count of the group which had received only irradiation was $12.06 \pm 2.35 \times 10^{11}$/l. Treatment with mitochondrial, lysosomal and microsomal fractions did not influence the platelet count of the animals; this agreed with that of the control group. In contrast, the peripheral platelet count of the mice treated with cytosol fraction on the 8th, 9th and

Table 1

The effect on megakaryocytopoiesis of platelet homogenate and its fractions obtained by ultracentrifugation

Treatment	Platelet count on day 14 ($\times 10^{11}$/l)	^{75}Se-selenomethionine incorporation ($\times 10^{-2}$%)
Controls	12.06±2.35 (11)	19.02±0.74 (7)
Mitochondria	12.10±2.76 (11)	19.73±1.02 (11)
Lysosomes	12.36±2.27 (11)	20.46±1.01 (11)
Microsomes	12.06±2.15 (12)	19.10±1.17 (11)
Cytosol	8.22±0.99 (10)*	17.88±0.88 (9)*
Full homogenate	7.37±1.37 (8)*	17.58±0.79 (8)*

±SD *$p < 0.01$ number of animals in brackets

10th days after irradiation was significantly lower ($p < 0.01$) in comparison with the control group on the 14th day, and agreed with the result of treatment with platelet homogenate. The results of ^{75}Se-selenomethionine incorporation and the platelet counts in the different experimental groups were similar (Table 1).

Discussion

For a long time only thrombopoietin was considered important in the regulation of thrombocytopoiesis [9]. Recently it has been observed that several other factors may play a role in it [10, 11, 22]. On the basis of a mathematical model, Wichman et al. [20] suggested that platelet homogenate may play a part in the regulation of megakaryocytopoiesis. In our earlier investigations [12, 19] we found in agreement with others [21, 23] that the platelets exerted their suppressive effect at a late stage of cell maturation and influenced the release of platelets.

According to the present results on ultracentrifugation, the megakaryocytopoiesis-suppressing substance passes into the cytosol fraction. Identification and isolation of this substance is the subject of our current investigation.

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Acute Leukemia with Lymphoid/Myeloid Cell Populations (Hybrid Leukemia). Case Reports

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Based on conventional and immunological cell markers the great majority of acute leukemias can be classified as of lymphoid or myeloid origin [1, 2]. However, in some instances, leukemic cells with both lymphoid and myeloid features are found [3, 4, 5, 6, 7]. For these cases the term of hybrid leukemia has been suggested; moreover, it has been shown that dual markers can be expressed by the same cell (biphenotypic leukemia) or myeloid and lymphoid cell populations coexist (biclonal leukemia) [4].

The accurate typing of acute leukemia is crucial in order to entail treatment and predict survival; therefore, the identification of acute hybrid leukemias may be important not only in terms of biological significance but also of management and outcome.

We report here two further cases of acute hybrid leukemia with poor response to chemotherapy and very short survival.

Keywords: acute leukemia, lymphoid-myeloid features, hybrid leukemia, accurate typing and identification

Case reports

Patient 1. A five month-old female first came to our observation on March 1983 because of pallor, petechiae and light hepatosplenomegaly. Hb was 8.2 g/l, platelet count was 10×10^9/l, WBC was 280×10^9/l with 99% blast cells. The bone marrow smear revealed more than 90% blast cells morphologically classified as L2 of FAB Classification. Blasts were PAS-positive and negative to other cytochemical stainings (Table 1). Cytogenetic analysis showed t (4; 11) chromosome rearrangement.

Induction chemotherapy consisting of vincristine, prednisone and daunorubicin was given but the patient failed to achieve complete remission. Subsequent, more aggressive chemotherapy was ineffective and the patient died four months later.

Patient 2. A twenty-nine year-old male was referred to our hospital with thrombocytopenic purpura, fever, weight loss, sweating, lymphadenopathy and moderate hepatosplenomegaly. Hb was 11.2 g/l, platelet count was 80×10^9/l, WBC was 61×10^9/l with 83% blast cells. The bone marrow smear disclosed more than 90% blast cells (L2 in FAB Classification), positive to PAS and acid phosphatase staining (Table 1). The karyotype was normal.

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Table 1

Morphological and cytochemical findings of bone marrow blast cells

	Pt.1	Pt. 2
FAB Classification	L 2	L 2
PAS	pos	pos
Acid phosphatase	neg	pos
NASDE	neg	neg
ANAAE	neg	neg
Peroxidase	neg	neg

Induction therapy consisting of vincristine, prednisone, daunorubicin and L-asparaginase led to complete remission within four weeks. However, one month later the patient was readmitted because of interstitial pneumonia; on this occasion bone marrow relapse was demonstrated and, in spite of intensive chemotherapy, the patient died four months after diagnosis.

Immunological characterization of bone marrow mononuclear cells was done by evaluating the number of E-rosette-forming cells (E-RFC) (T lymphocytes), immunoglobulin surface-bearing cells (SmIg) (B lymphocytes), and the percentage of blast cells reacting with the following monoclonal antibodies (MoAbs); OK series (Ortho), VIL A1 (anti-Common ALL antigen), VIM 2 and VIM D5 (anti-myeloid antigens), and VIB C5 (anti-B and anti pre-B cells antigen). VIL A1, VIM 2, VIM D5 and VIB C5 MoAbs were kindly provided by Prof. W. Knapp (University of Vienna). Terminal deoxynucleotidyl transferase positive cells (TdT) were assessed with commercially available antibody (Bethesda Research Laboratories). All tests were performed as recommended by the manufacturers.

Discussion

The present study deals with two patients affected with acute leukemia that was first classified as lymphoblastic (ALL) on the grounds of morphological and cytochemical findings. Additional cell marker investigations, however, demonstrated that myeloid and lymphoid components were present (Table 2).

In the first patient TdT was detected in all blast cells. It is well recognized that TdT-positivity in conjunction with PAS-positivity is a property of leukemic lymphoblasts [4]. In this case, however, 20% of cells reacted with VIM D5 MoAb; this percentage is accepted as significant for the myeloid lineage of leukemic cells [8]. All these features thus allowed to classify the case as biphenotypic hybrid leukemia. The t (4; 11) chromosome rearrangement was also found. Acute leukemias with this cytogenetic abnormality have been described in very young children and are characterized by poor response to chemotherapy [7].

Table 2

Immunological characterization of bone marrow blast cells

Markers	% Positive Cells	
	Pt. 1	Pt. 2
E-RFC	1	4
OKT 3	1	4
OKT 4	—	2
OKT 8	—	3
OKT 6	—	1
OKT 9	—	4
OKT 10	—	25
OK la1	84	72
SmIg	1	0
VIL A1	0	0
VIB C5	—	17
VIM 2	—	28
VIM D5	20	18
TdT	100	—

In the second patient both lymphoid (VIB C5+) and myeloid (VIM 2+ and VIM D5+) antigens were detected at MoAb analysis; however, at the relapse all cells expressed myeloid markers only (data not shown). This finding suggests that the initial blast population was biclonal and the treatment yielded selective eradication of the cell clone with lymphoid phenotype. In this case cytogenetic analysis was normal.

Acute leukemias with synchronous presence of myeloid/lymphoid blast cells are rarely reported, and few cases meet the criteria recently suggested for the hybrid type [4]. However, the increasing availability of immunological cell markers, including MoAbs, that are largely employed in assessing the differentiation of myeloid and lymphoid progenitors, might increase the frequency of these particular forms.

As far as management is concerned, conflicting evidences have been reported [2, 3, 5, 6, 7]. The present observations suggest that, although the age of the patients and high leukocyte counts could partially account as adverse factors for the clinical outcome, very intensive chemotherapy should be undertaken for this type of leukemia.

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Cerebrospinal Fluid Beta-2-Microglobulin Level as Marker of Central Nervous System Involvement in Lymphoma

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The cerebrospinal fluid (CSF) and serum beta-2-microglobulin (B-2-m) level was determined in 15 patients affected by lymphoma with and without central nervous system (CNS) involvement and in 15 normal controls. No statistical difference in CNS B-2-m was found between patients affected by lymphoma without CNS involvement and normal subjects. CSF B-2-m was significantly increased ($p < 0.001$) in patients with CNS involvement as compared to normal subjects or patients without CNS involvement. These findings suggest that CSF B-2-m can be used as a useful marker of CNS involvement in patients affected by lymphoma.

Keywords: beta-2-microglobulin (B-2-m), cerebrospinal fluid (CSF), central nervous system (CNS), lymphoma

Introduction

B-2-m is a low molecular weight protein (M. W. 11,700) present on the surface of nucleated cells as a constituent of HLA-A, B, C antigens [1, 2]. Synthesis and release in the vascular compartment of B-2-m is constant in healthy subjects. In the last few years, several authors have reported raised serum levels of B-2-m in lymphoproliferative disorders [3, 4, 5] related to an overproduction by neoplastic cells [6].

More recently, an elevated concentration of B-2-m has been found in the CSF of patients affected by lymphoproliferative or myeloproliferative diseases with CNS involvement [7, 8, 9].

The purpose of this study was to correlate CSF B-2-m levels to CNS-involvement in patients affected by lymphoma.

Materials and Methods

We examined 47 CSF samples collected from 15 patients affected by lymphoma (9 males and 6 females, age 19 to 72, mean 45 years); 13 suffered from lymphoma other than Hodgkin's disease and two from Hodgkin's disease. All subjects

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were suspected of having CNS-involvement because of symptoms such as headache, nausea and vomiting. Three patients had focal neurological signs.

All 15 underwent lumbar puncture and cerebral computerized tomography (CT) scan. Cytologic and chemical examinations were performed in all the CSF samples. Five patients (case Nos. 1, 2, 3, 5, 6) had a CT scan positive for CNS involvement; case No. 1 also had tumour cells in the CSF; in the other four the diagnosis was confirmed by brain biopsy. In case No. 4 the CT scan was negative, but tumour cells were found in the CSF (Table 1).

Table 1

Histological classification, CSF citological and chemical examinations, ACT and brain biopsy in lymphoma patients with CNS-involvement

				CSF					
Case	Sex	Age	Histological classification*	Total protein, dg/l	cells/mm³	Glucose, dg/l	ACT	Brain biopsy	Signs and Symptoms
1	M	72	DLPD	675	500 (blasts)	25	positive	not done	nausea, headache
2	M	62	DLPD	24	—	58	positive	positive	paraplegia
3	M	59	HD(mixed cellular)	71	2	58	positive	positive	athetoid movements, headache
4	F	67	DLPD	32	60 (blasts)	7	negative	not done	epileptic state, headache
5	F	19	DLPD	26	—	65	positive	positive	nausea, vomiting, headache
6	M	28	DU	64	3	96	positive	positive	headache, vomiting

* According to Eastern Cooperative Oncology Group (1980).

In the other nine patients, cytologic and chemical examination of CSF and the CT scan were negative. Follow-up of these patients confirmed the absence of CNS involvement.

Since CSF was not available from healthy subjects, fifteen patients with disk syndrome without organic lesions in CNS were used as controls (7 males and 8 females, age 34 to 57, mean 47 years). CSF samples were centrifuged at 2000 *g* for 10 minutes and stored at 40 °C until assayed for B-2-m.

Serum was obtained by centrifugation at 2000 *g* for 10 min of venous blood kept for 2 h at 37 °C. Serum samples were stored at −40 °C until assayed.

CSF and serum levels of B-2-m were determined by solid phase radio-immunoassay (Phadebas, Pharmacia Diagnostics, Uppsala, Sweden). Statistical methods: results are expressed as mean values ± standard deviation. Student's *t* test was used to analyse the difference between groups. Correlation between CSF B-2-m and CSF total protein, and CSF with serum B-2-m was evaluated with correlation coefficient.

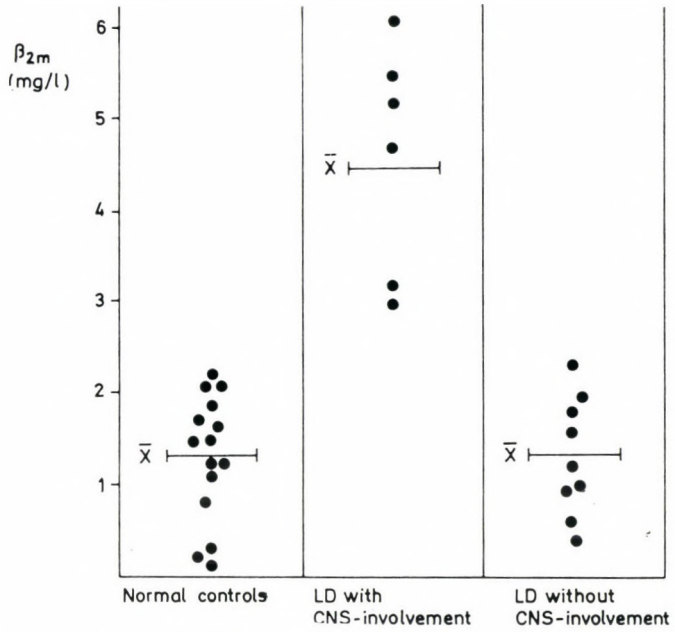

Fig. 1. CSF levels of B-2-m in different groups

Results

No statistical difference in CSF B-2-m was found between normal controls and patients without CNS involvement. CSF B-2-m was significantly increased ($p < 0.001$) in patients with CNS involvement as compared to those without CNS involvement and to normal controls (Fig. 1, Table 2).

Serum B-2-m was found significantly increased in patients without CNS involvement ($p < 0.001$) as well as in patients with CNS involvement ($p < 0.001$) as compared to normal subjects (Table 2).

No statistical difference was found between the serum B-2-m levels in the two groups of patients.

In all nine patients without CNS involvement, the CSF serum B-2-m ratio was lower than 1, whereas in the six patients with CNS involvement the ratio was lower than 1 in two cases and higher than 1 in the other four cases. No correlation

Table 2

Serum and CSF levels of B-2-m in normal controls and lymphoma patients

	No. of patients	CSF B-2-m (mg/l)	Serum B-2-m (mg/l)
Normal controls	15	1.31 ± 0.69	1.12 ± 0.45
Lymphomas with CNS-involvement*	6	4.61 ± 1.25	4.05 ± 1.09
Lymphomas without CNS-involvement**	9	1.33 ± 0.64	2.84 ± 0.78

* 5 NHL, 1 HD
** 8 NHL, 1 HD

was observed between serum and CSF B-2-m in both lymphoma groups (R 0.687, p = ns).

CSF protein concentration exceeded the normal range in only three patients with CNS involvement. No correlation was found between CSF B-2-m and total CSF protein (R 0.586, p = ns).

Discussion

CNS involvement is a frequent (10–26%) and very serious complication of lymphomas other than Hodgkin's disease [10, 11], while it is not often seen in Hodgkin's disease [12].

An early diagnosis is necessary to institute a timely therapy. Nevertheless, abnormal cells or elevated protein concentration are not always found in CSF of patients with CNS involvement. Therefore the availability of other tests is an important diagnostic aid.

Recently Mavlight et al. [7] and Koch et al. [9] reported an increase in CSF B-2-m concentration in patients affected by lymphoproliferative disorders with CNS involvement.

In the present study raised CSF B-2-m level has been found only in patients affected by lymphoma with CNS involvement.

In all patient groups no correlation was found between serum and CSF B-2-m levels. These data confirm that B-2-m does not cross the blood brain barrier even in the case of severe CNS involvement [9]. So it is likely that a raised CSF B-2-m level reflects a rapid turnover of neoplastic cells in the CNS.

It has been suggested that CSF/Serum B-2-m ratio higher than 1 is a sensitive index of CNS involvement [7]. In our study, too, this ratio seemed to be an index of CNS localization of the disease. In fact, the ratio was always <1 in patients without CNS involvement and >1 in 4 out of 6 patients with CNS involvement. Even if we consider the mean values of the two groups, the ratio is <1 in patients

without CNS lymphoma and >1 in subjects with CNS disease; furthermore the ratios are significantly different between the two groups of patients.

Nevertheless, our data suggest that a raised absolute value of CSF B-2-m is a reliable index of CNS disease. We found normal values of CSF B-2-m in the patients without CNS lymphoma and raised levels in all patients with CNS involvements; but we found the ratio of <1 in two subjects of this group, and often observed a CSF/serum B-2-m ratio >1 also in the normal group. Moreover, the lack of correlation between CSF and serum B-2-m levels suggests that they are produced in different compartments without any communication. This finding confirms the greater diagnostic value of the absolute CSF B-2-m value as compared to the CSF/serum B-2-m ratio.

According to our study, CSF B-2-m is a useful marker in patients affected by lymphoma with clinical symptoms suspect of CNS involvement, especially when other diagnostic findings yielded by cytologic and chemical examinations are normal.

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Induction of Granulocyte Differentiation in a Human Leukemia Cell Line Does Not Require Cell Division

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The human promyelocytic cell line, known as HL-60, provides a model for the study of myeloid differentiation. The HL-60 can differentiate to granulocyte-like cells in the presence of dimethylsulfoxide (DMSO). We investigated the requirement for DNA synthesis in the myeloid differentiation process and found that induction of differentiation of HL-60 cells did not require ^{3}H-thymidine incorporation as judged by autoradiography. The results indicate that the triggering of granulocyte differentiation of HL-60 can occur in the absence of DNA synthesis.

Keywords: cell division, myeloid differentiation

Introduction

Proliferation and differentiation of hematopoietic cells are incompletely understood. Study of murine erythroleukemia cells has provided insights into the cellular and molecular events involved in erythropoiesis [1, 2, 3]. Research involving human granulocyte-monocyte differentiation has been impeded because of the difficulties in obtaining enriched populations of myeloid precursor cells for study. Several human myeloid leukemia cell lines have been established. One of the lines, HL-60, provides pure populations of promyelocytes which can be used for biological study [4]. A major feature of the cells is their ability to differentiate to mature granulocytes following exposure to dimethylsulfoxide (DMSO) and several other compounds. Following DMSO induction the cells morphologically resemble metamyelocytes and granulocytes, phagocytize Candida albicans, undergo chemotaxis, produce superoxide and express Fc-IgG receptors [5].

The relationship of DNA synthesis and the differentiation program has been studied in a number of eukaryotic cells. Leder et al. [2] and Levenson et al. [6] suggested that Friend erythroleukemia cells can be triggered to differentiate in the absence of DNA synthesis. Other studies of erythroleukemia differentiation suggest that DNA synthesis is required for the transition to expression of differentiated characteristics [1, 7, 8, 9]. Controversy over the role of DNA synthesis in the regu-

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lation of differentiation has also been debated in other cell types such as fibroblasts, lymphocytes and muscle cells [10, 11, 12]. Phorbol esters can induce differentiation of HL-60 promyelocytes to macrophage-like cells; and Rovera et al. [13] and we [14] have shown that the phorbol ester-induced macrophage differentiation does not require DNA synthesis. The present study investigated the requirement for DNA replication in triggering the DMSO-induced granulocyte differentiation of HL-60 promyelocytic cells. Autoradiography allowed simultaneous examination of individual cells for both expression of differentiation characteristics and incorporation of ^{3}H-thymidine. The study was facilitated by the addition of amiloride to the cultures containing DMSO. Amiloride, an inhibitor of passive Na^+ flux, markedly potentiates DMSO-induced differentiation of HL-60 cells [15].

Materials and Methods

Cell culture conditions. The human myeloid cell line, HL-60, was established from a patient with acute promyelocytic leukemia. Characteristics of the cell line have been previously reported in detail [4]. Cells were grown in α-media (Flow-Labs, Oxnard, California) containing 10% fetal calf serum and penicillin/streptomycin, in a 5% CO_2 atmosphere, humidified 37 °C incubator.

Differentiation induction and autoradiography. Dimethylsulfoxide (1.2%) arabinosyl cytosine (araC (2×10^{-7} M)) and amiloride (20 μg/ml), generously supplied by Dr. Edward Cragoe, Jr. (Merck, Sharp & Dohme Research Labs, West Point, Philadelphia), were added to HL-60 cells (4×10^5/ml) on day 0. The araC was added to the cultures to partially inhibit DNA synthesis. The ^{3}H-thymidine (^{3}H-Td) was added simultaneously with the DMSO, amiloride and araC. Differentiation was assessed on days 1, 2, 3 and 4 following addition of the inducer. Amiloride alone had no effect on differentiation of HL-60. The araC at 2×10^{-7}M did not trigger differentiation of HL-60 cells; higher concentrations of the drug ($> 10^{-6}$M) have been reported to induce limited differentiation [16, 17].

The induction of differentiation was determined by morphology and the ability of the cells to reduce nitroblue tetrazolium (NBT) and to phagocytize Candida albicans. HL-60 cells must be at approximately the myelocyte and early metamyelocyte state of maturation to reduce NBT and phagocytize Candida albicans, respectively. For phagocytic assays, a solution of Candida albicans (2×10^6 yeast/ml) in Hank's balanced salt solution (Gibco, Grand Island, New York) was opsonized in 20% AB serum. One ml was then added to 1×10^6 cells. After 30 min at 37 °C, the slides were processed for ^{3}H-Td incorporation by autoradiography and subsequently stained with Giemsa. The number of cells completely engulfing 1 or more yeast cells was determined microscopically. The NBT stains were prepared as outlined elsewhere [18]. The cells were stained with Giemsa and counterstained with eosin after processing the autoradiographs. The reduction of NBT is dependent on the synthesis of superoxide, and a good correlation exists between myeloid maturation and the ability of the cell to reduce NBT [19].

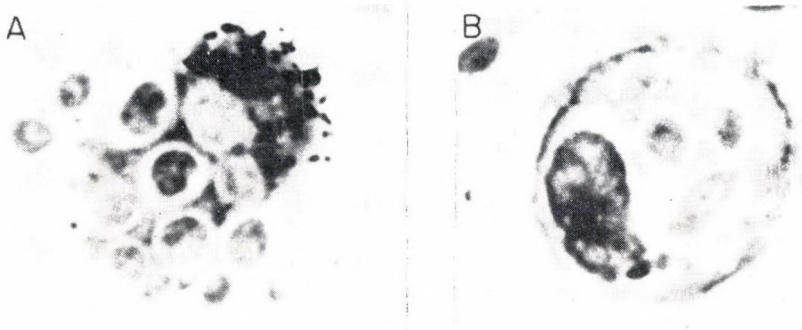

Fig. 1. HL-60 cells have phagocytized Candida albicans after exposure to 1.25% DMSO and 20 μg/ml amiloride for 3 days. "A" indicates a cell which has engulfed Candida albicans and has undergone DNA synthesis; "B" is a cell which is phagocytic, but has not incorporated ^{3}H-Td

The ^{3}H-Td (6.7 Ci/mM) (New England Nuclear, Boston, Massachusetts) was added to the cells at a final concentration of 1.0 μCi/ml on day 0 of culture simultaneously with the addition of DMSO, amiloride, and araC. Cells were harvested on day 1, 2, 3 and 4, washed with phosphate buffered saline and prepared for morphology, autoradiography was then performed and the cells were stained with Giemsa or Giemsa and eosin as described previously [20]. Labeled cells were defined as having 7 or more grains over their nuclei. Thymidine labeling and morphology, yeast ingestion or NBT reduction were evaluable simultaneously on the slides (Fig. 1). The NBT reduction and ^{3}H-Td incorporation on day 4 of culture was difficult to evaluate simultaneously because of the intensity of blue staining over the nucleus, therefore, day 4 NBT data were deleted.

Results

The combined phagocytosis/autoradiography data using HL-60 cells are summarized on Table 1. By day 3 of DMSO/amiloride/araC exposure, 29% of the HL-60 cells developed phagocytic capabilities, and 34% of the phagocytic cells did not incorporate ^{3}H-Td. At day 4, 39% of the cells developed phagocytic ability with 28% of the phagocytic cells not demonstrating ^{3}H-Td incorporation.

The combined NBT reduction/autoradiography data using HL-60 cells are shown in Table 2. By day 3 of DMSO/amiloride/araC exposure, 60% of the HL-60 cells developed the ability to reduce NBT; 20% of the cells that reduced NBT developed the NBT reducing capabilities with no ^{3}H-Td incorporation.

Cell division was not necessary for the triggering of morphological maturation to the myelocyte or metamyelocyte stage of development. By day 4 of culture 82% of HL-60 cells were morphologically at the myelocyte or older stage of maturity, and 17% of the mature cells incorporated no ^{3}H-Td.

Table 1

Combined phagocytosis and autoradiography data of HL-60

Days of HL-60 induction	Phagocytic cells		Nonphagocytic cells	
	No ^{3}H-Tdr incorporation %	^{3}H-Tdr incorporation %	No ^{3}H-Tdr incorporation %	^{3}H-Tdr incorporation %
Day 1	0	0	52	48
Day 2	1	3	37	59
Day 3	10	19	13	58
Day 4	11	28	8	53

Results are the mean of two separate experiments. The cells were cultured with 1.2% DMSO, 20 μg/ml amiloride and 2×10^{-7} M araC and the ^{3}H-Tdr was added simultaneously with the inducer. No phagocytosis was observed by untreated cells. Results represent the mean of 2 experiments and ≥ 250 cells were examined on each slide. In both experiments at least 2 slides were counted for each of the NBT, phagocytosis and morphology studies.

Table 2

Combined NBT reduction and autoradiography data of HL-60

Days of HL-60 induction	NBT positive cells		NBT negative cells	
	No ^{3}H-Tdr incorporation %	^{3}H-Tdr incorporation %	No ^{3}H-Tdr incorporation %	^{3}H-Tdr incorporation %
Day 1	0	1	52	57
Day 2	12	27	18	42
Day 3	12	48	4	36

Results are the mean of two separate experiments. The cells were cultured with 1.2% DMSO, 20 μg/ml amiloride, and 2×10^{-7} M araC, and the ^{3}H-Tdr was added simultaneously with the inducer. Less than 1% NBT reduction was observed in untreated cells. Results represent the mean of two experiments and ≥ 250 cells were examined on each slide. In both experiments at least 2 slides were counted for each of the NBT, phagocytosis and morphology studies.

Discussion

Controversy exists as to the relationship between proliferation and induction of differentiation of hematopoietic cells [1, 3, 21, 22]. Investigators using different inducers of differentiation and various cell types have provided conflicting evidence for the dependence of differentiation on DNA synthesis.

In this study, we examined simultaneously individual HL-60 cells for both granulocyte characteristics and ^{3}H-Tdr labelling of nuclei and found that DNA synthesis of HL-60 cells was not a prerequisite for the triggering of myeloid dif-

ferentiation. Ferrero et al. also showed that the HL-60 cells could terminally differentiate in the absence of cell proliferation [23]. The investigators used thymidine arrest of cell division and pulse exposure to the inducing agent during the block in DNA synthesis. We confirm their finding by using autoradiography of individual HL-60 cells exposed to inducer (DMSO), ^{3}H-Tdr and a concentration of araC (2×10^{-7}M) that decreased the number of cells going through DNA synthesis. Rather than attempt to inhibit DNA synthesis of the entire cell population our technique allowed us to determine which individual cells entered the S phase of DNA synthesis and differentiated. Our study clearly demonstrates that DNA synthesis was not required for differentiation of HL-60 because individual HL-60 cells could be triggered to differentiate without incorporating ^{3}H-Tdr. It remains to be studied if myeloid differentiation can be uncoupled from proliferation in less mature hematopoietic cells.

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Examination of Two Anti-HLA-DR-like Monoclonal Antibodies

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The cellular distribution of two anti-HLA-DR-like monoclonal antibodies was examined. The reagents showed typical affinity to E^- cells, adherent cells and activated T cells. Functionally, they affected the presentation of PPD antigen from APC to T cells in autologous and allogeneic mixed lymphocyte reaction. One of them significantly influenced the proliferation of T helper cells activated polyclonally with PHA.

Keywords: cell culture, monoclonal antibody, T cell activation, lymphocyte subpopulation

Introduction

Optimum response to most antigens usually needs interaction and collaboration of different cell types normally present in peripheral blood. Among them the most important are T, B cells and macrophages. T and B lymphocytes express humoral and cell-mediated immunity, respectively. They have specific recognition units on their surface playing a critical role in their cooperation during the response to antigens. The most important among them are genetically coded determinants of MHC. In humans, the counterpart of the mouse Ia region – HLA-DR, is a unit directing the answer to a variety of antigens.

We examined the reactions of two monomorphic determinants belonging to the HLA-DR family, using monoclonal antibodies. We studied their functional effect in antigen presentation and T cell activation.

Abbreviations: MLR – mixed lymphocyte reaction, APC – antigen-presenting cells, FCS – fetal calf serum, PBS – phosphate buffered saline, PHA – phytohaemagglutinin, PPD – purified protein derivate, PBM – peripheral blood mononuclear cells, FITC – fluorescein isothyocyanate, SRBC – sheep red blood cells, AET – S-2-aminoethylisothiourea bromide hydrobromide

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Materials and Methods

Monoclonal antibodies

Two monoclonal antibodies, called jD2 and mB1 were developed in our laboratory [5]. Briefly, Balb/c spleen cells, previously immunized with normal human peripheral blood mononuclear cells, were hybridized with Sp2/O mouse myeloma cells.

Screening

Culture supernatants were tested on a panel of highly purified target cells by indirect immunofluorescence. 5×10^5 cells were incubated at 4 °C for 40 min with monoclonal antibodies in the presence of 0.01 % NaN_3. As a second antibody, FITC-conjugated goat-anti-mouse IgG was used.

Isolation of the different cell populations

Isolation of E^+ and E^- cells

E^+ cells were obtained after rosetting of PBM cells with AET-treated SRBC [3]. The rosetting procedure was done at 4 °C for 1 h and after that the cells were centrifuged over Ficoll/Uromiro density gradient. The rosette positive cells were found as bottom fraction and SRBC were lysed with Gay's haemolysing solution. Rosette negative cells were collected from the interface. Both fractions were washed in RPMI containing 10 % FCS and used for further examination.

Isolation of adherent cells

PBM cells were allowed to adhere to plastic Petri dishes at 37 °C for 90 min, were then rinsed five times with medium and the remaining cells collected after 2 min incubation with 0.1 % EDTA in PBS. The cells were washed and resuspended in medium.

Cultured cell lines

K_{562} was a myeloid cell line with erythroid markers; Jurkat, a T cell line; and Raji, a B cell line.

Activated cells

PBM cells were maintained in RPMI 1640 (Serva) supplemented with 10 % FCS (Humán, Budapest) and 2mM L-glutamine. T cell blasts were induced by 5 μg/ml PHA-P (Difco), 3 μg/ml Con A (Difco), while B blasts were induced by 50 μg/ml LPS B E. coli (Difco). The blasts were used for assay on the fourth day after induction.

Functional in vitro assays

Cell separation

Normal heparinized human blood was used in all experiments. PBM cells were isolated by centrifugation over Ficoll/Uromiro density gradient (Ficoll, Pharmacia Fine Chemicals; Uromiro, Bracco, Milano). The cells were collected from the interface, washed in medium three times and resuspended in the same medium for culturing.

Preparation of T-cell-enriched population and nylon adherent cells

PBM cells at a concentration of 6×10^6 cells/ml in medium containing 10% FCS were incubated on nylon wool columns at 37 °C for 1 h. The resulting T cell population was collected by rinsing with medium while adherent cells were eluted with RPMI containing 50% FCS and collected by mechanical agitation [1]. To remove the last possible adherent T cells from the adherent population, rosetting of these cells with AET-treated SRBC was performed. Rosette-positive cells were collected and called E^+ cells. Rosette-negative cells containing B cells and adherent cells were called APC. The purity of both fractions was examined. The E^+ population was 80% positive with UCHT 1 anti-T cell monoclonal antibody [3] and the APC fraction was 50% positive when tested with goat-anti-human IgG (Humán, Budapest).

Technique for brief antigen pulsing

The APC fraction was incubated at 37 °C for 1 h with 5% CO_2 in medium in the presence of 50 μg/ml PPD (Weybridge Suney, England) at a concentration of 5×10^6 cells/ml. 40 μg/ml of mitomycin C (Sigma) were added and the incubation lasted another 1 h at 37 °C, 5% CO_2. The treated cells were washed three times in PBS and once in medium and resuspended for culturing [2].

T-cell proliferative response to antigen-pulsed APC

Autologous mixed lymphocyte reaction

10^4 antigen (PPD)-pulsed antigen presenting cells were incubated in 200 μl of medium containing 10% FCS with 10^5 E^+ cells for 5 days in U-bottom tissue culture plates (Greiner). 16 to 18 h before harvesting 0.5 μCi of (^{3}H) thymidine (UVVVR, Prague) were added to each well. Triplicate samples were harvested and counted in liquid scintillation counter (Packard). The results were expressed as total counts per minute (cpm).

Allogeneic mixed lymphocyte reaction

In the case of allogeneic MLR we used the same system with the same E^+: APC ratio. The only difference was that the E^+ cells were isolated from one donor, while the APC cells were obtained from another donor possessing different DR type.

At the beginning of incubation 5 μg/ml purified Ig from jD2, mB1 and control antibody were added to the various cultures. 10^4 APC-PPD pulsed cells co-cultured with 10^5 E^+ cells were used as positive control. 10^4 APC-PPD nonpulsed cells co-cultured with 10^5 E^+ cells were used as negative control in a case of autologous MLR. For allogeneic MLR, the negative control was the cpm value from 10^5 E^+ cells.

Isolation of E^+ cells and cultures with PHA

PBM cells were rosetted with AET-treated SRBC at 4 °C for 1 h. Rosette-positive cells were separated by Ficoll/Uromiro gradient centrifugation at 1000 *g* for 30 min [3]. Rosette-negative cells were again rosetted overnight. All rosette-positive cells were then collected and resuspended at 10^6 cells/ml or 2×10^5 cells/well respectively in U-bottom tissue culture plates. The cells were cultivated for 4 days in the presence of 5 μg/ml purified Ig from jD2, mB1 and control monoclonal antibody which were added to the cultures initially. 16 to 18 h before harvesting, 0.5 μCi (^{3}H) thymidine was added to each well. The triplicate samples were harvested and counted as described above.

Purity of the fractions

The purity of the E^+ fraction was tested with UCHT 1 anti-T-cell monoclonal antibody by indirect immunofluorescence. The average positivity was 80%. For the specificity of UCHT 1 see reference [3].

The control monoclonal antibody used in all functional assays reacted with E^- cells, adherent cells, granulocytes and 59% of the bone marrow cells, but had no anti-HLA-DR specificity [5]. Student's *t* test was used to evaluate the significances of the difference between monoclonal antibody-treated and untreated variants. Results with a p value of 0.01 and over are presented.

Results

The first approach to establish the reaction patterns of jD2 and mB1 monoclonal antibodies on different haemopoietic cells was done by indirect immunofluorescence. The expression of the molecules was studied on highly purified cell fractions isolated from the blood of various healthy donors. To avoid repetition in the DR specificities and to proof the public character of the determinants depicted by our monoclonal anti-HLA-DR-like antibodies, they were tested on cells from at least 20 donors; the average numbers from all the tests are given in Table 1.

Staining of PBM cells was 17% for jD2 and 25% for mB1; none of them showed any binding to E^+ cells. About 50% of the E^- cells reacted with jD2 and mB1 monoclonal antibodies. A positive reaction could be observed with 97–98% of the adherent cells and no reaction with granulocytes.

Screening with lymphoid cell lines failed to show binding to the cell line of T origin as well as to the cell line possessing erythroid markers such as Jurkat and K_{562}. On the other hand, they stained brightly the B cell line Raji.

The anti-HLA-DR-like character of the monoclonals was confirmed by their positive reaction with activated T cells. They bound to the T blasts activated with

Table 1

Reaction patterns of jD2 and mB1 monoclonal antibodies on different cell types

Cell type	jD2 (%)	mB1 (%)
PBM cells	17	25
E^+ cells	—	—
E^- cells	50	56
Adherent cells	97	97
Granulocytes	—	—
Jurkat	—	—
K_{562}	—	—
Raji	100	100
Con A blast	23	31
PHA blasts	27	22
LPS blasts	75	87
MLC blasts	25	24

Purity of the fractions was tested with anti-T cell monoclonal antibody UCHT 1 [3], the E^+ fraction; and with goat-anti-human IgG, the E^- fraction. The positivity was 80% and 50%, respectively. As a negative control human target cells with the second antibody — FITC conjugated goat-anti-mouse IgG were used. Such samples never showed more than 3% positivity.

PHA, Con A and to B blasts stimulated with LPS. The monoclonals also showed a high percentage of MLR activated cells.

We tested the effect of the jD2 and mB1 monoclonal anti-HLA-DR-like antibodies on the proliferative response of T cells to APC pulsed with PPD. First, the behaviour of the monoclonals in autologous MLR was tested, in order to establish their effect on self-DR restriction. T-cells from 5 different individuals (three gave typical reactions) were cultured with PPD pulsed autologous APC for 5 days in the presence of the monoclonals. As shown in Table 2, monoclonal antibody-dependent inhibition of the T cell proliferative response was observed with the first and second antibody, the two anti-HLA-DR-like antibodies. In contrast, the third monoclonal antibody, a molecule different from HLA-DR, did not show any significant inhibition. Both jD2 and mB1 showed concentration-dependent inhibition with the maximum effect between 1 μg/ml and 5 μg/ml.

It has to be stressed that we selected donors previously immunized with PPD, because human T cells show only secondary proliferative response to PPD.

Table 2

Blocking effect of jD2 and mB1 monoclonal antibodies on the proliferation of T cells when PPD antigen is presented by autologous APC

Cell cultures and monoclonal antibody added	Patient 1 DR type-2,8		Patient 2 DR type-6		Patient 3 DR type-5,7	
	(^{3}H)TdR uptake		(^{3}H)TdR uptake		(^{3}H)TdR uptake	
	cpm	% inhibition	cpm	% inhibition	cpm	% inhibition
Negative control (medium)	578±26		440±94		1 493±147	
Positive control (PPD control)	5737±841		5564±998		17 565±306	
+5 μl/ml jD2 mAb	2886±22	55	2757±258	59	11 152±250	40
+5 μg/ml mB1 mAb	3198±462	49	3234±47	45	10 914±1057	41
+5 μg/ml control mAb	5403±719	6	5066±11	9	17 336±1238	1.4

In order to detect the influence of our monoclonal anti-HLA-DR-like antibodies on the response of allogeneic T cells, combinations of donors with different DR types were selected. Using an experimental system including PPD pulsed APC and allogeneic T cells, we tested the effect of jD2 and mB1 in five different combinations. It is shown in Table 3 that the anti-HLA-DR-like antibodies inhibited to some extent the proliferative response of the allogeneic T cells to PPD.

To examine the connection of the determinants detected by our anti-HLA-DR-like antibodies to the help generally given by T cells, we purified E^+ cells and stimulated them with PHA. At the beginning of culturing, 5 μg/ml of the anti-HLA-DR-like antibodies were added. As shown in Table 4, monoclonal antibody mB1, an anti-HLA-DR-like reagent, had no effect on the proliferation of helper cells and showed a negligible or no blocking. The same results were obtained with the control antibody. On the contrary, jD2 antibody extended a 52% to 74% inhibitory effect on the different cell donors.

Discussion

Monoclonal antibodies proved to be valuable reagents to analyse functional relationships in many immunological systems. A large battery of anti-Ia and anti-HLA-DR monoclonal antibodies were used to document the immunological functions of class II products in various in vitro systems involving helper T cells [2, 4].

One consistent feature emerged from these studies, the inhibitory effect of anti-HLA-DR monoclonal antibodies on T cell recognition of the HLA-DR

Table 3

Blocking effect of the jD2 and mB1 monoclonal antibodies on the proliferation of T cells when PPD antigen was presented by allogeneic APC

Cell cultures and monoclonal antibody added	Combination 1		Combination 2	
	E^+ — DR 5,7	APC—DR 6	E^+—DR 2,8	APC—DR 2,7
	(^{3}H) TdR uptake		(^{3}H) TdR uptake	
	cpm	% inhibition	cpm	% inhibition
Negative control (medium)	2 119±1301		440±94	
Positive control (PPD control)	42 359±2457		7 323±1345	
+5 μg/ml jD2 mAb	27 797±709	36	4 956±593	34
+5 μg/ml mB1 mAb	25 706±632	41	5 742±799	23
+5 μg/ml control mAb	41 449±1344	2	10 604±292	—

Table 4

Blocking effect of jD2 monoclonal antibody in PHA-dependent T cell proliferation

Cell cultures and monoclonal antibody added	Patient 1		Patient 2		Patient 3	
	(^{3}H)TdR uptake		(^{3}H)TdR uptake		(^{3}H)TdR uptake	
	cpm	% inhibition	cpm	% inhibition	cpm	% inhibition
Negative control (medium)	1 918±594		1 918±598		1 964±355	
Positive control (PHA control)	17 381±4473		19 503±3606		19 743±2195	
+5 μg/ml jD2 mAb	9 375±3315	52	6 533±1839	74	8 617±621	63
+5 μg/ml mB1 mAb	18 636±5869	8	18 636±5869	5	16 107±655	20
+5 μg/ml control mAb	15 125±3269	14	16 973±956	14	20 146±4241	2

products. Such studies have led to the expectation that immunologically specific suppression of T cell recognition results from competing interactions of the monoclonal antibody with target structures.

Our monoclonal antibodies jD2 and mB1 showed a typical anti-HLA-DR cellular distribution and significantly blocked the presentation of PPD antigen to autologous T cells. Their inhibitory effect was also significant in antigen presentation to allogeneic T cells.

The interesting feature of our jD2 monoclonal antibody was its capability to block not only the helper activity of the T cells to autologous and allogeneic APC, but also the development of activated T helper cells when stimulated with polyclonal mitogen.

The above results suggested that the HLA-DR determinants on the surface of activated T cells, during the response to antigen or mitogen, govern interactions with APC as well as the general development of stimulated helper cells.

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Quantitative Problems in Bone Marrow Transplantation by Peripheral Blood Stem Cells

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Investigation into radiation bone marrow aplasia in mice, guinea pigs, dogs and clinical trials in man presented clear evidence of successful engraftment of autologous or allogeneic peripheral blood stem cells. The quantitative donation problems are discussed arising with the use of continuous cytopheresis to obtain a sufficient quantity of peripheral blood mononuclears (stem cells) for repopulation of aplastic bone marrow.

Although bone marrow repopulation is possible by using peripheral blood mononuclears (stem cells) in individual cases, the method can only be used in practice after discovering an apropriate stimulator able to augment several times the number of bone marrow stem cells in the peripheral blood, or a new method for stem cell multiplication in vitro.

Keywords: Bone marrow, blood stem cells

Introduction

Early experimental data in this century demonstrated that the fetal blood formation arises from one pluripotentional mesenchymal stem cell. The eminent German pathologist Rudolf Virchow was the first to teach that in the adult body blood cells also derive from one precursor. Later, in 1909, the well-known Russian embryologist and histologist, A. Maximov postulated that a pluripotent mesenchymal cell morphologically defined as a small lymphocyte is the stem cell of which all subsequent cells involved in myelopoiesis and lymphopoiesis originate. During the last twenty years many investigations confirmed these ideas. Now it is clear that bone marrow stem cells, not only differentiate into blood cells but also circulate in the blood and are "homing" in different parts of the haemopoietic stroma and through the mediation of the thymus and bursa of Fabricius (of its analogues in mammals) differentiate into B and T lymphocyte populations.

Based mostly on in vivo and in vitro cell cloning methods [4, 13, 22,] it is now evident that the kinetics scheme of haemopoietic stem cells is as follows [2 16, 17]:

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Haemopoietic stem cells are generated and settled in the bone marrow, and differentiate into haemopoietic and lymphoid cell lines. A small proportion of the stem cells enters the circulation. It is clear that there must be a system controlling stem cell division, and perhaps this system regulates the distribution of stem cells between bone marrow and the circulating pools. The presence of haemopoietic stem cells in peripheral blood was proved first indirectly by transfusing blood or leucocyte concentrates into heavily irradiated recipients or parabionts [8, 18, 19, 24, 26]. The results of these experiments encouraged some investigators to use haemopoietic stem cells from blood instead of bone marrow to restore the haemopoiesis of patients with bone marrow aplasia.

In the experimental and clinical studies on bone marrow transplantation by peripheral blood stem cells, two problems are important, 1) the immunological relations between the graft and the recipient, and 2) the quantity of the haemopoietic stem cells transfused for successful take of the graft. The second problem is the subject of the present paper.

Bone Marrow Transplantation by PBSC*

Animal experiments

As the blood contains stem cells, it is only logical to ask whether it is possible to repopulate the aplastic bone marrow and lymphoid tissues by transfusion of peripheral blood leucocytes or mononuclear cells. Congdon et al. in 1956 and 1960 gave the first positive answer to the question: on transfusing leukaemoid or leukaemic blood from mice into lethally irradiated syngeneic recipients, the latter escaped radiation death and their haematopoiesis was restored.

Later, in the sixties, the regeneration kinetics of the depleted myeloid and lymphoid tissues after leucocyte transfusion were studied by some authors [12, 19]. Experiments in dogs by transfusion of buffy coat cells were more exact and more close to the human trials [26].

In 1962 our laboratory reported on the repopulation of aplastic bone marrow in lethally irradiated dogs after massive allogeneic exchange transfusion [24]. Between 1964 and 1967 Storb et al. [26], Cavins et al. [5], Ebstein et al. [8] from the Seattle group presented more detailed data of successful "engraftment" of blood stem cells by cross circulation or transfusion of criopreserved autologous or allogeneic leukocytes in lethally irradiated dogs. In the last ten years the experiments carried out on dogs using the new separation technique with continuous or semicontinuous blood flow centrifugation by Fliedner et al. [9] and separately by our group [25] suggested that this kind of "bone marrow" transplantation had clinical perspectives. A good survival rate and haematopoietic recovery typical of bone marrow transplantation were observed after autologous

* Abbreviations: PBSC — peripheral blood stem cells; PBMC — peripheral blood mononuclear cells

or allogeneic peripheral mononuclear cell transfusion. After allogeneic transplantation, some of the dogs had later graft versus host disease (GVHD). Where immunosuppressors were used, the animals survived and chimerism could be proved [9, 13, 15, 22].

Our experiments with selenium-methionine radioisotope technique indicated that transfusion of a sufficient quantity of autologous mononuclear white blood cells resulted in haematopoietic recovery in lethally irradiated dogs, the great majority of transplanted dogs survived, while the irradiated non-transplanted controls all died within 12 days [25].

Clinical trials

Corroborative evidence of successful repopulation of bone marrow and lymphoid tissues in man by transfusion of a sufficient quantity of autologous or allogeneic leukocytes is based on limited trials. Levin et al. [18] were the first who have seen transplantation phenomena in three acute lymphoid leukaemia children with cytostatic-induced marrow aplasia, after being transfused with leukocytes from chronic myeloid leukaemia patients. Many years later Goldman et al. showed in 5 chronic myelogenic leukaemia patients that leukocytes harvested and cryopreserved during remission may restore haematopoiesis. These patients were treated with high dose cyclophosphamide and total body irradiation in blastic crisis and then transfused with cryopreserved autologous leukocytes [10, 11]. McCredie et al. [cited in 10] reported a case of bone marrow aplasia induced by chemotherapy where haematopoietic recovery was accelerated by transfusion of normal leukocytes taken from the patient's twin brother. Recently, Abrams et al. have reported a case of a 16 year old patient with Ewing sarcoma who, after 150 rads of total body irradiation and intensive combined chemotherapy, received as adjuvant treatment 6 transfusions of blood mononuclear cells taken from his twin brother. Due to the inappropriately low graft size, however, there was no detectable recovery in haematopoiesis, only the lymphocyte count improved 3–4 weeks after transplantation [1].

Quantity of PBSC and PBMC for successful bone marrow transplantation

It is well known that the main barriers for successful bone marrow transplantation are immunological incompatibility reactions and the quantity of applied stem cells. Here we shall concentrate only on the optimum graft size needed to transplant patients with peripheral blood mononuclear cells, or concentrated haematopoietic stem cells.

The quantity of peripheral blood mononuclear cells that have to be transfused for successful engraftment is determined by the stem cell content measured by the colony forming units (CFU-S, CFU-E or CFU-GM). Estimation of PBMC and CFU-GM has to be based on the marrow-transplantation experience in primates by Van Dieke et al. [7] and human clinical trials. Transplantation

of autologous bone marrow is likely to succeed if the bone marrow nuclear cells (BMNC) are not less than $50 \cdot 10^6$/kg body weight, and the stem cell content is between $15–40 \cdot 10^3$/kg body weight. In allogeneic conditions the cells have to be more than $200 \cdot 10^6$/kg body weight and the stem cell content over $200 \cdot 10^3$/kg body weight.

Table 1

Peripheral blood stem and mononuclear cells/kg b.w. needed for successful engraftment (using van Bekkum's and Van Dieke's experimental data)

Conditions	BMNC	SC (CFU-GM)	PBMC
Autologous	$50 \cdot 10^6$	$15–40 \cdot 10^3$	$1 \cdot 10^9$
Allogeneic	$200 \cdot 10^6$	$200 \cdot 10^3$	$4 \cdot 10^9$

The normal CFU-GM level in the blood is $0.5–8.0/10^6$ leucocytes or $2–32/10^6$ mononuclears. In mononuclear cell concentrates obtained by continuous flow centrifugation in dogs and man the number of CFU-GM is higher, 50–85 per 10^6 mononuclears [15, 25]. Thus, the amount of mononuclears needed for repopulation of the aplastic bone marrow and lymphoid tissues must be 20 times that of marrow cells (Table 1).

The data by Fliender et al. [9] and ours indicated that in autologous transfusion the minimum peripheral mononuclear cell requirement for marrow repopulation in dogs after lethal gama-irradiation has to be at least $0.20–0.22 \cdot 10^9$/kg b.w. (Table 2).

These findings show an at least one magnitude difference between the calculated and the experienced minimum graft size: for successful transplantation in dogs about five times less PBM were needed than it had been calculated. Knowing that one liter blood yields by leukopheresis only about 10^9 PBMC, it is evident that the minimum dose of PBMC or stem cells is received by leukopheresis of 0.5 liter donor blood per 1 kg body weight of patient treated. This means that in autologous conditions the minimum amount of blood required for the collection of a sufficient number of peripheral blood stem cells is about

Table 2

Minimum and optimum amount of PBM cells for successful b.m. transplantation in dogs (autologous conditions) [13, 22, 25]

Minimum	Optimum	Minimum	Optimum
$0.22 \cdot 10^9$	$0.40 \cdot 10^9$	$0.20 \cdot 10^9$	$0.40 \cdot 10^9$
per kg body weight			

30–35 litres per adult patient. For allogeneic conditions quantity of the blood passing through the cytopheretic machine has to be 180–200 litres, which is practically impossible to be realized.

In view of the quantitative difficulties, up to now the blood of chronic myeloid leukaemia patients was used as a stem cell source. This blood contains 20–40 fold or more leukocytes and the ten-fold of stem cells than that of healthy persons [10]. Thus in the experiment of Levin et al. [18] the amount of CML leukocytes transfused was $190–380 \cdot 10^9$ cell per patient [2.5 to 8-year old children]. Goldman et al. [11] transfused $1.7–3.9 \cdot 10^9$ leukocytes per kg body weight. The stem cell engraftment in the mentioned trials was successful. Transfusion of $2 \cdot 10^9$/kg body weight isologous leukocytes failed, because the stem cell content of the suspension was suboptimum ($6.6 \cdot 10^3$ CFU-GM).

To obtain more stem cells by leukopheresis there are two possible ways: to increase the blood volume passing through the machine or to increase the stem cell level in the blood. The first is practically impossible, but the second could be achieved by finding a stem cell mobilizing agent. So far, such an effect has been achieved with dextran-sulphate treatment in dogs. Even though never attempted in humans, this stimulator injected into dogs produced a ten-fold elevation of the blood CFU-GM level [15]. In our hands dextran-sulphate stimulation in canine cytopheresis increased to threefold the yield of PBMC and CFU-GM colonies [25].

Thus, for solution of the donor problem a stimulating agent should be found by which the quantity of bone marrow stem cells could be augmented several times over the normal level. Another way is to discover a new method for multiplying the peripheral blood stem cells in vitro. Then the difficulties of donation of these cells will be overcome and their engraftment in patients with severe bone marrow aplasia will be more realistic than it is nowadays.

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Frequency of Non-Secretor Types Among Stomach Cancer Patients

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The secretor type and the ABO blood group characteristics were studied in 293 patients suffering from cancer of the stomach. The results were compared with the corresponding data of 760 healthy persons. The frequency of non-secretors was found to be 8.8% lower among the stomach cancer patients, and the incidence of A blood group substance 10% higher than in normal ones.

Keywords: non-secretor type, stomach cancer

Introduction

More than 100 authors have investigated the connection between the ABO blood group system and stomach cancer [1], while hardly any publications are available on the connection between the ABH secretory type and cancer of the stomach [2].

The discovery of oncogenes, recognition of their chromosome localization and discovery of the chromosome aberrations in connection with human tumours put into new light the statistical studies devoted to the connection between ABO groups and stomach cancer. It has become clear that one of the oncogenes is closely localized in the genes of the ABO blood group [3]. The functions of this and other oncogenes are not sufficiently understood, though our knowledge about them is continuously increasing [4]. Their close localization on the chromosome may be of importance without having a clear picture about the interaction of these genes.

It is well known that the secretion of A, B and H is genetically controlled by the Se genes. In homozygotes, the Se genes and the A, B and H substances are not secreted in water soluble form in the body fluids. The genes determining the secretory and non-secretory type are localized on chromosome 19 in the neighbourhood of the Lewis (Le) and Lutheran genes [5]. On this chromosome, however, there is no oncogene, so the connection between secretory status and stomach cancer may indirectly shed light on the possible existence of a new oncogene. This was, therefore, the aim of the present study.

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Patients and Methods

Between 1966 and 1983, we investigated blood and saliva samples of 293 stomach cancer patients (206 males and 87 females) for ABO characteristics and secretory status. The Le blood group substances were studied in red blood cells and ABH substances in saliva. 760 healthy blood donors served as controls; their blood and saliva samples were taken at the surgical departments of our Hospital, at the Second Department of Medicine of University Medical School, Debrecen, and at the County Oncological Care Centre, Nyíregyháza. The diagnosis was established by endoscopical and histological examinations.

The ABO tests and the agglutination inhibition test for the H group substance were carried out after proper treatment of the saliva, according to the directives of the National Institute of Haematology and Blood Transfusion, Budapest.

The presence of Le^a and Le^b antigens on the red blood cell surface was also studied. As it is known, the Le^a antigen positive individuals are obligate non-secretors, those who are Le^b positive are secretors, just as the overwhelming majority of Le^a and Le^b negative individuals [6].

All the assays mentioned above were performed with ABO antisera (Humán Co., Budapest, Hungary) and using the sera and methods described by Biotest Serum Institute (Frankfurt), Orthodiagnostics and Cilag AG (Schaffhausen).

Results

The frequency of non-secretory type was 8.8% lower in the stomach cancer group than in the controls. Among 760 healthy subjects 150 (19.7%) proved to be non-secretor. The frequency of non-secretors in Hungary has not been determined, the European mean occurrence is about 22% (McConnel [7], Sneath and Sneath [8]). Of the 293 stomach cancer patients only 32 (10.9%) were non-secretors, of the whole patient material, 50.5% carried the A blood red cell group substance, 10% more than in our normal county population [9], thus the occurrence of red blood cell A substance was significantly higher in the patient group.

Discussion

A number of publications appeared about the possible connection between blood group substances and diseases. Aird et al. in 1953 were the first [10] to demonstrate the increased occurrence of blood group A among stomach cancer patients.

In addition, we studied the frequency of non-secretor type in the stomach cancer patient group. As to this question, there is some controversy in the literature. Berg et al. [11] investigating 47 patients found 7 non-secretors (15.2%), while Desai and Greger [12] found 24 non-secretors among 210 Japanese stomach cancer patients (11.4%) and 18 among 97 American white patients (18.5%).

In the past few years authors from Japan and other countries have extended their studies on blood group substances of the gastric mucosa. Attention has been paid to the occurrence of I(Ma) antigen in cancer of the stomach.

Walter et al. [13] were the first to publish a report on the connection of secretory type and cancer of the stomach.

In 1959 Glynn et al. [14] reported that the blood group substances were maintained in the stomach cancer tissue. On the other hand, Davidson et al. [15] and Sheahan et al. [16] concluded to a complete or partial loss of these substances in the tumour tissue. The loss was independent of the primary or secondary nature of the tumour, neither was there a correlation between cancer tissue differentiation and the preserved blood group substances. They used a highly sensitive immunoperoxidase technique as did also Slocomb et al. [17] who also reported the loss of the blood group substances, even in well-differentiated tumours. They supposed a blocking mechanism in the malignant cells which would hinder the setting up of the carbohydrate chain of the blood group substances.

Kapadia et al. [18] using immunocytochemical methods found disturbances in the gastric mucosa of non-secretor subjects and in the staining properties for A/H antigen in secretory: the normal of mucosa non-secretors possesses a weaker staining property for A/H antigen but a stronger one for I(Ma) antigen. The majority of secretor type cancer patients showed clearly a stronger staining with anti-I(Ma) antibody as compared to the normal secretor type mucosa. Their results were explained by an incomplete biosynthesis of the A/H determinants. In the stomach cancer patients with small bowel metastasis a significant loss of the A/H and I(Ma) antigens was detected in the non-secretor group.

We have also studied whether in patients in whom gastric cancer developed on basis of a preexisting ulcer, there occurred a change of the secretory type accompanied by an eventual loss of the Le^a antigen on the red blood cells. We were, however, unable to find a patient whose secretory type had changed and who had previously been Le^a positive or non-secretor in the saliva studies.

It is concluded that stomach ulcer patients of the secretory type carry a significantly higher than normal risk to develop cancer of the stomach.

Table 1

Genetical markers in stomach cancer patients and controls

Secretors/non-secretors. Relative incidence 2.007. χ^2 for difference from unity 11.9700.

	0	A	B	AB	Le^{a+}	Le^{b-}	Se	se	
Patients	70	156	51	16	19	274	261	32	
N = 293	23.88	53.24	17.4	5.46	6.35	93.65	89.0	10.9	per cent
Controls	237	305	153	65	141	619	610	150	
N = 760	31.15	40.12	20.10	8.63	18.55	81.44	80.2	19.7	per cent

One might speculate that the secretion of ABH substances may exert a protective function, at least in gastric ulcer patients, against a malignant transformation in the stomach. The question deserves further investigation and might be the subject of basic research.

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Acute Megakaryocytic Myelosis Preceded by Myelodysplasia. Report of a Case and Review of the Literature

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Acute megakaryocytic myelosis represents a distinct disease entity. As shown by a survey of the literature, it is a rapidly fatal disease, not preceded by a chronic myeloproliferative disorder, and mostly refractory to cytotoxic treatment. The first manifestation is pancytopenia with initial absence but quick development of hepatosplenomegaly. In contrast to "acute myelofibrosis", which is characterized by hyperplasia of all three haematopoietic lineages, a purely megakaryocytic proliferation develops together with a slight reticulinic fibrosis. Immature megakaryocytic cells may appear in the circulation, allowing classification of the disease as a variant of acute non-lymphatic leukaemia. This view is stressed by the observation of a preceding myelodysplastic phase in the case reported here.

Keywords: megakaryocytic myelosis, acute leukaemia, myelodysplasia, acute myelofibrosis

Introduction

The term acute megakaryocytic myelosis [23] refers to an ill-defined clinicopathologic entity, which most probably includes haematological disorders of different pathogenesis. While in some of these rare cases the immature megakaryocytic proliferation apparently constitutes the accelerated phase of chronic myeloid leukaemia [1–3] or myelofibrosis with myeloid metaplasia [1, 4], it seems to represent a variant of acute non-lymphatic leukaemia in others [5–29]. Recently, we observed a pure, maturing megakaryocytic proliferation involving the bone marrow, spleen, liver and lymph nodes in a patient who had presented with typical signs of myelodysplasia.

Materials and Methods

Tissue samples were fixed and decalcified [30]. After conventional paraffin embedding 3 μm thick dewaxed sections were stained. Blood and bone marrow smears were air-dried and fixed in a mixture of 60 ml acetone and 5 ml Schaefer's fixative [30] then made up to 100 ml with distilled water. The following staining

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procedures and cytochemical reactions were performed: H.E. and Giemsa (sections); Pappenheim (smears); PAS reaction; Perls' reaction for iron; Gömöri's silver impregnation for reticulin (sections); myeloperoxidase (MPOX) reaction using as substrate 3-amino-9-ethyl-carbazole [31] (smears); naphtol-AS-D-chloracetate esterase reaction [32]; leukocyte alkaline phosphatase (LAP) reaction [33] (blood smears); acid phosphatase reaction without (smears) and with tartrate inhibition [30].

Factor VIII antigen was demonstrated in paraffin embedded tissue samples with the PAP technique using rabbit polyclonal antibody (Dakopatts Lab., Denmark) diluted 1 : 50.

Results

Case report (Fig. 1.)

On admission on June 30, the 59-year-old male patient presented with a three months' history of weakness, fatigue and weight loss of 20 kg as well as fever, night sweats and cutaneous haemorrhages of a few days duration. He had pancytopenia with a hypercellular marrow aspirate (see below). His spleen and liver were not palpable. On the occasion of a hospitalization six months previously for diabetes mellitus and an episode of N VII palsy, no haematological abnormality had been apparent. Under steroid treatment peripheral blood cell values were unchanged. A control sternal aspirate and iliac crest biopsy were performed on July 20, and

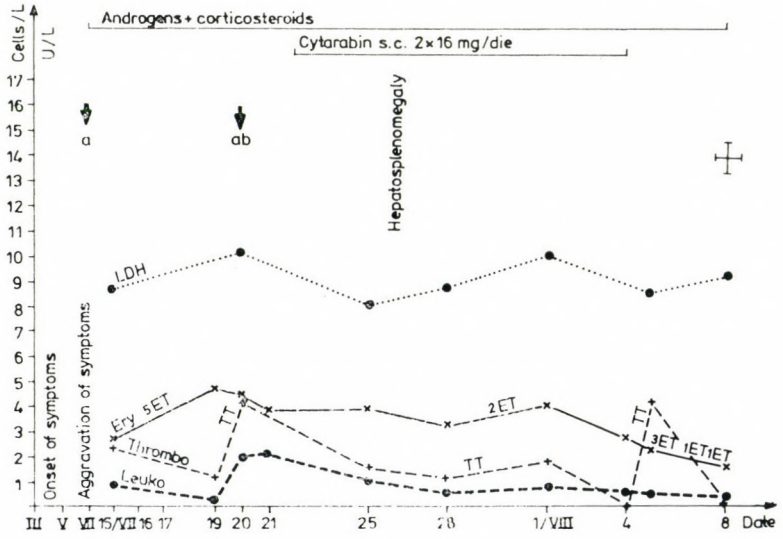

Fig. 1. Schematic representation of the important clinical data of the patient; ery = erythrocytes ($\times 10^{12}$/l), leuko = leukocytes ($\times 10^{9}$/l), thrombo = thrombocytes ($\times 10^{10}$/l), LDH = = lactate dehydrogenase ($\times 10^{2}$ U/l), ET = transfer of erythrocytes, TT = transfer of thrombocytes, a = sternal aspirate, b = iliac crest biopsy

subcutaneous application of cytosine-arabinoside was started on July 22, but it did not improve the clinical picture. Four days later the spleen had a craniocaudal diameter of 14 cm, the liver of 18 cm. Severe gastrointestinal bleeding and fever prompted withdrawal of the drug after 14 days. The patient died 4 days later (August 8) with the signs of haemorrhagic shock. Prominent findings at autopsy were: a markedly enlarged spleen (690 g) of firm consistency and complete replacement of fat tissue in the femur shaft by wax coloured bone marrow. The lymph nodes were not enlarged. The liver weighed 1900 g. Widespread cutaneous and visceral haemorrhages, intestinal bleeding and a fibrinous-haemorrhagic pneumonia of the right lower lobe were additional findings.

Morphology during clinical course

The initial bone marrow aspirate on July 1 was severely hypercellular; no fat cells were found in marrow particles. The myeloid/erythroid ratio was 1 : 1 (lymphocytes 3%, plasma cells 4%). Erythropoietic cells displayed prominent megaloblastosis with a nuclear maturation defect. Often nuclear budding and atypical mitoses were visible. In addition giant erythroblasts with multiple or bizarre-shaped nuclei were also present. The cytoplasm of immature erythroblasts exhibited a strong granular PAS-reaction, while the more mature forms were negative or diffusely positive (Fig. 2a). Sideroblastosis was not apparent; the siderin content of marrow reticulum cells was moderately increased. The quantitatively less affected granulopoiesis was shifted to the left. Mature cells often showed Pelger-like nuclear deformities. Megakaryocytes were seen only rarely; typically they consisted of small forms with unlobulated or bilobulated nuclei and finely granular PAS-positive cytoplasm (Fig. 2b).

This morphological picture was unchanged in the second sternal aspirate (July 20). However, marrow cells were not well spread, suggesting marrow fibrosis. Iliac crest biopsy indeed revealed a slight, patchy reticulinic fibrosis (Fig. 3b). Marrow hyperplasia with reduction of fat cells, which equally involves erythropoiesis and granulopoiesis, was seen at this marrow site, with sparsely micromegakaryocytes (Fig. 3a). These as well as a few blast cells exhibited a diffuse to finely granular cytoplasmic positivity for factor VIII antigen.

In peripheral blood smears (88% mature neutrophils, 2% myelocytes, 3% monocytes, 7% lymphocytes), in addition to Pelger-like cells, an abnormal coarse and irregularly distributed MPOX granulation of mature neutrophils could be demonstrated (Fig. 2c). The LAP index was maximally decreased to 0.

Morphology post mortem

Necropsy revealed a complete change in bone marrow sections from the iliac crest, sternum, thoracic vertebrae and femur shaft. The marrow cave was occupied by an atypical, ASD-chloracetate esterase negative cell population including many giant cells (Fig. 4). Some of them could be identified as mature megakaryocytes by their finely granular, PAS-positive cytoplasm. The nuclei were either chromatin-dense, irregularly lobulated and folded, or immature, with small nucleoli. Other

giant cells displayed a moderately basophilic cytoplasm. Most of the cells were smaller in size, but still larger than immature granulopoietic or erythropoietic cells. Dissociation of maturation was characteristic between nucleus and cytoplasm resulting in cells with mature cytoplasm and immature, unlobulated or bilobulated nuclei, with interspersed small blast cells. There were numerous atypical mitoses. Factor VIII antigen could be demonstrated in the cytoplasm of most of the giant

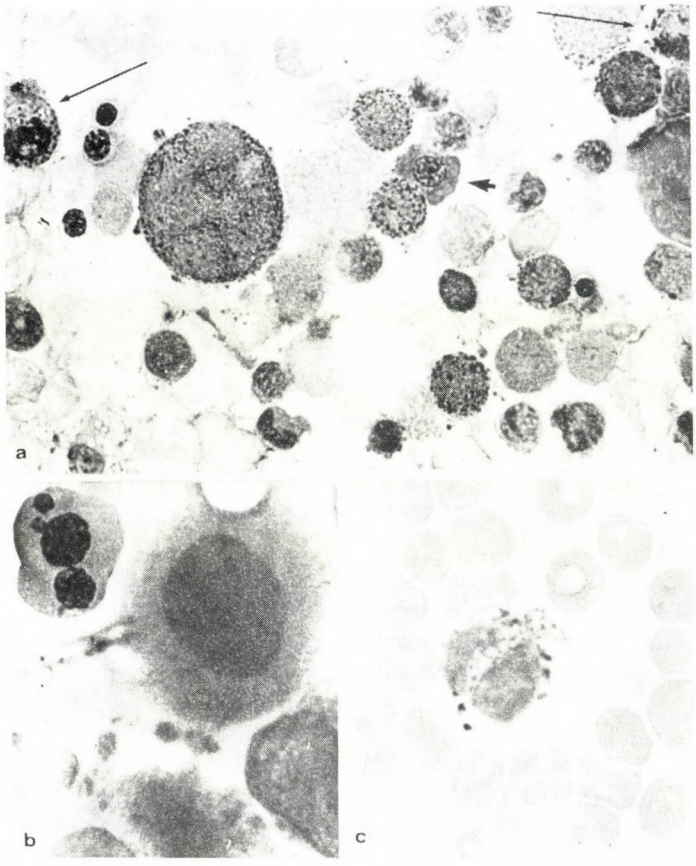

Fig. 2. Cytological and cytochemical atypias of blood and bone marrow cells during the initial phase of the disease

(a) Hypercellular and megaloblastic erythropoiesis in sternal aspirate (July 1); PAS, ×850. Immature erythroblasts (→ = atypical mitosis) display a strong and coarsely granular PAS reaction, while mature forms are negative or diffusely positive (→). On the left, a multinucleated, PAS-positive, immature giant erythroblast

(b) Micromegakaryocyte with mature, finely granular cytoplasm and unlobulated nucleus, and binucleated, mature megaloblast with nuclear budding and fragmentation. Sternal aspirate (July 1); Pappenheim, ×1200

(c) Mature neutrophilic granulocytes with Pelger-like nuclear deformity and irregularly distributed, coarsened granulation. Blood smear (July 20); MPOX, ×1200

forms and micromegakaryocytes as well as of some blast cells. In the latter the activity was diffuse, in the more mature cells mostly finely granular. In all marrow sections reticulin fibres were slightly increased. In the spleen the red pulp was completely infiltrated by the same atypical megakaryocytic cell proliferation. In the liver only the sinuses were filled with atypical megakaryocytes, as were the subcapsular sinuses of some abdominal lymph nodes.

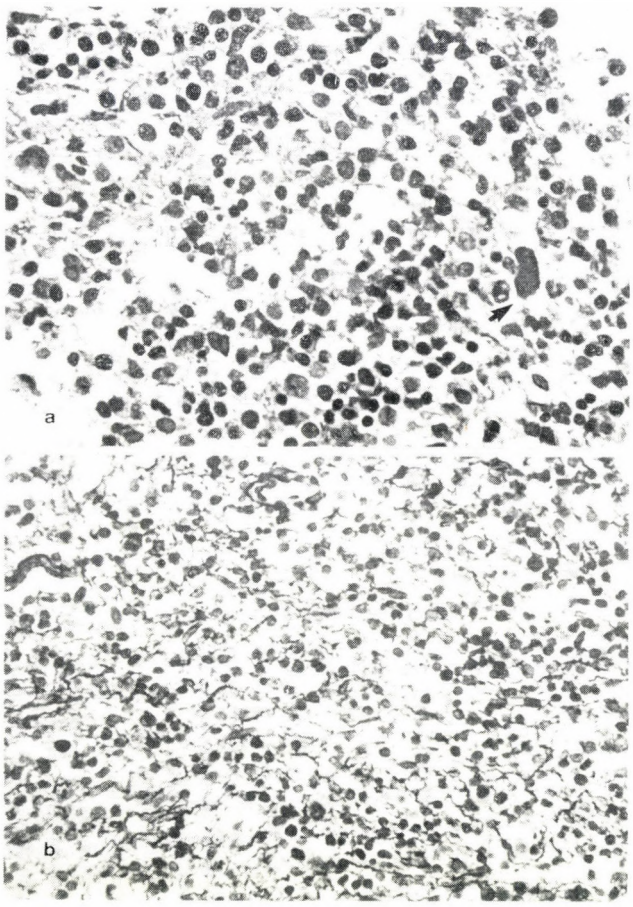

Fig. 3. Iliac crest biopsy (July 20) reveals a hyperplastic erythropoiesis and granulopoiesis, including very few micromegakaryocytes (→), and slight reticulinic fibrosis. (a) PAS, × 600 (b) Gömöri's silver impregnation, × 400

Table 1

Summary of clinicopathological features of cases with acute megakaryocytic myelosis reported in the literature

Ref. No.	Sex	Age	Aleukaemic phase				Leukaemic phase	
			Hb g/dl	RBC 10^{12}/l	WBC 10^9/l	Plt 10^9/l	WBC 10^9/l	Bl %
5	m	72	2.8	—	8.6	115	—	—
6	m	67	6.4	—	0.6	26	—	—
7	m	67	6.7	—	1.9	16	—	—
7	m	74	5.7	—	3.5	40	—	—
7	m	45	4.1	—	5.0	131	—	—
7	f	14	5.5	—	4.1	1	—	—
8	m	52	—	—	9.5	8	—	—
9	f	59	—	—	—	—	2	16
9	m	21	—	—	—	—	6	24
10	f	24	—	—	—	—	187	77
11	m	80	—	2.8	2.6	12	2	80
12	f	43	—	—	—	—	2	76
13	m	72	—	2.2	1.5	3	1	46
14	f	26	—	2.2	2.2	45	80	90
14	f	45	—	1.6	4.2	5	20	90
14	m	55	—	1.4	4.7	2	60	80
15	f	59	—	—	—	—	7	35
16	m	66	—	—	—	—	49	64
17	m	55	—	1.8	2.3	27	10	51
18	f	70	—	—	1.0	7	—	—
19	m	60	—	1.3	3.1	1	—	—
20	m	77	6.7	—	5.0	136	4	18
20	m	66	—	—	—	—	3	19
20	m	52	11.5	—	5.0	30	—	—
21	f	43	—	2.0	0.7	65	—	—
21	f	61	—	3.9	4.2	0	—	—
22	f	60	—	—	—	—	100	90
22	f	34	—	—	—	—	2	15
23	m	28	—	3.2	4.1	44	—	—
24	m	52	9.5	—	5.6	195	—	—
25	f	15m	—	—	—	—	14	19
25	f	18m	7.2	—	5.1	8	—	—
26	m	68	7.7	—	2.6	20	—	—
27	f	55	—	—	—	—	5	75
27	m	14	10.1	—	2.4	30	2	14
27	m	79	9.9	—	4.4	140	6	11
28	m	61	8.3	—	1.9	15	—	—
28	m	69	10.1	—	3.2	43	—	—

C/*1 = immature megakaryocytic proliferation at terminal stage;

Bone marrow findings			Spleen weight	Infiltrated organs	Observ. time from onset of		Response to cytotoxic therapy
1	2	3*	g		sympt.	diagn.	
+	–	–	605	all organs	–	5w	n.d.**
+	–	+	360	liv. spl. ln.	1m	1d	n.d.
+	–	++	–	–	4m	5w	n.r.
+	–	+	–	–	7m	5m	n.d.
+	–	+	–	–	19w	12w	n.d.
+	E	+	–	–	6m	4m	yes
+	E	–	1010	liv. spl.	–	4m	n.r.
+	–	+	260	spl. ln.	–	–	n.d.
+	–	+	–	all organs	–	8m	n.d.
+	–	–	310	liv. spl. ln.	5w	16d	n.d.
+	–	+	120	spl.	4m	2m	n.r.
+	–	–	270	no	1m	10d	n.r.
+	E	++	920	liv. spl. ln.	16m	9m	n.r.
+	E	+	2070	all organs	9m	5m	n.r.
+	E	–	1160	all organs	9m	3m	n.r.
+	G, E	–	2690	all organs	11m	7w	n.r.
+	–	+	290	all organs	5m	–	n.d.
+	G	+	950	all organs	5m	–	n.r.
+	–	+	160	all organs	15m	13m	n.r.
+	E, G	–	180	spl.	–	10d	n.d.
+	–	+	160	liv. spl. ln.	30d	–	n.d.
+	E	–	–	liv. spl.	–	46d	n.d.
+	E	–	–	spl.	–	60d	n.d.
+	–	–	–	liv. spl.	–	9d	n.d.
+	–	–	350	all organs	20d	–	n.d.
+	–	+	190	all organs	3m	–	n.d.
not reported			–	–	–	–	n.r.
+	E	+	–	–	–	–	n.r.
+	–	+	1250	liv. spl. ln.	3m	–	n.d.
+	E	–	–	ln.	6m	3m	n.r.
+	–	+	–	–	17m	14m	yes
+	–	+	–	–	11m	10m	yes
+	E	+	500	spl.	23m	18m	n.d.
+	–	+	–	spl.	–	14m	n.r.
+	–	+	–	–	–	23m	n.d.
+	–	+	–	spl.	–	30m	n.d.
+	–	+	–	not reported	6m	4m	n.d.
+	–	+	–	not reported	12m	6m	n.r.

2 = dysplastic signs of erythropoiesis (E), granulopoiesis (G) before terminal stage; 3 = fibrosis. **n.d. = not done; n.r. = no response.)

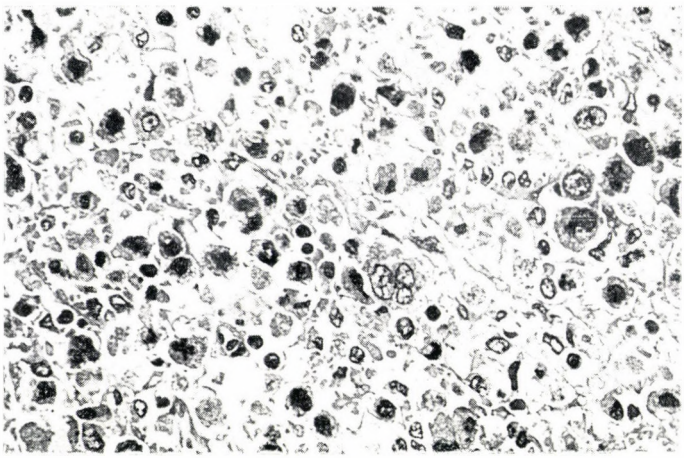

Fig. 4. Complete replacement of the bone marrow at autopsy (August 8) by large cells with a finely granular PAS-positive cytoplasm. Nuclei are either hyperchromatic and irregularly folded, or blast-like with small nucleoli and rounded or bilobulated; PAS, ×600

Discussion

Reports on neoplastic proliferations of purely megakaryocytic lineage are rare, but have become more numerous since histochemical techniques and electron microscopy reliably allow the identification of lymphocyte-like megakaryocytic precursor cells [1, 7, 9, 11, 18, 20, 22, 26–29]. In contrast to cases where a megakaryocytic proliferation represents the terminal phase of chronic myeloproliferative disorders [1–4], our patient had no haematological abnormalities six months before the first manifestation of his rapidly fatal disease. He showed pancytopenia from the onset, and an initial absence of hepatosplenomegaly which, however developed soon. Reticulinic fibrosis in the bone marrow was only slight, and low-dose cytosine-arabinoside treatment had no effect. Our case was comparable to a recently published series of 12 patients [29] and a survey of the literature yielded 38 additional cases (Table 1). In several of these cases the neoplastic cells appeared in the blood allowing classification as megakaryocytic leukaemia, though in comparison to other subtypes of acute non-lymphatic leukaemia the proportion of aleukaemic or subleukaemic cases is conspicuously higher. This may be due to the rapidly fatal course, which mostly was refractory to cytotoxic treatment.

The relation to acute non-lymphatic leukaemia was stressed in our case by preceding haematological changes designated now as myelodysplasia [34]. Among the published cases preceding myelodysplasia has rarely been noted [8, 26, 29], but could be deduced from the descriptions of some authors [7, 13, 14, 16, 18, 20, 22, 24].

Acute megakaryocytic myelosis has to be distinguished from "acute myelofibrosis" [35]. In both conditions medullary fibrosis is only one symptom common

to a variety of haematological disorders with a prominent proliferation of megakaryocytes [1, 36], which are known to synthesize factors capable of activating fibroblast precursors [37]. Acute myelofibrosis in contrast to acute megakaryocytic myelosis shows hyperplasia of all three cell lines [35], and is thought to represent a myelodysplastic condition [38] with a special connection to acute myeloblastic leukaemia [35, 38].

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Obituary

Professor István Cserháti, chairman of the Szeged University School of Medicine, and a member of the Editoral Board of *Haematologia* passed away in January 1986, aged 56 years.

He studied medicine at the Szeged University School of Medicine, where he graduated "summa cum laude" in 1955. As a young medical student he worked in the Pathophysiological Institute. From 1955 to 1973 he worked at the 1st Department of Internal Medicine, gradually moving up the ranks. Dr. Cserháti received his specialist's degree in Internal Medicine in 1959. He achieved his PhD in 1967 and became Doctor of Sciences in 1981. Dr. Cserháti was nominated University Professor by the Hungarian Ministry of Health in 1973 and was named, at the same time, Director of the 2nd Department of Internal Medicine.

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From 1976 he was deputy chairman and from 1984 chairman of the Szeged University School of Medicine.

Dr. István Cserháti was not only a gifted research worker, but an excellent teacher beloved by his students and co-workers.

Dr. Cserháti's scientific work can be divided into two fields: in his earlier years he was primarily interested in clinical bacteriology (he was head of the 1st Department of Internal Medicine). Later, his research work has been concentrated on clinical and experimental haematology. He was one of the pioneer workers in the research of thrombopoietin.

Dr. Cserháti was a member of the European Society of Haematology, the Hungarian Society of Allergology, the Hungarian Society of Internal Medicine, the National College of the Institute of Internal Medicine. He was also a member of the Executive Committee of the Hungarian Society of Haematology.

Dr. István Cserháti, appreciated, loved, and depended on by his colleagues and friends will be much missed.

Abstracts

Transferrin endocytosis and iron uptake by developing erythroid cells in the chicken (Gallus domesticus). B. C. Lim and E. H. Morgan (Department of Physiology, University of Western Australia, Nedlands, Western Australia). *J. Comp. Physiol. B. 155*, 201 (1985).

The mechanism of iron uptake by avian erythroid cells was investigated using cells from 7 and 15-day chicken embryos, and chicken serum transferrin and conalbumin (ovotransferrin) labelled with ^{125}I and ^{59}Fe. Endocytosis of the protein was determined by incubation of the cells with pronase at 4 °C to distinguish internalized from surface-bound protein. Iron was taken up by the cells by receptor-mediated endocytosis of transferrin or conalbumin. The receptors had the same affinity for serum transferrin and conalbumin. Endocytosis of diferric transferrin and conalbumin and exocytosis of apo-protein occurred at the same rates, indicating that iron donation to the cells occurred during the process of intracellular cycling of the protein. The recycling time was approximately 4 min. The rate of endocytosis of diferric protein varied with incubation temperature and at each temperature the rate of endocytosis was sufficient to account for the iron accumulated by the cells. These results and experiments with a variety of inhibitors confirmed the role of endocytosis in iron uptake. The mean cell volumes, receptor numbers and iron uptake rates of 7-day embryo cells were approximately twice those of 15-day embryo cells but the protein recycling times were approximately the same. Hence, the level of transferrin receptors is probably the main determinant of the rate of iron uptake during development of chicken erythroid cells. Transferrins from a variety of mammalian species were unable to donate iron to the chicken cells, but toad *(Bufo marinus)* transferrin could do so at a slow rate. The mechanism of iron uptake by developing chicken erythroid cells appears to be similar to that described for mammalian cells, although receptor numbers and iron uptake rates are lower than those reported for mammalian cells at a similar stage of development.

A. Egyed

Experiment to determine the effect of riboflavin deficiency at weaning on iron economy and heme synthesis. H. J. Powers (Medical Research Council Dunn Nutrition Unit, Cambridge, UK). *Ann. Nutr. Metab. 29*, 261 (1985).

21-day-old female Norwegian Hooded rats were fed a riboflavin-deficient diet for 7 weeks. A control group consisted of individually weight-matched rats fed a complete diet. Reticulocytosis was induced by phlebotomy and heme synthesis measured in a reticulocyte-rich preparation in vitro. Concentrations of circulating iron and liver stores of ferritin iron and non-heme iron were measured. Riboflavin deficiency significantly impaired the process of accumulation and maintenance of hepatic iron stores but did not appear to influence the rate of heme synthesis in an in vitro system. A primary lesion in iron metabolism in young ribo-

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flavin-deficient rats may be at the level of iron absorption so that assimilated iron is diverted to the erythroid marrow at the expense of repleting iron stores.

A. Egyed

Monensin stimulates sugar transport in avian erythrocytes. I. Bihler, P. Charles and P. C. Shaw (Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Canada). *Biochim. Biophys. Acta 821*, 37 (1985).

The cell-medium distribution of the non-metabolized glucose analog, 3-O-methyl-D-glucose was studied in pigeon erythrocytes. The sodium ionophore monensin increased in parallel and in a dose-dependent manner the influx of hexose and of Na^+. These effects were independent of external Ca^{2+} and there was no alteration in ^{45}Ca influx. If, as suggested previously, hexose transport in these cells is modulated by cytoplasmic Ca^{2+}, the stimulatory effect of monensin on hexose transport may be due to increased mitochondrial Ca^{2+} efflux via $Na^+ - Ca^{2+}$-exchange, owing to the elevation of cytoplasmic Na^+. Such a mechanism is consistent with the observed failure of monensin to affect 3-O-methyl-D-glucose transport in cells partially depleted of Ca^{2+}. Monensin also depressed cellular ATP levels but the data favour a Ca^{2+}-dependent mechanism of hexose transport regulation rather than a direct effect of metabolic depletion. The inhibitor of specific-mediated hexose transport, cytochalasin B was found to inhibit equally basal and stimulated 3-O-methyl-D-glucose uptake but there was a cytochalasin B-insensitive uptake component in excess of L-glucose uptake. This appears to reflect a greater diffusional permeability of 3-O-methyl-D-glucose than of L-glucose.

G. Gárdos

Evidence for a Na—K—ATPase-inhibitor in erythrocytes of patients with essential hypertension. U. Walter and S. Müller (St. Hildegardis Hospital, Mainz, F.R.G.). *Eur. J. Clin. Invest. 15*, 209 (1985).

ATPase activities were determined in haemolysed and dialysed erythrocytes and in haemoglobin-free membranes of twenty patients with essential hypertension and twenty normotensive controls. Ouabain-sensitive ATPase (Na-K-ATPase) activity of haemolysate but not that of membranes was decreased in hypertensives whereas ouabain-insensitive ATPase (Mg-ATPase + some residual Ca-ATPase) activity was increased in both enzyme preparations when measurements were performed in the absence of Ca^{2+}-chelating substances. In haemolysed erythrocytes ouabain-sensitivity as a percentage of total ATPase activity was a good discriminator between both groups and may be a possible marker for essential hypertension. The decreased activity of Na-K-ATPase in haemolyaste is apparently due to a non-dialysable inhibitor of Na-K-ATPase which is either tightly bound to the erythrocyte membrane or dissolved in the cytoplasm. Following haemolysis with subsequent centrifugation the Na-K-ATPase inhibitor is removed, at least in part, and thus differences in Na K-ATPase activvity demonstrable in haemolysed and dialysed erythrocytes are no longer apparent in haemoglobin-free membranes.

G. Gárdos

The influence of cellular ATP levels on dimyristoylphosphatidylcholine-induced release of vesicles from human erythrocytes. P. Bütikofer and P. Ott (Medizinisch-chemisches Institut der Universität, Berne, Switzerland). *Biochim. Biophys. Acta 821*, 91 (1985).

Release of membrane vesicles from human erythrocytes was induced by modulation of red cell ATP levels, by incubation of erythrocytes with sonicated dimyristoylphosphatidylcholine (DMPC) suspensions, or by a sequential combination of both procedures. When red blood cell ATP levels were decreased prior to incubation with DMPC, the lag-time between addition of the lipid and beginning of vesiculation was reduced. Furthermore, the rate of vesicle release itself was accelerated. Experiments carried out with a rapid ATP depletion technique showed that the onset of vesiculation and the release were most evidently accelerated in these cases where echinocytes has been formed prior to the addition of DMPC. The

results suggest that red blood cell with reduced cellular ATP levels or an altered cell shape are more susceptible to a further perturbation of the membrane by addition of exogenous DMPC.

G. Gárdos

Ca^{2+}-induced polyphospoinositide breakdown due to phosphomonoesterase activity in chicken erythrocytes. P. J. Raval and D. Allan (Department of Experimental Pathology, School of Medicine, University College London, London, UK). *Biochem. J. 231*, 179 (1985).

Treatment of chicken erythrocytes with ionophore A23187 and Ca^{2+} caused the breakdown of a large proportion of the cellular polyphosphoinositides. Since no diacylglycerol or phosphatidate was generated, but there was a small increase in the level of phosphatidylinositol, it was concluded that breakdown occurred as a result of phosphomonoesterase activation. Experiments with subcellular fractions showed that the phosphomonoesterase activity was present in the cytosolic fraction of the cells.

G. Gárdos

Calcium-dependent association of a protein complex with the lymphocyte plasma membrane: Probable identity with calmodulin-calcineurin. P. D. Chantler (Department of Anatomy, Medical College of Pennsylvania, Philadelphia, Pennsylvania). *J. Cell Biology 101*, 207 (1985).

A protein complex is shown to participate in a calcium-dependent association with plasma membranes purified either from pig mesenteric lymph node lymphocytes or from human lymphoblastoid cell lines. Plasma membranes prepared in the presence of calcium possess this complex; those prepared in the absence of calcium (5 mM EGTA) do not. The complex associates itself with the inner cytoplasmic surface of the plasma membrane. This complex is referred to as the "acidic protein band" because of its location during migration upon alkaline-urea gel electrophoresis. The complex dissociates from the plasma membrane during electrophoresis on 8-M urea gels, irrespective of calcium levels during electrophoresis; at intermediate urea concentrations (4—6 M), the complex is not dissociated in the presence of calcium. Upon purification of the acidic protein band, SDS acrylamide gel electrophoresis, immunoblotting, and radioimmunoassay techniques suggest that the acidic protein band is composed of at least four peptides (designated 68K, 59K, 20K, 20K): two of these (68K, 20K) are immunopositive for calcineurin and one (20K) is immunopositive for calmodulin. Immunoblots of urea gels also indicate that the calcineurin heavy chain (68K) can also appear at three different locations on the urea gel. Patches and caps induced in human peripheral blood lymphocytes by fluorescein-conjugated goat anti-human IgG are not coincident with the location of calcineurin, which remains distributed throughout the cell.

Ilma Szász

Activation of human platelets by ADP causes a rapid rise in cytosolic free calcium without hydrolysis of phosphatidylinositol-4,5-bisphosphate. G. J. Fisher, S. Bakshian and J. J. Baldassare (Division of Laboratory Medicine, School of Medicine, Washington University, St. Louis, MO). *Biochem. Biophys. Res. Comm. 129*, 958 (1985).

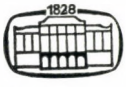
Phosphatidylinositol-4,5-bisphosphate decreased 40% within 10 seconds after the addition of thrombin to platelets. This thrombin-induced loss was accompanied by a corresponding increase of inositol phosphates. In contrast, within the first 60 seconds after exposure of platelets to ADP there was no detectable change in the amounts of phosphatidylinositol-4,5-bisphosphate or inositol phosphates. Both thrombin and ADP, however, caused a very rapid rise of cytosolic free calcium, as measured by quin2. The magnitude of this rise of calcium was similar for the two agonists. These results suggest that in platelets, agonist stimulation may lead to increased cytosolic free calcium independently of phosphatidylinositol-4,5-bisphosphate degradation.

Ilma Szász

Inhibition by forskolin of cytosolic calcium rise, shape change and aggregation in quin2-loaded human platelets. S. O. Sage and T. J. Rink (Physiological Laboratory, Cambridge, England). *FEBS Letters, 188*, 135 (1985).

The adenylate cyclase stimulator forskolin was used to study the inhibitory effects of elevated cAMP on the activation of washed human platelets loaded with the fluorescent Ca^{2+} indicator quin2. In the presence of 10 μM isobutylmethylxanthine forskolin inhibited rises in $[Ca^{2+}]_i$ evoked by thrombin and platelet-activating factor (PAF) due to both Ca^{2+} influx and release from internal stores with similar potency. Aggregation evoked by thrombin and PAF was suppressed whilst partial shape-change persisted even in the absence of a measurable rise in $[Ca^{2+}]_i$. Forskolin did not effect the rise in $[Ca^{2+}]_i$ evoked by Ca^{2+} ionophore; aggregation was suppressed but shape-change persisted.

Ilma Szász



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BUDAPEST

TRANSMURAL PLASMA FLOW IN ATHEROGENESIS

by H. JELLINEK, Z. DETRE and B. VERESS

In English. 1983. 190 pages. 125 figures. 17×25 cm. Hardcover $25.00/DM 63.—/£ 13.80
ISBN 963 05 3205 0

This monograph discusses the hypothesis on atherogenesis suggested by Professor Jellinek and his collegues. The structure and function of the different factors participating in transmural plasma flow are discussed first under normal conditions, then in a pathological sate. Special chapters deal with the methods suitable for studying transmural plasma flow. Among the risk factors the effects of hypertension and atherogenic diet on permability were extensively examined. Mural effects on local and generalized anoxia, mechanical injury and immuno-mediated lesions are discussed as well. These investigations are completed with the study of cell cultures derived from the vessel wall.

AKADÉMIAI KIADÓ
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The Use of Monoclonal Antibodies in Graft Versus Host Disease Prevention

M. K. BRENNER, J-P. GROB, H. G. PRENTICE

Department of Haematology, Royal Free Hospital, London, NW3 2QG, England

(Received 14 November 1985; accepted 18 November 1985)

One of the most important causes of procedure related death after bone marrow transplantation (BMT) is graft versus host disease (GvHD) in which donor T-lymphocytes recognise alloantigens in the recipient and attack and damage the cells bearing them. Even when donor and recipient are matched at all loci of the major histocompatibility complex (MHC) 40%—70% of recipients develop severe graft versus host disease after conventional BMT: in a third of those affected the outcome is fatal [1]. When donor and recipient are less than identical at the MHC the incidence and severity of acute graft versus host disease are correspondingly higher [2].

The morbidity and mortality associated with acute GvHD has limited the application of bone marrow transplantation in two ways: first by restricting the procedure to patients with serious haematological disease and second by excluding individuals who might benefit from BMT but who lack an MHC identical sibling. In this review we discuss briefly the theoretical work that led us to our protocol for T-cell depletion for GvHD prevention and then describe the results of our own and other groups undertaking T-cell depleted bone marrow transplants. Finally, we discuss some of the new problems — and benefits — associated with T-cell depletion and outline the improvements in techniques now taking place.

Keywords: graft versus host disease, monoclonal antibody, T cell depletion

Introduction

Soon after it became clear in the '50s that the lethal effect of irradiating mice could be prevented by transplanting marrow cells [3, 4], the problem of graft versus host disease became apparent [5]. The mechanism of GvHD was clarified when Billingham & Brent [6] showed that a similar syndrome could be produced in neonatal mice by transplanting them with allogeneic spleen cells. Lymphocytes present in the donor grafts were able to recognise tissue alloantigens in the recipient and the severity of GvHD and the rapidity with which it appeared depended on the numbers of lymphoid cells in the graft [7]. Subsequently graft versus host disease was shown to occur not only when donor and recipient differed at Class I and Class II loci of the major histocompatibility complex, but also if they differed at minor loci. By the late 1960s it was established that lymphocytes fell into two phenotypically and functionally distinct groups, the T-cell maturing in the thymus, and the B-cell which matured in the bone marrow. Studies in solid organ transplantation showed that the T-lymphocytes were responsible for graft

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rejection and evidence that they were similarly responsible for GvHD soon followed [8]. Investigations ensued employing antisera to the antigen, θ present on mature mouse T but not B-lymphocytes. They showed that removal of the cells bearing this antigen abrogated GvHD in allograft recipients whilst the speed of onset and severity of GvHD was proportional to the number of θ positive cells added back [9, 10, 11, 12]. Subsequently these observations were shown to apply to other rodents, to dogs and to monkeys [13]. These data can be summarised by saying that GvHD is a consequence of transplanting mature, alloreactive T-lymphocytes, and that the phenomenon is prevented if T-cells are removed from the donor graft.

Methods of Removing T-cells in Man: Effects on Acute GvHD

An understanding of the theoretical basis of GvHD suggested that its prevention by post-transplantation immunosuppression with methotrexate, cyclophosphamide or cyclosporin A would be superseded by pre-transplantation depletion of T-lymphocytes from the donor marrow. This theoretical prediction has been amply supported by practical experience [14, 15, 16]. T-depletion has been achieved by either physical, immunological or immuno-pharmacological methods.

1. *Physical separation*

Successful separation of lymphocytes from marrow progenitors was first described in 1978 using discontinuous albumin gradients [13]. Although this method has been used in man [17] it has now been replaced by more selective techniques. Two examples are cell elutriation [18] in which cell fractionation depends both upon cell size and density, and a rather more demanding procedure developed by Reisner et al. [19] which depletes T-lymphocytes using soybean agglutination and sheep erythrocytes. Unfortunately these techniques are relatively time consuming and have largely given way to simpler methods.

The combination of monoclonal antibodies with physical separation procedures allows a very high degree of selectivity. The monoclonal antibody may be fixed on a plate (panning) or on a column. More recently magnetic colloid to which antibody to the target antigen is attached has been used to remove T-lymphocytes by passing the marrow through a magnetic field [20].

2. *Immunological depletion using monoclonal antibodies*

Monoclonal antibodies can also be used to destroy or disable T-cells directly. Initial studies at this centre used anti-human T-cell antibodies which coated the target cells in the donor marrow after harvest and the treated marrow was then infused into the recipient: T-cell depletion thus depended either upon in vivo cytolysis or phagocytosis by the host reticulo-endothelial system [21]. The mono-

clonal antibody used in the two larger studies was OKT3, which produced moderate improvement in the incidence and severity of acute GvHD [15, 21]. Nevertheless the desired aim of totally preventing GvHD was not achieved and the studies proceeded to investigate in vitro cytolysis.

In vitro cytolysis – Rabbit complement

Studies by Granger and co-workers [22] showed that adding single monoclonal antibodies to lymphocytes in vitro rarely achieved total target cell lysis with rabbit complement. When at least two monoclonal antibodies reacting with two different antigenic sites were combined more than 99.9% lysis could be achieved. On the basis of these observations our own group proceeded to treat matched donor marrow with a combination of MBG6 (CD6) (more recently RFT12) and RFT8 (CD8). Using two courses of baby rabbit complement treatment we achieved a $> 98\%$ T-cell lysis in 39 of 43 donor marrows [14]. No concurrent comparative group has been studied but the degree of GvHD prevention has been almost total. Of 51 evaluable patients transplanted, from matched donors for the treatment of leukaemia 37 had no GvHD and only 3 have suffered greater than Grade I GvHD. These three individuals were recipients of marrow in which treatment in vitro had been technically unsatisfactory, lysing $< 90\%$ of T-cells.

In vitro cytolysis – Human complement

Further studies with another monoclonal antibody Campath 1 have confirmed that T-cell depletion is the optimal method of GvHD prevention [16, 23]. Campath 1 has the advantage that it fixes and is lytic with human complement and consequently total T-cell lysis is readily achieved without using screened batches of rabbit complement and without extensive washing of the treated marrow. The disadvantage of Campath 1 is that the antigen detected is also expressed on B-lymphocytes and some accessory cells, which are therefore also destroyed. Studies from our group suggest that B-cell immunity can be transferred from the donor after specific T-cell depletion, and this benefit may be lost where donor marrow is additionally depleted of B-lymphocytes. Many groups are trying to produce human complement fixing antibodies which react exclusively with T-cells and no doubt such antibodies will be available soon.

3. *Immuno-pharmacological depletion using monoclonal antibody toxin conjugates*

Although complement mediated lysis appears to be effective at producing near complete T-cell depletion, an alternative approach is to use toxins coupled to appropriate monoclonal antibodies for target cell lysis. By far the most experience has been gained with the naturally occurring toxins such as ricin. Whole ricin may be coupled to monoclonal antibodies and non-specific binding avoided by chemically modifying the toxin or by using lactose to block the beta chain

binding site. Alternatively the ricin A chain alone may be coupled to the antibody molecules. Preliminary studies using intact ricin with lactose blocking has demonstrated efficiency of target cell lysis, lack of host toxicity and apparent prevention of GvHD [24].

Effects of T-cell Depletion on Chronic GvHD

While there is no doubt that acute GvHD is prevented whatever technique of effective T-cell depletion is adopted, evidence that it will similarly prevent chronic GvHD has only recently become available. Of 31 evaluable patients in the Royal Free Hospital only 5 had GvHD, 4 of skin and 1 of skin and liver, indicating that T-depletion is effective at preventing this disabling problem.

Effects of T-depletion in Mismatched BMT (1 Haploidentical)

Studies using soybean rosetting or rosetting techniques alone in transplantation for severe combined immunodeficiency suggested that GvHD could be prevented by T-cell depletion even when donor and recipient were only haploidentical [25]. Preliminary studies in our own group have tended to confirm this, demonstrating that the major problems have been failure of engraftment or late rejection of an initially successful marrow graft [14]. 13 out of 19 patients who received a haploidentical transplant engrafted successfully; 6 had no GvHD, 4 had Grade I GvHD, 1 had Grade II and 2 had Grade III. In general the incidence and severity of acute GvHD seems somewhat lower than that seen with other methods of GvHD prophylaxis in *matched* BMT.

Problems and Benefits of T-cell Depletion

Any novel technique brings with it subsidiary problems and benefits. Effective T-cell depletion is no exception.

1. *Graft rejection*

Prior to bone marrow transplantation, recipients undergo conditioning with cytotoxic drugs and often with total body irradiation as well. This treatment is designed to eliminate recipient marrow and render the patient sufficiently immunosuppressed to accept an allogeneic marrow graft. When the patient has leukaemia the conditioning fulfills the additional purpose of eliminating residual disease and is therefore correspondingly more intense. After conventional bone marrow

transplantation for aplastic anaemia or thalassaemia, graft rejection occurred in about 20% of recipients overall and was particularly common if recipients previously required extensive and prolonged blood product support. Rejection was, however, rare in leukaemic recipients – an observation attributed to the immunosuppressive effects of leukaemic therapy and the intensive conditioning regimens which included total body irradiation.

A number of centres rapidly discovered that the previously rare problem of graft rejection by leukaemic recipients became much more frequent when T-depletion was introduced as a method of GvHD prevention [16, 26]. The risk of graft failure varied enormously from centre to centre. In a preliminary study at the RFH failure was only seen in 2.6% of the patients but in collaborating centres where the marrow treatment was identical, but the conditioning regimens were different, a failure rate of 69% (9 of 13 BMTs) occurred. The only difference between the RFH and its collaborating centres was the method of TBI conditioning used. The RFH used a single dose of 750cGy TBI at 15cGy/min received (mid-plane) dose rate. The other centres used a *fractionated* slow dose rate treatment of up to 1200cGy. There is accumulating data that the former TBI regimen is more immunosuppressive [27]. The only patients at the parent centre who rejected had all received < 690cGy mean total body dose. Campath 1 users have experienced similar problems: here again, relatively modest changes in conditioning regimens permit engraftment and success has been reported following the addition of total lymphoid irradiation [28].

In summary we feel that allograft resistance may be overcome by a graft versus host immunity phenomenon and that this may be lost in T-cell depletion of donor marrow. This loss should be compensated for by increasing immunosuppressive conditioning of the recipient. Enhanced immunosuppression need not require increasing doses of cytotoxic drugs or TBI: the in vivo use of anti-T-cell mAbs may well prove equally effective.

2. *Graft versus leukaemia*

One potential weakness associated with effective GvHD prevention in patients receiving BMT for leukaemia is that there might be a concomitant loss of the graft versus leukaemia effect [29], for leukaemic cells carry host alloantigens and would therefore also be a target for alloreactive cytotoxic T-cells. Whilst we are in no doubt that this phenomenon is real, studies in our own programme have shown only one leukaemic relapse up to 36 months after 21 bone marrow transplantations for 30 patients in first CR of acute leukaemia or first chronic phase of CGL. Relapse, however, was seen in 11 of 13 patients with more advanced disease – a figure typical of BMT using conventional conditioning [14]. We feel that to exploit GvHD to prevent leukaemic recurrence after transplantation for advanced disease is unacceptable and it seems preferable either to transplant patients in 1CR or to rely on more intensive conditioning rather than the potentially lethal phenomenon of GvHD which is difficult to predict and control.

3. *Delayed engraftment after T-cell depletion*

There are theoretical reasons why T-depletion with monoclonal antibodies might delay bone marrow engraftment: the procedure could damage pluripotent stem cells, or removal of T-lymphocytes with a capacity to produce a range of bone marrow colony stimulating factors might significantly delay the time taken for the patient to reach safe levels of neutrophils and to become platelet independent. In practise these fears do not seem well founded. The time taken to engraftment (neutrophils > 500 and/or platelet independence) has been prolonged by about five days compared with cyclosporin A prophylaxis, but is identical to that seen with methotrexate.

4. *Immune reconstitution*

Immune recovery following allogeneic BMT *without* T-depletion is slow. The capacity to mount specific antibody responses, to produce delayed-type hypersensitivity reaction and to show effective cytotoxic T-cell responses may take 6–12 months or longer to return [30, 31]. Although the mechanisms for this extended delay are uncertain, three factors have been implicated. First, after conventional BMT there is an overshoot in T8/CD8 (cytotoxic/suppressor) cells to substantially higher levels than normal: second, a high proportion of patients have GvHD which is itself immunosuppressive and increases circulating T8 cell numbers: finally, almost all patients studied to date have received long term immunosuppressive drugs as GvHD prophylaxis or treatment [30]. None of these factors apply to most recipients of T-depleted marrow in whom T4: T8 ratios normalise rapidly [32] and significant GvHD with the attendant requirement for immunosuppressive drugs is rare. Paradoxically then, T-depletion might actually increase the rapidity of immune reconstitution and there is accumulating evidence that this is indeed the case.

a) *NK cell function.* Natural killer activity returns rapidly after T-depleted BMT [33] even though the T8 subset removed during the procedure contains a significant proportion of cells with NK activity [34]. Moreover NK cells post-transplant are activated and kill targets that are not regularly susceptible to NK activity. This activation may be an important component of immune reconstitution, for these cells have been shown to secrete a range of lymphokines including gamma interferon, IL1, IL2 and B-cell differentiation factor.
b) *B-cell function.* B-cell function is undamaged by our T-depletion protocol: serum IgG, IgM and IgA remain at low-normal level throughout the post-transplant period [35], and specific antibody production is also relatively unimpaired. In a study by Wimperis et al. [37], 38 donors were immunised 5–7 days pretransplant with tetanus toxoid and immunity was shown to be adoptively transferred to the recipient who

developed a significant rise in specific antibody. Similarly donor memory B-cells could be reactivated if B-cells taken from a donor immunised months or years previously were transferred to a recently immunised recipient. Here a late rise in serum antibody was seen. If both donor and recipient are immunised pretransplant, then serum antibody responses are enormously increased in titre and in duration.

c) *T-cell recovery*. Little data on functional recovery of T-cells after T-depleted allografts are yet available, but surface phenotyping data indicate that T8 overshoot is uncommon and that in most patients T4 numbers become normal within 5 months of BMT [32].

In conclusion these results indicate that NK function is rapidly restored after T-depleted BMT and that an effective B-cell immune system is transferred when specific T-depletion is the sole method of GvHD prophylaxis.

Future Advances in GvHD Prevention Using mAb

Specific removal of alloreactive T-cells

One of the most striking features of the immune system is its specificity and the ultimate aim in developing techniques to manipulate the immune system is to achieve a similar precision. For GvHD prevention this would involve depletion only of alloreactive T-cells. Although at present there is no way of attaining quite this degree of specificity in man, techniques are available which could reduce the degree of generalised damage to the immune system which might be consequent upon depleting all T-cells. These techniques are dependent on a more accurate understanding of the phenotype of the T-cells responsible for GvHD. Animal models indicate that when transplantation takes place across class 1 loci then only suppressor/cytotoxic cells (Lyt 1–2+) induce GvHD. If the differences are across class 2 loci then both Lyt 1+2-helper and Lyt 1–2+suppressor cells are important [36]. In other words for haploidentical transplants it would be necessary to continue to remove helper and suppressor subsets. Most transplants in man, however, are carried out between siblings who are identical for all major histocompatibility loci but have numerous differences in minor transplantation loci gene products. Precisely analogous murine models have been difficult to find, but transplantation between B10.BR and CBA/J may offer the closest resemblance to human BMT [36]. Here removal of cytotoxic cells completely blocks GvHD, but removal of helper cells has a weak, though significant, effect only.

These results do not offer much guidance for any attempts to narrow the target of anti-T-cell antibodies in man. While it could be argued that anti-suppressor/cytotoxic cell monoclonal antibodies would suffice for matched transplants between siblings, the murine model is used is some way from the human

equivalent and even here there is evidence for a role to be played by helper T-cells. In practice, since immune recovery after T-cell depletion is relatively rapid (see above), such attempts to carry out T-subset depletion may be unnecessary.

Technical Improvements

There are a number of technical improvements that could be made – in particular the development of anti-T-cell antibodies that are human complement fixing. Until recently the only way of achieving this goal was by laborious screening of hybridomas until one was found that secreted antibody of the appropriate specificity and subclass. However, it is now possible to add human Fc fragments to Fab molecules of the desired specificity, thereby producing a chimeric antibody molecule with the necessary specificity and effector function. There is little doubt that this approach will be increasingly exploited. It also seems likely that such antibodies will be used in conditioning protocols, thereby avoiding the morbidity and mortality that are inevitably associated with the increased doses of cytotoxic drugs and total body irradiation now required for successful engraftment of T-cell depleted marrow.

Conclusion

The effective prevention of GvHD is one significant advance in the practise of BMT. We believe there is little doubt that over the next decade the use of mAbs in conditioning regimens and in prevention of post-transplant infection will make a substantial impact on the safety of BMT, finally allowing the procedure to achieve its full therapeutic potential.

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Heterogeneity of Sickle Cell Disease as Shown by Density Profiles: Effects of Fetal Hemoglobin and Alpha Thalassemia

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Factors that modify the intraerythrocytic concentration of hemoglobin S may influence the clinical expression of the disease. Using the phthalate ester method, the red blood cell density has been studied as a function of the mean corpuscular hemoglobin concentration. Four parameters have been used to compare the density distribution of the erythrocytes: D50 (median cell density), R60 (density range in which the middle 60% of the cells are found), F4 and F5 (proportion of cells with density greater than 1.110 and 1.120 g/ml). Compared to normal controls the density distribution of sickle red cells is heterogeneous, reproducible for the same patient (except in case of crisis), while different from one to another. The R60 is correlated with the percentage of dense cells, and the highest values for both R60 and dense cells are found when hemoglobin F is less than 10%. The highest values for the median cell density and dense red cells, but not for R60 which is normal, are observed in S/C patients. In sickle cell anemia patients, the median cell density values are not very different from the normal ones. The highest levels of hemoglobin F are found in this median subpopulation of red cells, while they are very low in the densest cells. R60 and the percentage of dense cells are not affected by the association of sickle cell disease with the deletion of one α gene. Their values are very near the normal ones in the case of an association with β thalassemia or homozygous α thalassemia. In this series, the associated α thalassemia seems to affect the red cell distribution only when it is present at the homozygous state, and the level of hemoglobin F is higher in sickle cell patients without α thalassemia.

Keywords: sickle cell anaemia, red cell density, fetal hemoglobin, α thalassemia

Introduction

Sickle cell disease is characterized by an extreme clinical and biological heterogeneity. It has been shown that the sickle mutation arose, in Africa, at least three times in three different geographical areas (Atlantic West Africa, Central West Africa and Equatorial Central Africa) and expanded by malaria selection [1]. Two genetic factors, at least, seem to have epistatic effects: a) the α thalassemia gene and b) the expression of fetal hemoglobin. Alpha thalassemia is a common feature in the normal Black African, the frequency being 0.10 among normal controls and higher in sickle cell patients of some areas [2]. Fetal hemo-

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globin is quantitatively higher and qualitatively different in the patients of Atlantic West Africa [3]. These two factors are known to influence the concentration of hemoglobin S within the red blood cells and therefore to modulate the extent of polymerization [4].

All the studies on sickle cell disease have demonstrated the heterogeneity of the hematological parameters among the red cell population. This is of paramount importance concerning the intraerythrocytic hemoglobin concentration which varies from normal to values as high as 50 g/ml or even more in the irreversibly sickled cells. The usual hematological techniques give mean values which do not reflect this heterogeneous distribution.

Since a convenient and easy method for determining the density distribution has been described by Danon and Marikovsky [5], this approach has been largely applied to the study of various hematological disorders. In normal individuals, the cell distribution is gaussian, the densities ranging from 1.080 to 1.120 g/ml with a mean value of 1.110 g/ml. In 1978, Oda et al. [6] reported that in sickle cell anemia there was an increase in the proportion of both the heaviest and lightest cell fractions and that in hemoglobin S/C disease a small fraction was shifted to heavier cells. Both sickle cell trait or thalassemia minor showed a normal density distribution.

Recently, Fabry et al. [7, 8] and Bunn et al. [9] have studied the density profiles more thoroughly, using Percoll Stractan isopycnic centrifugation. This method is potentially preparative but requires more expensive reagents and equipment.

In order to investigate the epistatic effects of α thalassemia and fetal hemoglobin, we compared the profiles of the distribution of density among the erythrocytes of sickle cell patients using the phthalate ester method of Danon and Marikovsky.

Material and Methods

Normal volunteers and patients with sickle cell disease followed as outpatients in Paris hospitals have been selected for this study.

Venous blood specimens (5 ml) were collected in EDTA, kept at $+4\,^{\circ}C$ and analyzed the same day.

Determinations of red cell density distributions were made according to Danon and Marikovsky [5]. Fourteen density markers, ranging from 1.075 to 1.135 g/ml, were prepared by mixing phthalate dimethylester (PDM) ($d = 1.190$ g/ml) and phthalate dibutylester (PDB) ($d = 1.045$ g/ml). The appropriate ratio for each density point (d) was determined as:

$$\%\text{PDB} = 100 \times (1.190 - d)/0.145$$

For each cell density point, a microcapillary tube was loaded with phthalate ester marker and filled with blood. The ester end was sealed with clay and the tube was spun in a microhematocrit centrifuge for 15 min at room temperature.

The fractions of packed erythrocytes under and above each phthalate mixture were measured. The phthalate ester profile was obtained by representing the percentage of cells lighter than the marker as a function of the density. The histogram for the density distribution corresponded to the first derivative of the previous curve.

We characterized the profiles using essentially the parameters selected by Noguchi et al. [10]:

- D50, the median cell density of the distribution,
- R60, the density range in which the middle 60% of the cells were found,
- F4 and F5, proportion of cells with density higher than 1.110 and 1.120 g/ml, respectively. The percentage of cells above the value of 1.110 g/ml was in good agreement with the dense fraction F4 as defined by Fabry et al. [7].

When cell fractionation of erythrocytes was required for further studies on subpopulations, it was done by isopycnic centrifugation in discontinuous Stractan gradient [11].

The percentage of fetal hemoglobin was determined by alkali denaturation resistance [12].

The diagnosis of heterozygous (α-/$\alpha\alpha$) or homozygous (α-/α-) α thalassemia was ascertained by restriction endonuclease analysis of DNA. DNA obtained from peripheral blood leukocytes was digested with Bam HI. Following agarose electrophoresis, the DNA fragments were transferred to nitrocellulose filter [13]. α globin specific plasmid JW101, was ^{32}P nick-translated and used for gene mapping [14]. After hybridization the blots were analyzed by autoradiography. Fragments of 10.5 and 14 Kb indicated, respectively, the presence of one and two α genes per chromosome.

Results

As described previously, the density distribution obtained for homozygous sickle cell patients differs from that found in controls (Fig. 1). Besides normal cells, a subpopulation with a very high density and another one below the normal range are observed [6, 15].

In remission, the density profiles are almost identical from one examination to another. In crisis we have observed, as described by Fabry et al. [16], a decrease in the proportion of dense cells and a parallel increase in the light population related to reticulocytosis.

In contrast to the reproducibility of the density profile in the same patient, we observed a heterogeneity of this pattern from one patient to another. Therefore, in patients in remission, the density profiles, as defined by the various parameters described above, were evaluated in relation to the hemoglobin F level and the presence of α thalassemia.

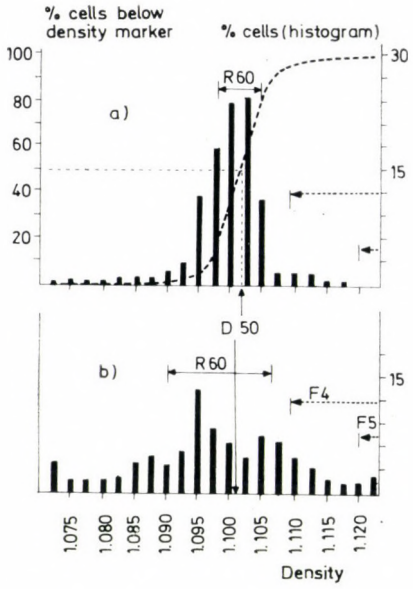

Fig. 1. Erythrocyte density distribution
The different characteristic parameters are indicated: D50, median cell density of the distribution; R60, density range in which the middle 60% of the erythrocytes are found; F4 and F5, percentages of cells with density higher than 1.110 and 1.120. *a)* normal subject: the dotted line represents the experimental curve plotting the percentage of cells observed below each density marker. The histogram corresponds to the first derivative of this curve; *b)* sickle cell anemia individual: the D50 is almost similar to the normal; high density cells are present; R60 is clearly broader

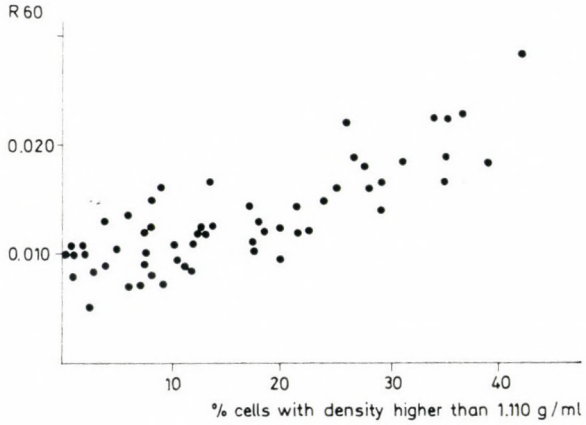

Fig. 2. Spread of the cell distribution (R60) as a function of dense cell percentage (F4)

1) Spread in density distribution (R60)

As illustrated in Fig. 2, the spread in density distribution is highly correlated with the percentage of dense cells. This indicates that the patients with the higher R60 are those with the greater level of irreversibly sickled cells and in whom the fraction of hemoglobin S polymers is high, even in remission [17]. We indeed, observed, in all the severe forms, a coincidence with a broad distribution.

Factors inhibiting polymerization are therefore expected to narrow the cell distribution. This is shown for fetal hemoglobin in Fig. 3: when a high R60 value was observed, the level of fetal hemoglobin was always below 10% and, conversely, when the fetal hemoglobin was higher, the R60 approached the normal value.

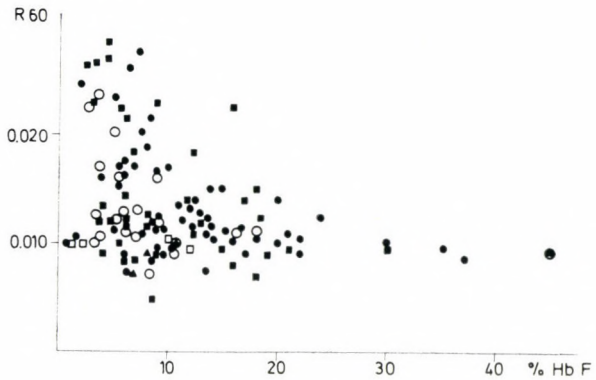

Fig. 3. Spread of the cell distribution (R60) as a function of the hemoglobin F level All the patients are represented When determined the number of α genes and/or the association with β thalassemia are indicated: undetermined: ● ; presence of 4α genes: ■; presence of 3α genes: ○ ; presence of 2α genes: □ ; association with β thalassemia: ▲; The symbol ▲ indicates two siblings with associated α and β thalassemia. The one with 40% of fetal hemoglobin is homozygous for the α gene deletion. His brother is a heterozygote α thalassemic. The highest R60 values always correspond to low fetal hemoglobin levels

Table 1

	Hb F	D50	R60	F4	F5
S/S					
4α (n = 36)	10.5±5.9	1.1011±0.004	0.0135±0.006	15.5±12.6	5.5±7.4
3α (n = 20)	7.1±4.1	1.0997±0.007	0.0135±0.004	15.6±12.6	6.1±8.1
2α (n = 6)	6.1±4.9	1.097±0.005	0.0093±0.002	5.3±4.7	0.4±0.2
S/β Thal (n = 4)	15±16.8	1.1025±0.003	0.008±0.0025	5.5±2.3	0.7±0.7
S/C (n = 10)	1.45±1.7	1.108±0.0054	0.0074±0.0005	43.4±28.7	4.9±2.2
Controls (A/A and A/S) (n = 20)		1.1023±0.002	0.0072±0.001	6.4±8.1	0.2±04

The number of α genes was determined for 62 individuals. As shown in Table 1, R60 was not different between patients carrying 3 or 4 α genes. In contrast a clear difference was observed when there was either a homozygous α thalassemia (2 genes deleted) or a β thalassemia.

2) Median density (D50)

The highest values were found in the individuals carrying both hemoglobin S and hemoglobin C.

In sickle cell patients a slight decrease of the D50, proportional to the number of deleted α genes was observed. Nevertheless, the D50 values remained

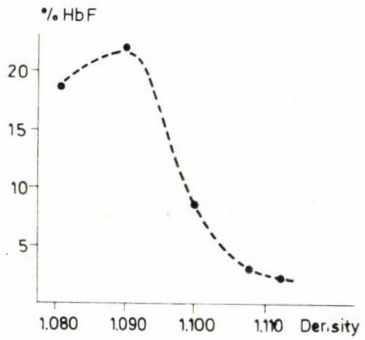

Fig. 4. Distribution of fetal hemoglobin among the cell subpopulations fractionated by discontinuous density stractan gradient

close to the normal. These results were in good agreement with the fact that the erythrocytes which have the best protection against sickling correspond to a subpopulation with almost normal hematological parameters.

This point was evidenced when the level of fetal hemoglobin was measured in subpopulations isolated by isopycnic centrifugation. As shown in Fig. 4, the median population presented the higher level of fetal hemoglobin, in contrast with the light one which contained young cells, heterogeneous for the fetal hemoglobin level, and with the dense one containing almost no fetal hemoglobin. A similar heterogeneity in the erythrocyte life span is expected.

3) Dense fractions (F4 and F5)

As for the D50 the highest amount of F4 cells was observed in hemoglobin S/C individuals. However, when considering F5, the values found were similar to that of homozygous sickle cell patients.

Again no difference was observed between S/S subjects carrying 3 or 4 α genes. It was only when 2 α genes were deleted, or when a β thalassemia was associated, that the amount of dense population was in the normal range.

Discussion

Determination of the density profiles by the simple phthalate ester method confirmed the biological heterogeneity of sickle cell disease and the effects of fetal hemoglobin and thalassemias.

The spread of distribution seemed to be the most significant parameter, its value being a function of both the amount of dense cells and of the degree of reticulocytosis. In the severe sickle cell anemia patients these two subpopulations are often increased and lead to a wide range distribution of the cell density. In contrast, S/C hemoglobin is a model in which the mean density is higher and the dense cells more abundant, but in which the densities are spread over a normal range.

It appears that the overall effect of fetal hemoglobin is clearly to narrow the density distribution when its level is above 10%. Nevertheless this effect is difficult to study more precisely for two reasons. First, the fetal hemoglobin is heterogeneously distributed among the cells. The second reason is that the measured quantity of fetal hemoglobin is the result of two phenomenons: a true synthesis and/or a selection of the cells containing the highest levels of fetal hemoglobin.

Unlike Embury et al. [18] and in agreement with Noguchi et al. [10] we found in this series no significant differences in the erythrocyte density distribution between the patients carrying 3 or 4 α genes.

Another fact that arises is that the level of fetal hemoglobin, in our results, is higher in the sickle cell anemia patients without α gene deletion than in those with α thalassemia. This has also been observed by Higgs et al. [19].

Finally, comparing our results with other similar investigations, one has to emphasize the broad variety of results. Two explanations, at least may explain the discrepancies:

- Sampling of patients is different from one place to another, the genetical epistatic factors and the environmental and socio-cultural conditions vary. For instance, it has been observed in West Atlantic Africa that an increased synthesis of fetal hemoglobin exist together with a low frequency of deletional α thalassemia [3].
- Our investigation, as well as the others, has taken into account only the two best known modulating factors, fetal hemoglobin and α thalassemia, but it is clear that others exist which are not as yet well characterized and are still under study.

It seems nevertheless that the determination of density profiles, integrating all the interfering factors, is an interesting part of the phenotypic description of the disease.

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Circulating Immune Complexes in Sickle Cell Anaemia

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Circulating immune complexes were investigated in sera of 100 patients with sickle cell anaemia (SCA) in different clinical stages, using inhibition of complement-dependent rosette formation (EAC rosette inhibition), anticomplementary activity, polyethylene glycol 6000 precipitation, immunoconglutinin titre, total haemolytic complement, C4 and C3d. A high positivity was observed in patients with hepatic crisis (75%) and cases with leg ulcers (43%) compared with painful crisis (12%) and asymptomatic (7%) patients. These results provide evidence for the presence of circulating immune complexes during leg ulcers and hepatic crisis in SCA.

Keywords: anticomplementary activity, complement breakdown products, EAC rosette, immune complexes, immunoconglutinin, PEG 6000 precipitation, sickle cell anaemia

Introduction

The presence of immune complexes in a variety of human disorders is now well established. They are found in numerous malignant and non malignat diseases [1–4]. In sickle cell anaemia (SCA) an immune complex nephritis occurs in some patients because of in situ complex formation composed of autologous renal tubular antigens and specific antibodies [5]. The possibility, however, that these deposits result from trapping of soluble complexes derived from the circulation with the glomerulus acting as an innocent bystander cannot be eliminated. Liver dysfunction and leg ulcers are common complications in SCA, but the pathophysiology of both is still uncertain [6, 7]. It has been reported that circulating immune complexes are present in SCA patients during hepatic crisis [8] and leg ulcers [9]. The present report attempted at evaluating, by several methods, the immune complexes in sera of SCA patients in different clinical stages: asymptomatic, painful crisis, leg ulcers and hepatic crisis.

Material and Methods

100 adult SCA patients (range: 16–40 years, mean: 28 years of age) were studied. The diagnosis was substantiated as detailed elsewhere [6]. The patients were grouped according to the clinical stage as follows: I – asymptomatic

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(n – 42); II – painful crisis (n = 8); III – leg ulcers (n = 14) and IV – hepatic crisis (n = 16).

Sickle cell hepatic crisis was diagnosed on the basis of the clinical course, liver function tests and differentiated from hepatitis by liver biopsy [7, 10]. None of the patients had received blood transfusions in the month preceding this study.

Peripheral blood samples were obtained by venipuncture. Sera were assayed on the day of preparation or stored at −80 °C until tested. All control sera were collected and stored under the same conditions.

Measurement of immune complexes in serum was performed by three methods: inhibition of complement-dependent lymphocyte rosette formation, anticomplementary activity and precipitation with polyethylene glycol 6000 at 3.75% final concentration.

Inhibition of EAC rosetting was measured as described previously [8]. Normal and patient's sera were treated with deoxyribonuclease [11] (BDH Chemicals, Poole, England) and EDTA-Na_3 [12]. This treatment allowed the elimination of interference of DNA and C-creative protein complexes, respectively, thereby avoiding false positive results. The presence of antilymphocyte antibodies was determined by a microcytotoxicity test following the positivity criteria proposed by Terasaki et al. [13]. Antibodies found in 3 patients were absorbed with a healthy donor lymphocyte pool. The inhibition test was performed in each positive case, before and after antilymphocyte antibody absorption.

Anticomplementary activity was measured by the method of Johnson et al. [14]. Results were expressed as CH_{50} units left in the assay system.

Precipitation with polyethylene glycol 6000 at a final concentration of 3.75% was performed according to the method described by Hašková et al. [15]. C3d, the breakdown product of C3, was quantitated by single radial immunodiffusion of the supernatant obtained after treating the patient plasmas with polyethylene glycol 6000 at a final concentration of 11% [16], using a monospecific antiserum (Central Laboratory of the Red Cross Blood Transfusion Service of The Netherlands). Immunoconglutinin levels were measured by the micromethod of Hautanan et al. [17], and total haemolytic complement was determined by the method of Mayer [18]. C4 was measured by single radial immunodiffusion [19] using a monospecific antiserum (Hyland Laboratories, USA). Normal values were obtained by performing the tests described above in healthy adults.

Results

The percentages of positivity demonstrated in groups I and II were significantly lower than the ones obtained in patients with leg ulcers and hepatic crisis. Circulating immune complexes were detected by the EAC rosette inhibition in 12 (75%) patients in group IV, 6 (43%) patients in group III, 7 (25%) in group II and in 5 (12%) of group I; the anticomplementary activity was increased in four and nine cases in groups III and IV, respectively, while groups I and II showed

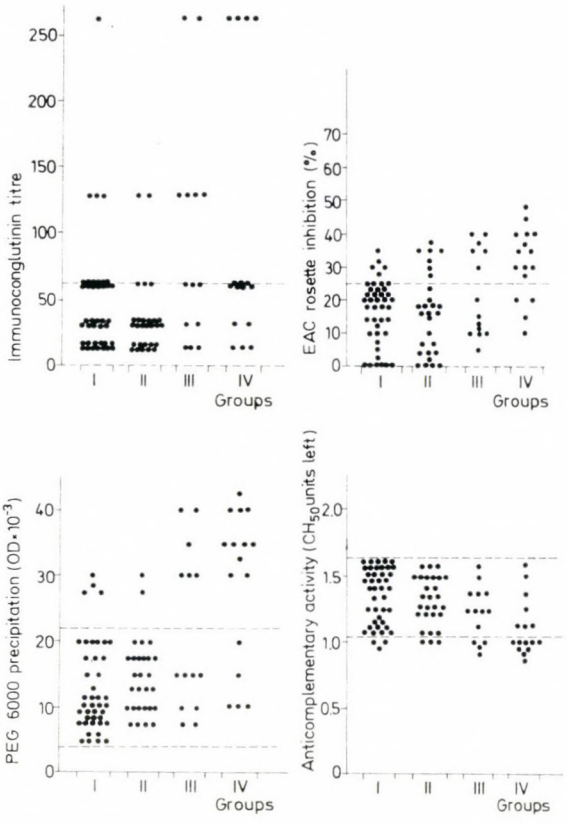

Fig. 1. Results obtained in SCA patients. Normal ranges are indicated by dotted lines

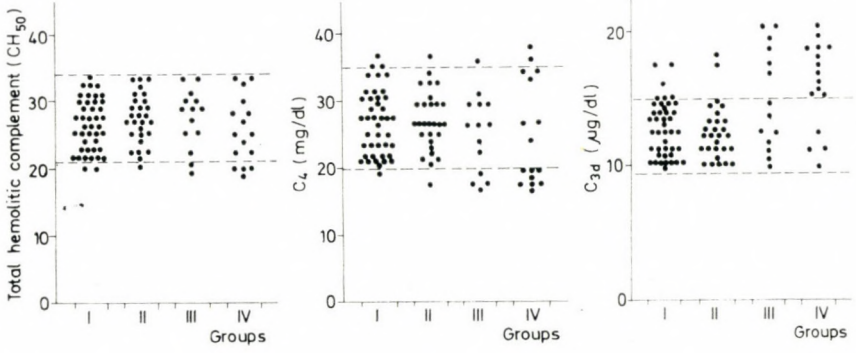

Fig. 2. Results obtained in SCA patients. Normal ranges are indicated by dotted lines

values below the control range only in 3 patients. High levels were found, mainly in groups III and IV with the polyethylene glycol 6000 precipitation test as well as high titres of immunoconglutinins. Twelve samples had C3d fragment values above the normal range in group IV, 6 in group III, 2 in group II and 3 in group I. Eight cases had marked consumption of C4 in group IV and four in the leg ulcers group, only one patient had low levels of the complement component in groups I and II respectively. Total haemolytic complement was decreased in four patients in group IV, 2 in group III, 1 in groups II and in two of the asymptomatic groups (Figs 1–2).

In group III and group IV significant differences were found in some ass ay between patients and normal controls. No differences were found in any of th methods employed in the other groups (Table 1).

Table 1

Methods	Sickle cell anaemia patient groups				
	Asympto-matic	Painful crisis	Leg ulcers	Hepatic crisis	Control group
EAC rosette inhibition (%)	16.2±9.7 (n = 42)	15.8±12.1 (n = 28)	21.7±13* (n = 14)	30.2±10.2** (n = 16)	12.5±12.5 (n = 30)
Anticomplementary activity (CH_{50} units left)	1.34±0.2 (n = 42)	1.26±0.2 (n = 28)	1.19±0.21 (n = 14)	1.05±0.22** (n = 16)	1.35±0 3 (n = 30)
PEG 6000 precipitation (OD · 10^{-3})	13.0±7.1 (n = 42)	14.5±5.9 (n = 28)	21.0±12.2* (n = 14)	28.6±11.6** (n = 16)	13.0±9.0 (n = 30)
Immunoconglutinin (Dilution reciprocal)	M = 32 (16–256) (n = 42)	M = 32 (16–128) (n = 28)	M = 64* (16–256) (n = 14)	M = 64* (16–256) (n = 16)	M = 32 (0–64) (n = 30)
Total hemolytic complement (CH_{50})	26.5±3.7	27.1±3.5	26.8±4.48	25.0±4.84	27.5±6.5
C4 (mg/dl)	26.8±4.3 (n = 42)	26.8±4.0 (n = 28)	25.2±6.19 (n = 14)	25.0±7.56 (n = 16)	27.5±7.5 (n = 30)
C3d (μg/dl)	12.8±2.1 (n = 42)	12.6±2.0 (n = 28)	16.2±3.6* (n = 14)	16.0±3.2* (n = 16)	12.0±3.0 (n = 30)

* – $p < 0.05$
** – $p < 0.01$
M – median

Results obtained in SCA patients in different clinical stages

A follow-up study was made in three SCA patients with hepatic crisis with high levels of circulating immune complexes. In all cases there was a gradual decrease of the serum glutamic pyruvic transaminase level and a normalization of circulating immune complexes after exchange transfusion and/or plasma exchange therapy as shown in Fig. 3.

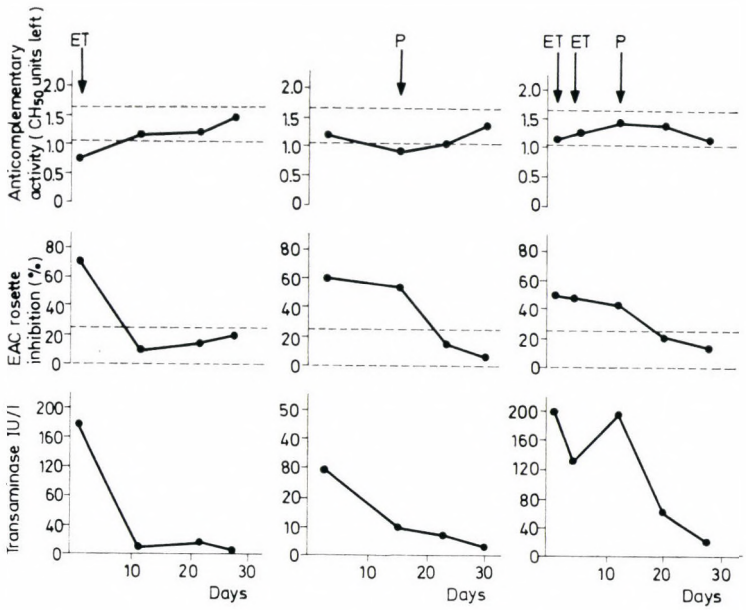

Fig. 3. Follow-up study in 3 SCA patients with hepatic crisis. ET-Exchange Transfusion; P-plasmapheresis

Discussion

Our findings provided evidence for the presence of circulating immune complexes in a high percentage of SCA patients with hepatic crisis and leg ulcers. This was not the case with the asymptomatic and painful crisis groups where the number of positive patients was significantly lower. These findings correlate with the results of our group in a previous study performed in a few SCA patients during hepatic crisis [8]. Several explanations can be postulated for the presence of circulating immune complexes in SCA patients with hepatic crisis: the possibility of in situ complex formation with subsequent solubilization by the complement system [20]; release of immunogenic material into the circulation, secondary to liver damage with the production of specific antibodies; decreased clearance of the complexes by the liver due to saturation or blockade of the mononuclear phagocytic system; decreased circulation through hepatic sinusoids [8, 20, 21]. These explanations consider immune complexes as a secondary event related to liver damage.

The hypothesis that the complexes could be the primary cause of liver disease is questionable, because in several SCA patients with hepatic crisis immune complexes could not be demonstrated. However, this depends on the sensitivity of the methods used and also the moment in which the patient sample was with-

drawn. Striking improvement of an intrahepatic sickling crisis in haemoglobin SC disease occurred after treatment by partial exchange transfusion probably by diminishing the concentration of S hemoglobin-containing cells [22]. Successful treatment of sickle cell intrahepatic cholestasis by partial blood-plasma exchange transfusion has been described. This treatment led to regression of hepatic encephalopathy, impending bleeding diathesis and noticeable clinical improvement within 48 hours. Whether the improvement resulted from the transfusion of normal red blood cells or from fresh-frozen plasma or both remained unknown [23]. Using exchange transfusion we have also obtained good results in the treatment of SCA hepatic crisis. The substantial improvement obtained in our SCA patients with hepatic crisis and in a case reported by Svarch et al. [24] after plasmapheresis suggest that certain elements present in patient plasmas could also participate in liver damage. Although a previous paper [8] mentioned that circulating immune complexes may have had no role in the pathogenesis of hepatic crisis, taking in account the almost ubiquitous presence of variety of complexes in liver diseases of different types [8], the results of the present study suggested the possibility of participation of these complexes to a certain degree. This possibility should be further explored.

The presence of circulating immune complexes in SCA patients with leg ulcers could be explained by some of the mechanisms mentioned above, but we must keep in mind the abnormal susceptibility to acute bacterial infections seen in these patients [25]. Morgan et al. [9] demonstrated the presence of cryoprecipitates in sera of ulcer patients, indicative of immune complexes in the circulation as a consequence of the liberation of antigenic material from infection, this being the result rather than a cause of leg ulcers.

In our study a low percentage of patients with complement consumption was obtained in all groups. These results may be contradictory to the number of cases with low levels of C4 and high titres of C3d demonstrated mainly in groups III and IV. This might be explained by an increased rate of synthesis of the complement components that equilibrate the consumption up to normal limits. Infection and immune complex formation in these patients could theoretically be responsible of the complement system activation basically through the classical pathway.

The causes of immune complexes in SCA hepatic crisis and leg ulcers remain unclear. It appears that additional work is needed to understand the significance of these findings.

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First Occurrence of Hb H in Hungary

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Blood sample from a 52 year old woman of mild anaemia was investigated for thalassaemia. Based on the haematological data and the results of the restriction enzyme analysis we concluded that the patient was heterozygote for α-thal-1 and α-thal-2.

Keywords: α-thalassaemia, Hb H disease, rightward deletion

Haemoglobin H disease is characterized by a moderately severe haemolytic anaemia. It is caused by interaction of α thal-1 and α thal-2 defects resulting in a single functional α gene per diploid genome [1].

α-thalassaemia is a very rare haematologic disorder in Hungary and Hb H was detected only in this single patient during a 20 year period.

The propositus was first admitted to hospital with complaints of weakness, and was found to have a mild anaemia and hepatosplenomegaly. The present haematological data were determined by standard methods [2, 3]. RBC 4.1 T/l, Hb 97.0 g/l, PCV 0.37, MCH 23.6 pg, MCV 90.2 fl, MCHC 26.2%, serum iron 1.15 mg/l, iron saturation index 0.39. Non-electrolyte permeability: glycerol t/2 42 sec, thiourea t/2 136 sec (normal range: 18–25 sec and 110–130 sec, respectively). Starch gel electrophoresis at pH 8.6 and isoelectric focusing revealed a fast moving fraction corresponding to Hb H (4.3–8.2%). Hb A_2 level was 2.06% and Hb F 1.08%. In vitro chain synthesis ratio $\alpha/\beta = 0.51$ has shown diminished α chain production. The methaemoglobin level varied between 2.6–8.9% in spite of normal NADH-methaemoglobin reductase activity.

Morphology of red cells showed marked anisopoikilocytosis and a number of target cells. Supravital staining with brilliant-cresyl blue resulted in typical Hb H inclusions. Electron microscopic investigation has shown that the inclusions were bound to the inner surface of the red cell membrane eliciting its protrusion [2]. Intracytoplasmic inclusions were present in approximately 35% of the bone marrow erythroblasts and reticulocytes as well [3].

The family history did not reveal any haematologic disorder or hydrops fetalis. The parents of the propositus were deceased. The brother and the daughter of the propositus appeared to be normal with respect to clinical and laboratory findings, therefore they could only be carriers for α thal-2. No other relatives were available.

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Restriction endonuclease mapping of the patient's DNA was carried out by Southern blot technique [4]. Hind III and Bgl II enzymes were separately used for digestion of the normal and the patient's DNA. The fragments were electrophoresed through 0.8% agarose. Transfer to nitrocellulose filter was done as usual and the filter was hybridized to nick-translated probe prepared at a specific activity of 10^8 cpm/μg from genomic DNA fragment that included an α structural gene.

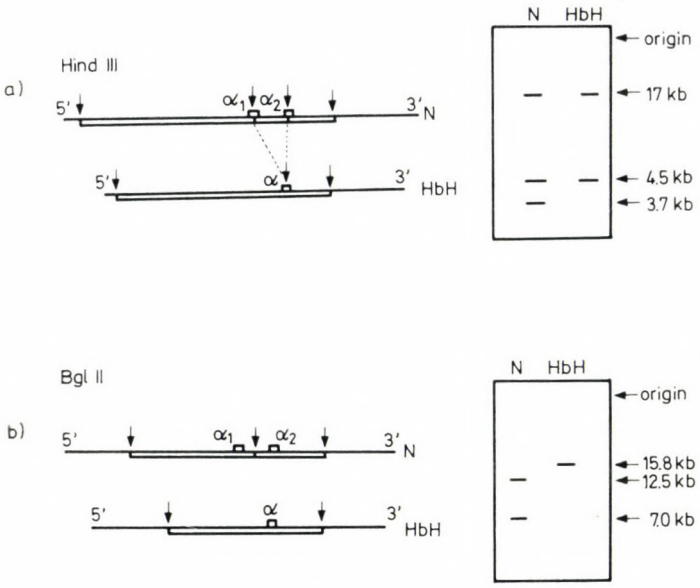

Fig. 1. Restriction sites of Bgl II (a) and Hind III (b) enzymes and schematic picture of the fragments, from normal and Hb H patient's DNA

HIND III cleavage sites are in normal DNA within both α genes at codons 90/91 [5] and so digestion results in three specific fractions: 4.5 kb, 3.7 kb, 17 kb. The propositus' DNA gave two HIND III fragments 4.5 kb and 17 kb, the third one was missing. (Fig. 1a)

For the localization of the deletion we used a second enzyme. Bgl II cleaves between the two normal α loci and produces two fragments of 12.5 kb and 7 kb. The size of the single α specific Bgl II fragment obtained was 15.8 kb which 3.7 kb less than the sum of the two normal Bgl II fragments and showed the deletion of the intergenic Bgl II site. (Fig. 1b)

These data are diagnostic of the rightward [6] deletion type Hb H in which the deletion resulted from an unequal but homologous crossover between the α_1 and α_2 genes [7].

Formally, with the blotting technique homozygosity for an α thal-2 chromosome cannot be ruled out, but this is highly unlikely because of presence of Hb H.

The rightward deletion occurs frequently in Asian, black and Mediterranean populations in α thal-2 [6] and in Hb H [8]. Our Hungarian propositus obviously has no genetic relation to these populations and in the absence of the pedigree data we cannot detect the origin of the chromosome deletion.

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Relationship Between Maternal and Infant Iron Stores: 1. Full Term Infants

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Serum ferritin, serum iron and total iron binding capacity were determined in 218 pregnant women at term and in the cord blood of their normal term infants.

Both the mean weight (3210 g) and serum ferritin level (geometric mean 81 μg/l) of the neonates of iron deficient mothers were significantly lower than those of mothers with "normal" iron stores (weight 3390 g; cord ferritin level 115 μg/l).

A weakly significant correlation was found between the logarithm of the maternal and neonatal serum ferritin concentration ($r = 0.15$, $p < 0.05$) and the maternal log serum ferritin and the newborn's weight ($r = 0.15$, $p < 0.05$). The weak correlations were supported by the differences between the values of cord samples from babies of iron deficient mothers and those whose mothers had "normal" iron values.

Adequate iron supplementation during the early period of pregnancy is suggested.

Keywords: fetal iron stores, iron status, maternal iron stores, serum ferritin

Introduction

The high iron demand of pregnant women and the frequent prevalence of iron deficiency during pregnancy are well known [Fenton et al. 1977; Sturgeon 1959; Van Eijk et al. 1978; Lányi et al. 1979]. It is still debated, however, whether this iron deficiency has any effect on the iron stores of the newborn infant. Several authors have examined the effect of maternal iron stores reflected by the serum ferritin concentration on the cord blood ferritin level. The results of these studies were contradictory.

In our laboratory the problems of anaemia during pregnancy have been studied for nearly a decade. Our examinations have shown that while the incidence of anaemia increased with the advance of pregnancy [Simonovits et al. 1977] a frequent decrease in Hb concentration was found only in the first trimester. Normal or slightly decreased Hb values of a woman in the 30–34 weeks of pregnancy may mask severe iron deficiency or even a complete exhaustion of the iron stores [Német et al. 1981]. With these observations in mind, the relationship between the maternal and neonatal iron status and the newborn's weight was studied.

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Table 1

Comparison of maternal and cord blood parameters of mothers with low and normal ferritin level

		Maternal blood					Cord blood				
		Age (years)	Gravidity	Serum iron (μmol/l)	TIBC (μmol/l)	Transferrin saturation	Birth weight (g)	Serum* ferritin (μg/l)	Serum iron (μmol/l)	TIBC (μmol/l)	Transferrin saturation
Maternal ferritin $10 \geq \mu g/l$	Mean	26.1	2.5	14.2	98.7	0.15	3210	81.3	20.8	39.9	0.54
n = 48	SD	5.0	1.2	5.7	26.3	0.09	442	13–200	6.5	9.1	0.19
Maternal ferritin $20 < \mu g/l$	Mean	27.2	2.3	17.2	83.3	0.21	3390	114.8	23.1	46.7	0.52
n = 108	SD	4.6	1.5	8.3	19.8	0.10	442	15–205	9 0	15.8	0.20
Student's *t* test		1 34	1.22	2.28	4.04	3.56	2.35	3.20	1.46	2.62	58
Probability (p)		>0.1	>0.1	<0.05	<0.001	<0.001	<0.05	<0.01	>0.1	<0.05	>0.1

* Values are recorded as geometric mean and range

Materials and Methods

Blood samples were obtained by puncture of an antecubital vein of healthy females immediately after delivering term mature babies, and also from the umbilical blood of the newborn just after clamping of the cord. Data of women who had suffered from any disease during pregnancy were omitted from analysis.

After clotting at room temperature blood samples were centrifuged. The obtained serum was stored at –20 °C until use. Serum iron, total iron binding capacity (TIBC), transferrin saturation and serum ferritin values were determined.

The serum iron and total iron binding capacity were measured by the modified method of Ceriotti and Ceriotti (1980), using ferrozine colour reagent. Photometric evaluation was carried out in 3 × 10 mm vials and a 0.8 ml sample was sufficient for testing both serum iron level and total iron binding capacity. To eliminate the interference of copper, thiourea was used instead of thiosemicarbazide.

Serum ferritin was determined by immunoradiometry as described previously [Mann et al. 1979].

Statistical analysis

With respect to the skewed distribution of serum ferritin values, all statistical analyses were performed as logarithms and were retransformed to antilogarithms to obtain the original value. Accordingly, the data for serum ferritin were expressed in terms of the geometric mean and the range [Martin, 1969]. Statistical evaluation of the data was assessed by Student's *t* test and correlation analysis.

Results

The data of mothers with depleted iron stores (serum ferritin below 10 μg/l) and of their babies were compared with those with serum ferritin values within the normal range (> 20 μg/l).

The data of mothers with 10.1–20 μg/l serum ferritin level were not included since these could mask the difference between the iron deficient and the healthy groups. Results are summarized in Table 1.

Beside serum ferritin, other iron values of the mothers (serum iron, TIBC and transferrin saturation) also pointed to iron deficiency. In all parameters significant differences were found between the iron deficient groups and those with normal values.

The weight of infants of iron deficient mothers (3210 g) was significantly less than those of mothers with normal iron stores (3390 g). The cord serum ferritin of infants of iron deficient mothers was also significantly lower (81 μg/l

Table 2

Comparison of maternal and cord blood parameters of new-borns with low and normal ferritin level

		Cord blood				Maternal blood					
		Birth weight (g)	Serum iron (μmol/l)	TIBC (μmol/l)	Transferrin saturation	Age (years)	Gravidity	Serum iron (μmol/l)	TIBC (μmol/l)	Transferrin saturation	Serum* ferritin (μg/l)
Cord blood ferritin ≤56 μg/l n = 22	Mean	3135	20.0	46.1	0.47	26.1	2.6	16.5	88.2	0.20	15
	SD	442	7.7	12.4	0.20	4.6	1.6	9.9	28.9	0.13	
Cord blood ferritin ≥56 μg/l n = 196	Mean	3335	22.9	43.1	0.55	26.7	2.4	16.6	89.5	0.19	22.2
	SD	432	7.9	13.3	0.18	4.7	1.4	7.4	22.0	0.10	
Student's *t* test		2.05	1.64	1.01	2.00	0.57	0.72	0.06	0.25	0.43	2.26
Probability (p)		<0 05	>0 1	>0.1	<0.05	>0.1	>0.1	>0.1	>0.1	>0.1	<0.05

* Values are recorded as geometric mean and range

and 115 μg/l, respectively). Hardly any difference was found in the values of iron deficient and healthy newborns' serum iron, TIBC and transferrin saturation.

The results were analysed also with respect to maternal age and previous pregnancies, to exclude the interference of these factors with the iron status. Age and the number of previous deliveries were practically the same in the two groups (no difference in $p > 0.1$).

Next, the parameters of newborns with low iron stores (56 μg/l, which means one standard deviation below the mean) and the data of their mothers were compared with those of normal infants (Table 2).

Babies of the low serum ferritin group weighed less (3135 g) than neonates of the normal serum ferritin group (3335 g). Although there was only a slight difference between the serum iron and TIBC values of the two groups, the transferrin saturation showed a significant decrease: neonates with low ferritin level had lower transferrin saturation (0.47) than those with high ferritin level (0.55).

As to the maternal data, only the serum ferritin level showed a slight difference between the two groups (15 μg/l in the low ferritin group and 22 μg/l in the normal group).

The relationship between maternal and neonatal parameters was also studied. Table 3 indicates the correlation coefficients (r).

Table 3

The correlation (correlation coefficients) between the parameters of maternal and neonatal iron status

Maternal blood	Cord blood				
	Log serum ferritin	Serum iron	TIBC	Transferrin saturation	Birth weight
Log serum ferritin	0.15 *	0.09	0.22 *	−0.10	0.15 *
Serum iron	−0.03	0.17 *	0.06	0.10	0.01
TIBC	0.07	0.07	−0.02	0.05	−0.02
Transferrin saturation	−0.12	0.04	0.04	0.04	0.02

$n = 218$, significant correlation * $p < 0.05$

A weakly significant correlation was found between the logarithm of the maternal and neonatal serum ferritin levels and between the log maternal serum ferritin and the newborn infant's weight. A similar correlation was found between the maternal and neonatal serum iron values ($r = 0.17$, $p < 0.05$). A linear correlation was observed between the logarithm of the maternal ferritin level and the total iron binding capacity of the newborns.

Discussion

The serum ferritin concentration reflects the total body iron stores [Jacobs et al. 1972; Cook and Finch, 1979]; it is therefore a sensitive indicator of iron deficiency and iron overload.

Maternal blood volume increases markedly during pregnancy with an increase in plasma and red cell volume. Consequently, to a certain degree (17% haemodilution), anaemia is a physiological phenomenon [de Grouchy, 1978]. Iron stores decrease rapidly in the first trimester. According to Svanberg et al. (1975) in this phase of pregnancy iron absorption is not yet increased. This means that fetal iron requirements are greatest at a time when the iron stores are decreased to a minimum.

In nearly 60% of pregnancies, the iron transferred to the fetus completely exhausts the maternal iron stores [Fenton et al. 1977; Taylor et al. 1982; Serfass and Liu, 1983; Romslo et al. 1983]. The crucial question is whether the maternal iron sources are sufficient to fill the iron stores of the fetus.

The results of our investigation suggested a negative answer. Mean serum ferritin level and birth weight of the neonates of severely iron deficient mothers were significantly lower than those of newborns of mothers having "normal" iron stores.

A correlation was found between the maternal and neonatal log serum ferritin concentration and between the maternal log serum ferritin and the newborn's weight. This means that increased maternal iron stores are associated with increased fetal iron stores and higher birth weight.

The observed correlation between log maternal ferritin and TIBC of the newborn has no biological meaning.

Different research groups in developed countries analysing the relationship between maternal and neonatal iron stores obtained conflicting results. Table 4 summarizes these findings in chronological sequence. Rios et al., 1975; Van Eijk et al., 1978; Jansson et al., 1979; and Zittoun et al., 1983, did not find any difference between the children of iron deficient and non iron deficient mothers. Hussain et al., 1977; Bratlid and Moe, 1980; and Blot et al., 1982, failed to find a correlation between the serum ferritin concentration of the mother and the cord blood. In contrast and in accordance to our results, Fenton et al., 1977; Kelly et al., 1978; and Kaneshige, 1981, found that the low serum ferritin level measured at delivery was associated with a significantly lower serum ferritin concentration in cord blood.

Many authors have examined the relationship between serum ferritin values in maternal and cord blood by correlation analysis. Kaneshige (1981) observed a close correlation ($r = 0.75$, $p < 0.001$) of the serum ferritin level in maternal and cord blood, while Kelly et al. (1978) could find no such correlation. When, however, the logarithmic value of maternal ferritin was compared to that of cord ferritin, a low but significant correlation ($p < 0.01$, $r = 0.254$) was found. The latter finding may explain the significant differences between the conclusion of different authors. As the distribution of serum ferritin values is log-normal,

Table 4

Relationship between maternal and cord blood ferritin

No. of Neonates	(Reference)	Maternal serum ferritin at birth	City	Ferritin conc. (μg/l) geometric mean	Range (μg/l)	Relationship between maternal and cord ferritin
6		9 μg/l	Seattle (USA)	100	77–131[c]	no
20	(18)	9 μg/l		117	82–166[c]	no
33	(1)	12 μg/l	Cardiff (GB)	174[a]	–	yes*
96		12 μg/l		245[a]	–	yes*
51	(21)	58[a] (16–201) μg/l	London (GB)	183[a]	62–313	no
23		12 μg/l	Glasgow (GB)	155	±63[c]	yes*; **
61	(24)	30 μg/l		218	±120[c]	
15	(3)	Without Fe suppl. 5[a] ± ±6[c] μg/l	Rotterdam (NL)	168	±44[c]	no
15		Fe suppl. 22[a] ± 12[c] μg/l		174	±45[c]	
4		12 μg/l	Malmö (S)	87[b]	57–170[b]	no
16	(19)	12 μg/l		72[b]	15–160[b]	
10		20 μg/l	Johannesburg	59	26–134	yes*; **
	(11)		(ZA)			
93		20 μg/l		83	44–156	
54	(22)	29[a] (6–86)	Tromsö (N)	144[a]	46–400	no
33	(25)	27[a] ± 166[c]	Okayama (J)	287[a]	±180[c]	yes***
100	(23)	9 (1–80) μg/l	Clamart (F)	75	10–286	no
48		Placebo suppl. (Ø anaemic) 20 (5–74) μg/l		214	60–762	
42	(20)	Fe suppl. (Ø anaemic) 34 (6–201) μg/l	Paris (F)	166	52–530	no
31		Fe suppl. (anaemic) 33 (6–186) μg/l		181	41–798	
48		10 μg/l	Budapest (H)	81	13–200	yes*; **
108 (present results)		20 μg/l		115	15–205	

Notes: [a] Median; [b] estimated from figure; [c] standard deviation
* significant difference between the means; ** weak correlation; *** strong correlation

.e. the logarithm of the data gave Gauss' normal distribution, in the calculations the logarithm of the serum ferritin values should be used. Means are affected slightly, correlation calculations somewhat markedly.

MacPhail et al. [1980] also sought for a correlation between log serum ferritin and other haematological indices on the one hand, and between the maternal and cord blood values on the other. They found a weak correlation between maternal and neonatal log-ferritin, and maternal and neonatal serum iron concentration.

Our results confirm the earlier general observation that severely iron deficient mothers deliver mature babies somewhat smaller in weight and deficient iron stores.

The borderline between the physiological and pathological level of iron deficiency in pregnant women is the aim of a WHO sponsored prospective study for the determination of adequate iron stores during periods of life with increased iron demand.

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Transplantation of Chemically Induced Mouse Leukaemia into Newborn F_{344} Rats

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Chemically induced lymphoid mouse leukaemia was successfully transplanted into newborn F_{344} rats. The developing rat-leukaemia contained not only donor cells but donor and recipient cells, or only recipient cells.

Keywords: Chemically-induced-xenotransplantation-donor-recipient cell-type

Introduction

Tumor transplantation represented one of the most widely used methods of experimental cancer research in the fifties. A number of chemically induced tumors have been rendered transplantable and maintained in this way. Since chemical carcinogenesis is being intensely studied even nowadays, the so-called classical and some more recent transplantable tumors amount to a considerable number. These models can be used for studying metastasis formation, kinetic and chemotherapeutic purposes quite efficiently, mainly because of their rapid development.

In our tumor-transplantation experiments, chemically induced lymphoid mouse leukaemia maintained by serial in vivo passages similar to human B cell leukaemia [5] was transplanted into newborn, untreated Fischer 344 rats. Surprisingly, the mouse leukaemic cells were not only accepted by the newborn rats, but an unusually great number of leukaemic blasts and a massive infiltration were observed in almost every organ. Such an extensive cell proliferation raised the question whether it represented an uncontrolled proliferation of the injected tumor cells (donor cells) or the host cells were transformed in some way and proliferated.

In this paper we have attempted to answer this question.

Materials and Methods

Leukaemia

U-867 leukaemia was obtained some years ago by urethane treatment in AKR × C3H/He-mg F_1 hybrid mice.

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On the basis of earlier experiments one seems to be B cell type [5]. This leukaemia-line was maintained by serial in vivo passages in AKR × C3H/He-mg F_1 male hybrid mice.

Animals

AKR × C3H/He-mg F_1 ($AKCF_1$) mice (maintaining leukaemia) and inbred female + male Fischer 344 rats were used. The animals originated from our own animal breeding stock and strain.

Transplantation studies

Leukaemia was maintained, transplanted into rats and retransplanted into mice according to Kiss et al. [17]. Cell suspension was prepared from the spleen of moribund leukaemic animals, this was filtered through four gauze layers, then the cell number was adjusted to 10^7/ml. Of this cell suspension 0.05 ml (5×10^5) was injected intraperitoneally (i.p.) into newborn animals, and 0.1 ml into the adult ones. Newborn F344 rats of similar age, treated i.p. with 0.1 ml physiological saline were used as controls.

Newborn rats received the leukaemic mouse spleen-cells within 24 h after birth. Sex distribution was determined by the litter itself. During retransplantation, the suspension obtained from rat-spleen was reinjected into 6 male and 6 female F344 rats, two months of age, as well as $AKCF_1$ hybrid mice of the same age. Untreated siblings of the leukaemic rats as well as newborn $AKCF_1$ male and female animals were also retransplanted with the rat tumor cells. During the investigation the experimental animals were kept on standard laboratory chow and tap water ad libitum. The leukaemia developed in the newborn rats after 7–9 days. Then peripheral blood smears were prepared, stained with May-Grünwald-Giemsa staining, the condition of the animals was recorded, and histological sections stained with haematoxilin-eosin were prepared from the thymus, spleen, lymph-nodes, liver, kidney, brain, muscle, bone marrow, heart and lung.

Enzimological methods

The pyruvate-kinase activity in the mesenteric lymph-node cells was determined by the kinetic method of Gutmann and Bernt [13], and thymidine-kinase activity was determined according to Machovich and Greengard [25]. The activity was expressed in the first case as mE/mg protein, and in the second case as nmol phosphorylated thymidine/min/mg protein.

Flow-cytometric method

Cell suspension (10^7/ml) was also prepared from the mesenteric lymph-node cells of leukaemic rats in Parker solution (TC-199 medium) for comparison with mouse leukaemic lymph-node cells [17, 34]. The intensity of scattered light, being

proportional to cell size, was measured in order to differentiate between the infiltrating, large blasts and cells of normal size [34].

Chromosomal examination

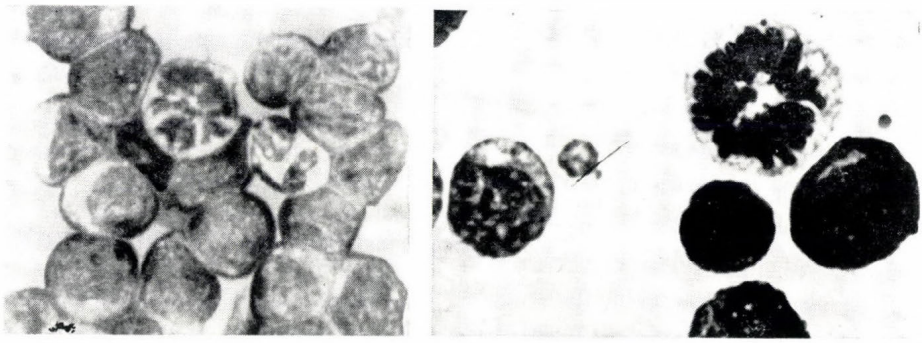
Chromosome examinations were carried out on the spleen cells, by direct procedure and in 24 hour short lifetime cultures, applying colchicine treatment. The metaphases were examined after G-banding (ASG-trypsin) [5].

Statistical comparisons were carried out by using Student's two sample *t* test.

Results

Successful xenotransplantation

During the first series of the experiments, 5×10^5 U-867 type leukaemic mouse spleen cells were injected i.p. into F_{344} rats, on the first day of their life. The experimental animals were sucking and growing normally up to the sixth day, from the seventh day, however, anaemia, icetrus and increasing weakness could be observed.



a) b)

Fig. 1. Peripheral blood smear of U-867 leukaemia in newborn F_{344} rat

Table 1

Results of xenotransplantation study from $AKCF_1$ hybrid mice into newborn F_{344} rats

Type of leukaemia	Newborn F_{344} rat	
	male	female
U-867	81/91	77/89

Their leukocyte count was over 100 000, while the number of red blood cells was hardly two million per mm^3. The blood smear was crowded with large blast-type cells, and proliferating forms were found surprisingly frequently. Two typical blood smears are shown in Fig. 1 (a–b). The success of xenogenic transplantation is shown in Table 1.

After the death of the animals (7–9 days following the tumor transplantation) an enlarged whitish spleen and liver as well as swollen supracoecal (mesenteric),

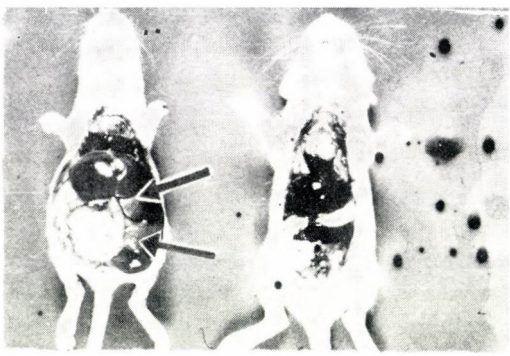


Fig. 2. Gross morphology of leukaemic and control rats. On the left leukaemic and on the right a control mouse. Arrows indicate enlarged spleen and liver

and paraaortical lymph nodes, and a bladder infiltrated by blood was found at autopsy. In Fig. 2 the gross morphological changes of a leukaemic rat are shown as compared to a healthy control.

Proofs of malignancy

Histological examination revealed a massive, monomorphic lymphoid infiltration in the liver, lung, bone marrow, spleen, lymph node and even in the muscles. An infiltrated muscle section is shown in Fig. 3.

Besides the histological findings, the infiltrative tendency of the cells was indicated also by the increased diameter of cells obtained from the lymph node (populational, flow-cytometric examination of 10^7 cells) (Fig. 4). This method is suitable for the separation of healthy and leukaemic blasts, but not for differentiating rats cells from mouse cells. The leukaemic infiltration was also verified by the increased pyruvate-kinase as well as thymidine-kinase activities in lymph node cells, since both enzyme activities correlate well with the development of various tumorous processes [1, 34]. The results regarding pyruvate-kinase are shown in Fig. 5, while those of thymidine-kinase in Fig. 6.

On the basis of the unusual finding that the peripheral blood smear of tumor-injected rats of 7–9 days resembled an infiltrated bone marrow rather than a peripheral blood smear, and that the infiltration was present in almost all the organs, the question arose whether the donor mouse cells were proliferating excessively or the rat cells, i.e. next rat blast-cells appeared and proliferated.

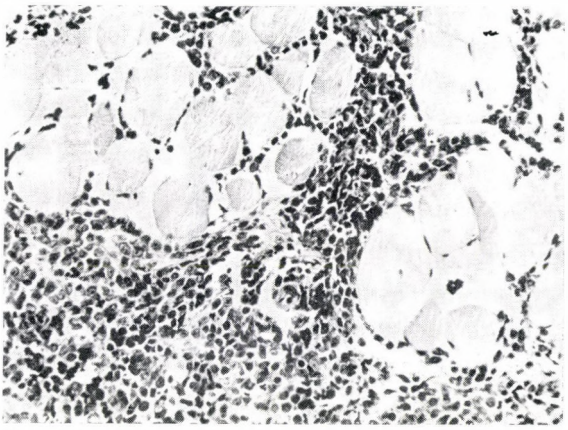


Fig. 3. Histological sections of leukaemic rat muscle with lymphoid, monomorphic infiltration

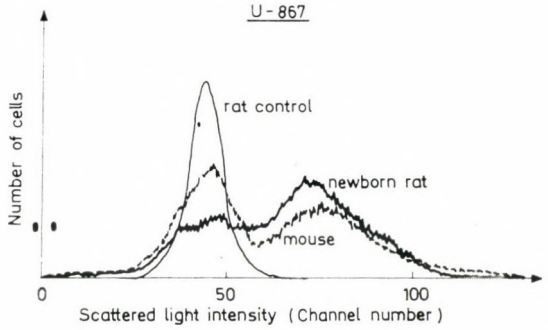


Fig. 4. FACS-III. Histograms (Fluorescence-Activated Cell-Sorter).
The continuous line shows the rat control, the upper line the newborn rat. The lower line corresponds to mouse leukaemic cells. The first peak represents the normal-size lymphocyte population, the second peak the large lymphoblasts

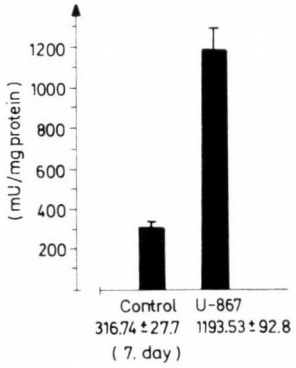


Fig. 5. Pyruvate—kinase activity of mesenteric lymph-node cells in newborn leukaemic and control rats

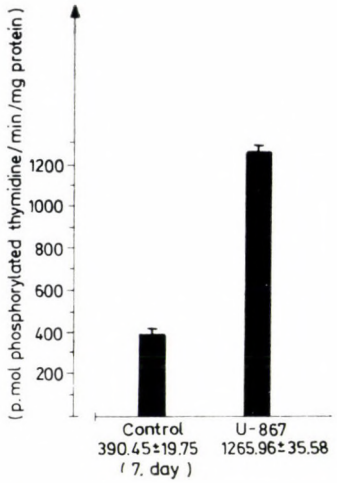


Fig. 6. Thymidine-kinase activity of mesenteric lymph-node cells in newborn leukaemic and control rats

Origin of the leukaemic cells in the rat

To answer this question, blast cells obtained from the spleen of 7–9 days leukaemic rats were injected into newborn and adult $AKCF_1$ mice as well as into newborn and adult untreated F_{344} rats of the same litter. We supposed that mouse blast cells adhere in newborn and adult mice and also in newborn rats, while they do not adhere in sucking or in young adult rats. On the other hand, if the leukaemic blast cells developing in rats originate from rat tissue, their proliferation could be expected in newborn mice and rats, also in the siblings and young adult rats of the same litter, but not in adult mice. These experiments proved that the blasts originated from mice. The results of these examinations are shown in Table 2.

Table 2

Results of retransplantation studies

	Newborn F_{344} rats (female + male)	F_{344} Litter number (female + male)	Young adults F_{344} rats (female + male)	Newborn $AKCF_1$ hybrid mice (female + male)	Young adults $AKCF_1$ hybrid mice (female + male)
U-867 type leukaemia from newborn F_{344} rat	8/8	0/14	0/12	9/10	9/12

In the last series of the experiments we attempted to support this finding by chromosome analysis. Obviously, one expected to find mouse karyograms in the blood cells found in the rats. Surprisingly, however, in some of the leukaemic rats the blast cells proved to be of rat origin, while in others the blast cells displayed a mixed origin (mouse and rat type). These date are shown in Table 3 while Fig. 7 shows the rat and mouse karyogram of the same animal (besides the xenogeneity, the sex is also different).

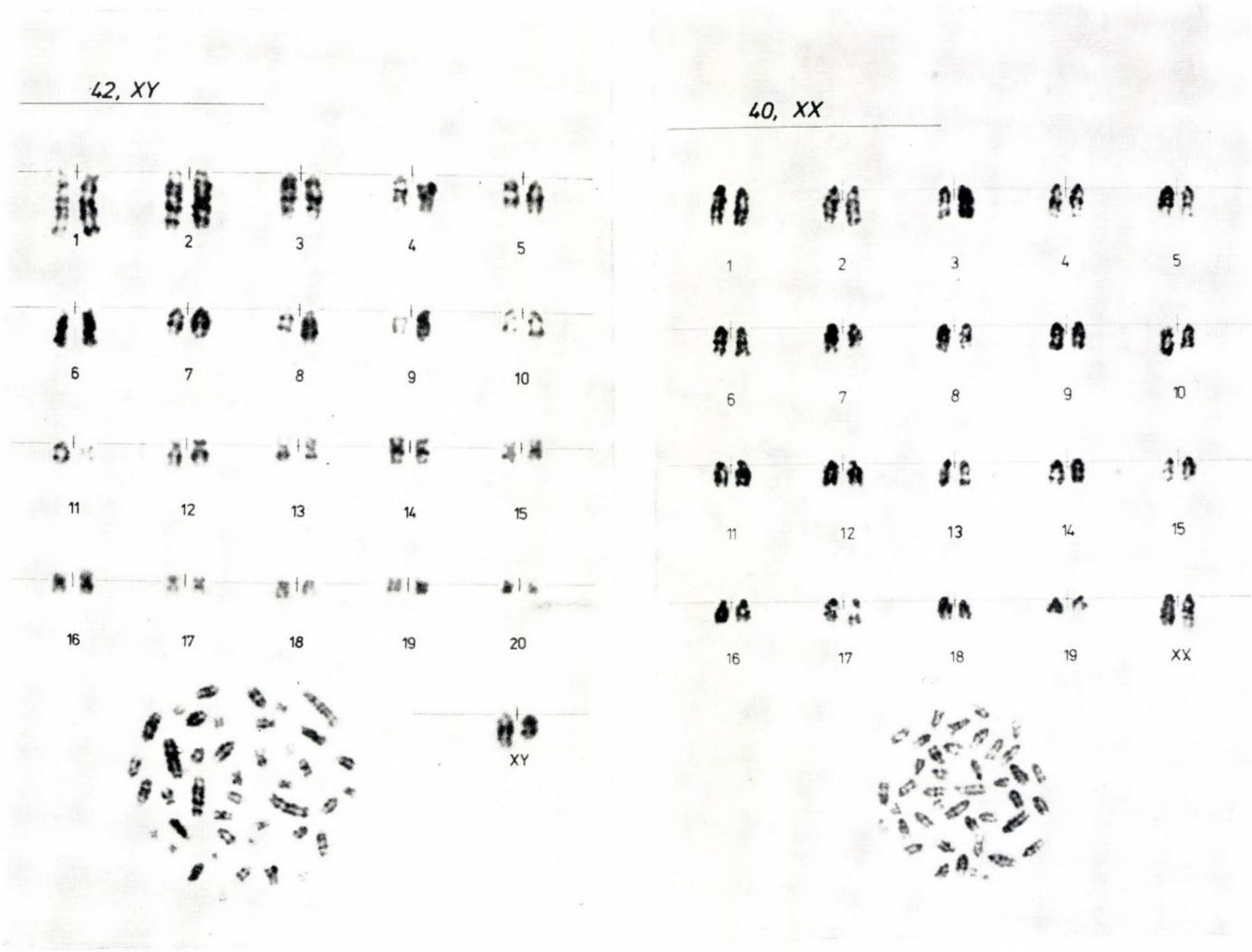
Table 3

Cytogenetic investigation of lymphoid mouse leukaemia transplanted into newborn F_{344} rat

Type of leukaemia	No.	No of inv. meta-phases	Distribution of meta-phases		Distribution of cell type on the basis of chromosome examination										No. of abnormal cells	
					mouse					rat						
			mouse	rat	38	39	40	41	42	40	41	42	43	44	mouse	rat
U-867	1.	10	—	10	—	—	—	—	—	1	—	8	1	—	—	2
	2.	3	2	1	—	—	2	—	—	—	—	1	—	—	—	—
	3.	6	2	4	—	2	—	—	—	—	—	4	—	—	2	—
	4.	12	3	9	—	—	3	—	—	—	1	9	—	—	—	1
	5.	31	23	8	—	—	21	2	—	—	1	7	—	—	2	1
	6.	25	—	25	—	—	—	—	—	—	2	23	—	—	—	2
Total		87	30	57	—	2	26	2	—	1	4	52	1	—	4	**6**

Discussion

During the past decades, experimental tumors were transplanted from animal to animal with varying success. The importance of immune processes in the transplantation of leukaemias became known after various inbred mouse and rat strains had been raised [14, 22, 30, 39]. However, efforts had been made for transforming allogen and xenogen leukaemias inspite of these experiences [7, 11, 23, 27, 28, 29, 32]. Gross [11] inoculated AKR leukaemic spleen cells into newborn C3H mice and observed that either early lymphosarcoma or late leukaemia developed in the animals. After retransplantational experiments it was found that lymphosarcoma was of the donor, while leukaemia was of the recipient cell type. Salaman et al. [29] had similar findings. In 1956, Furth et al. tried to synthetize the results [8]. They reached the conclusion that after transplantation the leukaemias induced chemically or by radiation may be of donor as well as of recipient cell type. Later, experiments were carried out with hybrid mice [29, 34] and it



a) b)

Fig. 7. Karyograms from leukaemic rat; Rat karyogram; Mouse karyogram

was found that the leukaemia which developed in F_1 hybrids could be reinoculated into the parental strains and when inoculated again into hybrids, they may be either of donor or of recipient type.

Shay et al. were among the first to work with xenogeneic systems and to inject lymphoid mouse leukaemia induced by methylcholantrene into newborn Wistar as well as Long-Evans rats [32].

Kirsten et al. [15, 16] had transplanted AKR leukaemia into Wistar rats. Early leukaemia was observed in some of the animals after 15–20 days of incubation, while in the other group late leukaemia was observed after 13–30 weeks of incubation. The early leukaemia could be retransplanted only into AKR mice, while the late one only into rats. The early leukaemia was thought to be caused by the multiplication of mouse cells, while the late one by the proliferation of rat cells induced by Gross-virus. In 1972 Goldenberg successfully transplanted L_{1210} leukaemia into the cheek-pouch of Syrian-golden hamsters [9].

To investigate the origin of allogeneic and xenogeneic leukaemias in transplantation systems, the possibility to analyze the chromosomal count had come up early. Foca et al. [6] transplanted chemically induced mouse sarcoma into rats, and gradual adaptation of the tumor was detected by chromosome examinations. Goldenberg [9] found L_{1210} karyotype and hamster karyotype cells in the metastasizing leukaemia.

Chromosome investigations may lead to different results even in syngeneic systems, on the basis of experiments with sexual or marker chromosomes. Kodama and his coworkers found that characteristics of the donor are decisive after transplantation [21], whereas solely the recipient feature was proved by Selypes and Kertai [31]. In 1981, in experiments with Moloney leukaemia, Spira and his coworkers found that after transplantation of trisomic and non-trisomic cells, thymus and bone marrow cells can induce donor type, while spleen cells can produce donor and recipient type leukaemias as well [33].

Summing up the results of our experiments, we can say that U-867 type mouse leukaemia when successfully injected into newborn rats induced there a chimeric form of lymphoblastosis (leukaemia) of rat origin.

There are two possible explanations for these findings:

1. Chemically activated viruses had been injected into the newborn rats together with the mouse leukaemic cells, and these led to the development of leukaemia within an unusually short time.

2. According to our hypothesis, some kind(s) of humoral differentiational factor(s) is/are required for the maturing of rat lymphoblasts. These factors may be bound by the injected mouse-leukaemia blasts, so that without these hypothetic factors the non-matured host lymphoblasts of rats are unable to differentiate, they can only reproduce themselves, overflowing the organism.

Further experiments are needed to clarify the real cause of these observations.

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Primary Lymph Node Plasmocytoma of IgD-Class

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An extramedullary plasmocytoma in a right inguinal lymph node was observed in a 62-year-old woman. The tumor showed monoclonal proliferation of plasma cells which revealed highly positive stainings of both IgD and lambda light chain using the PAP-technique. Monoclonal IgD-lambda was also secreted in serum and urine. Clinical data, lymphography and bone biopsy showed a tumor spreading in the right inguinal, left iliacal and lumbar regions without systemic involvement of bone. In contrast to extramedullary plasmocytomas of other immunoglobulin classes the disease progressed rapidly and the patient died 8 months after the onset. The present case is the first reported IgD-plasmocytoma in lymph nodes.

Keywords: IgD, lymph node, plasmocytoma

Introduction

IgD myeloma is beside the IgE, IgM and non-secreting plasmocytoma a rare variety of the disease (0.25–2.5%). Rowe and Fahey [1] published the first case in 1965, and since then 286 patients with IgD plasmocytoma have been described [2, 3]. Diagnostic difficulties, in particular unusual symptoms and poor diagnosis are characteristic of this type of myeloma. According to the Kiel classification, the lymph node plasmocytoma is an extramedullary one, which develops primarily in lymphoid tissue. Within the group of NHL diseases this is a rare one (0.8%) [4]. A primary lymph node plasmocytoma of IgD-type has not yet been reported, and we describe the first case of an IgD-lambda-lymph node plasmocytoma. An unusual clinical picture and the poor prognosis enriched our current knowledge of paraprotein secreting lymphomas.

Case Report

Clinical and laboratory observations

B. G., a 62 year old female was admitted to a district hospital in September, 1984, with weight loss and swelling of the right leg. In April, 1984, she had noticed a tumor in the right inguinal region for the first time. In July a needle biopsy

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showed tumor cells of unknown origin. On admission to the hospital a right inguinal tumor (10 × 5 cm size) was visible. Partial surgical removal was done, because the infiltration into the retroperitoneal space and minor pelvis was evident. Histological examination by means of Giemsa-, PAS-, Hematoxylin-eosin, Gomori-staining showed a lymph node plasmocytoma. Immunohistochemical investigations with monospecific anti-H and L-chain sera suggested the diagnosis by the detection of monoclonal plasma cells of IgD-lambda specificity (Fig. 1).

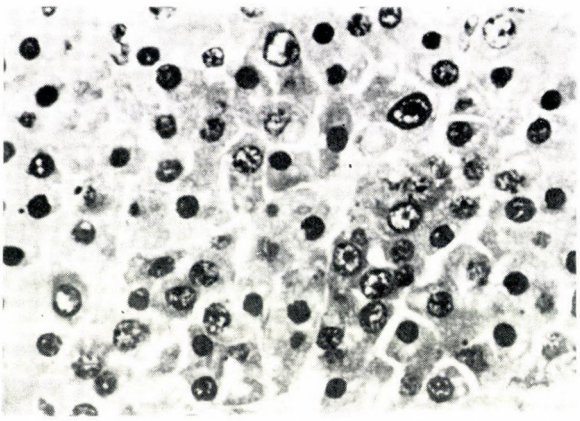


Fig. 1. Lymph node biopsy obtained from an inguinal tumor. Staining with Giemsa solution. Note the dense infiltration by plasmocytoma cells in different mature stages. (Original magnification × 832)

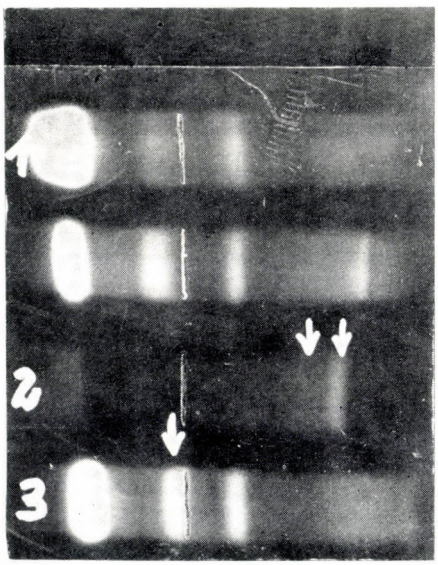


Fig. 2. Agargel electrophoresis in patient B. G. with IgD-plasmocytoma. 1 normal serum, 2 patient's urine, 3 patient's serum. Note monoclonal bands (arrows)

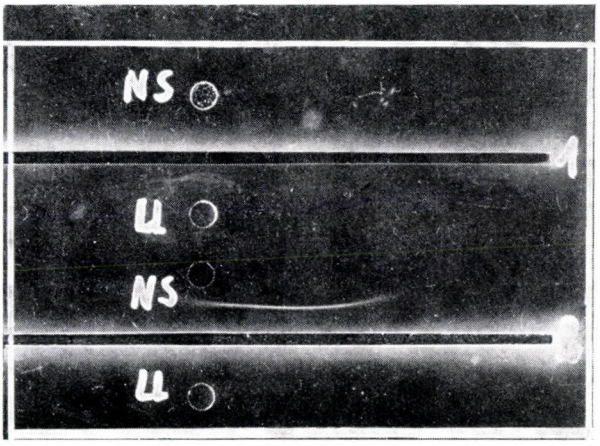


Fig. 3. Immunoelectrophoresis with anti-IgD (1) and anti-IgA (2) in troughs
NS normal serum; U patient's urine. Note prominent IgD arc with urine of patient B. G.

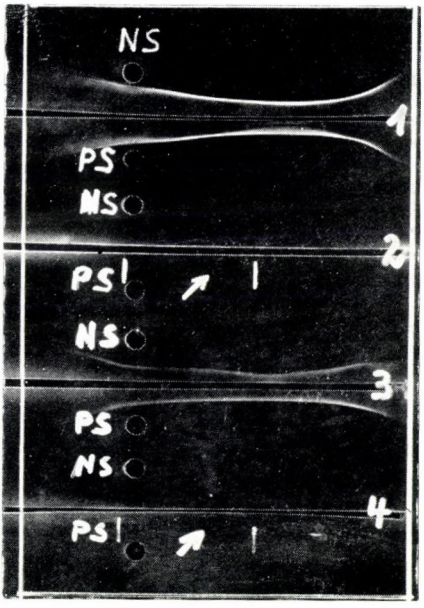


Fig. 4. Immunoelectrophoresis with different anti-immunoglobulins in troughs
1 anti-IgG; 2 anti-IgD; 3 anti-kappa; 4 anti-lambda; NS normal serum; PS patient's serum;
Note prominent arcs with anti-IgD and anti-lambda (arrows) of patient's serum (B. G.)

The patient was first seen by one of us (HST) in November, 1984. She reported weight loss, leg pain and weakness. The whole right leg was swollen. Two tumors (8×5 cm and 3×4 cm in diameter) could be observed in the right

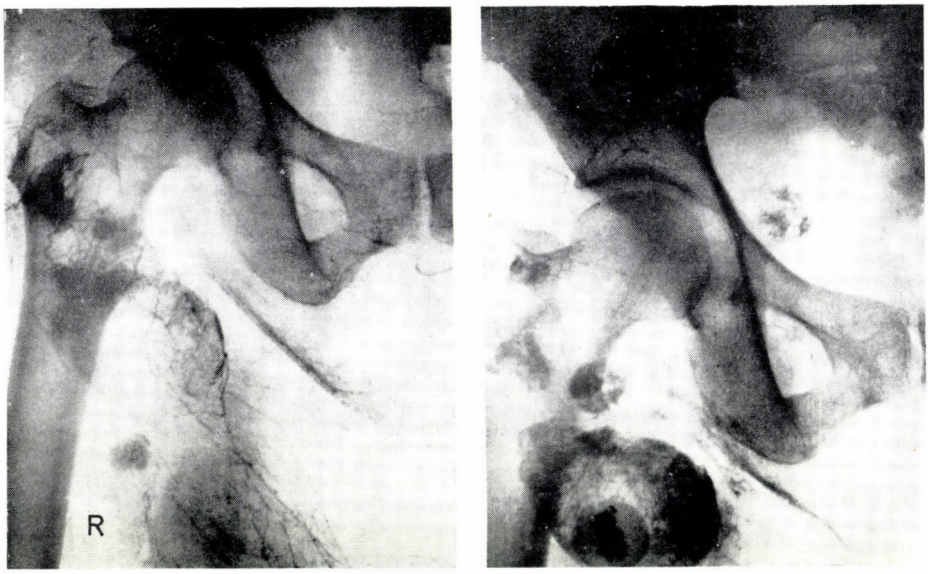
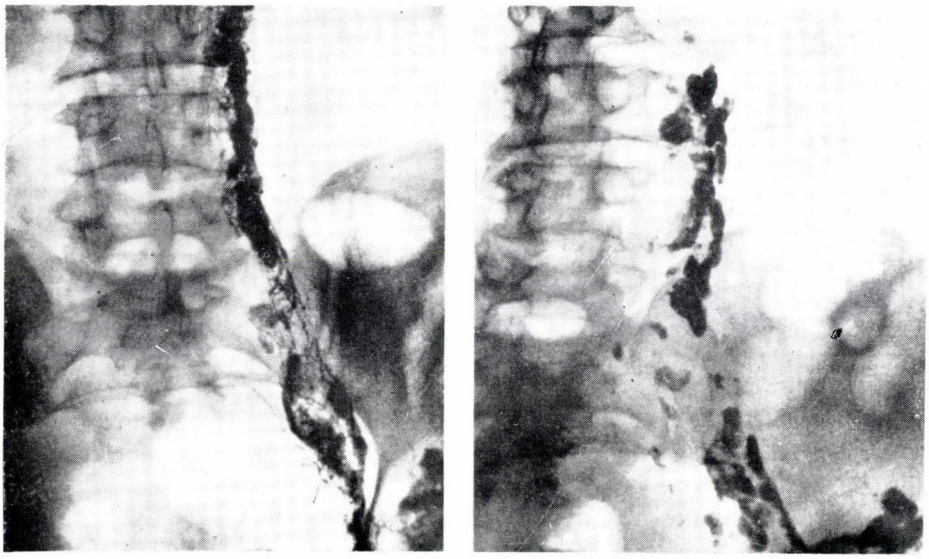


Fig. 5. Lymphogram of the right regio inguinales of patient B. G
a) Secondary lymphedema in the upper leg with filling of lymph vessels in the regio pubis; b) 24 hours later. Note the marked enlarged inguinal lymph nodes with numerous disseminated and lacunar filling defects. Consume of parenchyma and filling defects in external iliacal lymph nodes above the femur head

inguinal region. An X-ray survey indicated the absence of lytic lesions in the skull, thorax, spine and leg bones. Laboratory data showed a hemoglobin level of 8.1 mmol/l; WBC 6.4×10^9/l. The blood smear showed rouleau formation, with normocytic and normochromic RBC, but a normal differential count of WBC and platelets. The serum creatinine value was 160 μmol/l, the blood clotting tests and serum electrolytes were normal. The total serum protein and serum albumin values were 74.5 and 42.4 g/l, respectively. Agar gel electrophoresis in serum showed a dense, monoclonal band in the α_2 region and the fast γ-region. In urine electrophoresis 2 monoclonal bands in the slow γ-region were detected (Fig. 2). Immunoelectrophoresis demonstrated prominent precipitin arcs of IgD and lambda-chains in urine and serum (Figs 3 and 4). Serum immunoglobulin determination gave the following values: IgG 10.0 g/l; IgA 2.4 g/l; IgM 0.5 g/l; IgD 6250 U/ml. Trephine biopsy showed a hypoplastic marrow with a small infiltration of plasma cells of IgA, IgG, IgM, kappa and lambda light chains containing cells. Bipedal lymphography was performed with Lipoidol 10 and 8 ml, respectively. In lymphograms of the right leg marked abnormalities of the inguinal lymph nodes were seen with secondary lymph edema. The nodes showed extensive enlargment with lacunar and disseminated filling defects (Figs 5 and 6).



a) b)

Fig. 6. Bipedal lymphogram of patient B. G.
a) Filling phase. Normal left lymphogram a complete failure of contrast uptake in the right iliacal and lumbar nodes due to inguinal block; b) Storage phase after 24 hours. At L II left lacunar filling defects in lumbar nodes due to tumor infiltration. No contrast uptake on the right side

The lymphograms in the iliacal and lumbar region demonstrated, due to the inguinal block, no fillling on the right side but lacunar defects on the left, indicating involvement with tumor cells. Palliative radiation therapy of the right inguinal region was instituted in December, but no improvement was seen after 6 irradiations (total 18 Gy, 3 times 3 Gy per week). She developed progressive cardiovascular failure in the terminal phase and died January 1st, 1985, in a district hospital. No autopsy was performed.

Comment

Our patient had well-documented extramedullary plasmocytoma of IgD type with lambda light chains in the lymph nodes. A myeloma has been excluded by bone biopsy and skeletal X-rays. The case report illustrates the devasting course of an IgD-lambda-plasmocytoma. The time from first symptoms to death was 32 weeks. The poor prognosis of IgD myeloma with a mean survival of 13.7 month is well documented [2, 3]. In several aspects as younger age, predominate males, lymphadenopathy, hepatomegaly, splenomegaly, amyloidosis,

extraosseous spread, severe anaemia, low therapeutic response, azotemia and myelofibrosis this myeloma type differs from myeloma of other immunoglobulin classes [2, 3, 5].

Extramedullary plasmocytomas (EMP) are unusual tumors which occur most frequently in the upper airways and the oral cavity [6, 7]. The median survival is 100.8 months [8].

EMP in lymph nodes occur as metastases of myeloma disease [9] or in connection with EMP of the respiratory tract [7], and primarily in the lymphoid tissue of the lymph nodes. The sex ratio (males/females) is 2.6 : 1. Mostly the cervical and axillary, rarely other regions of lymph nodes are involved [4, 7, 10, 11]. Histological examinations show highly differentiated plasma cells without immunoblasts, plasmablasts and lymphocytes. The monoclonal immunoglobulins in plasma cells are of the $\alpha > \gamma > \mu$-type [11, 12]. Our first reported IgD case finished the chain, which is contrary to ontogenetic development. The prognosis of lymph node plasmocytoma can be evaluated better than in multiple myeloma [4, 13].

There is a remarkable preponderance of lambda type light chains in IgD myeloma (90%) and EMP, respectively, in contrast to their lower frequency in other monoclonal immunoglobulin classes [2, 3]. It was, however, this type that was detected in our patient. Comparison of patients who had IgD kappa type M-components with patients who had IgD lambda type did not show significant clinical differences [3, 14].

In the literature there exists an agreement that the poor prognosis of IgD myeloma is due to risk factors like azotemia, anemia and urinary light chain excretion more than the paraprotein type [2, 15, 16].

Our case demonstrates that the outcome may have been related to the IgD specificity. Another report with the same bad prognosis in an IgD EMP involving the sphenoid sinus [17] supports this suggestion. The direct unfavourable effect of the IgD paraprotein or the immaturity of every IgD B-cell clone are the possible reasons. The ontogenetic maturing process and the increment of incidence of the corresponding immunglobulin classes in lymph node EMP shows remarkable differences in the sequence of IgA and IgG. This is an argument for the different pathogenesis of both entities.

The prognostic significance of IgD paraprotein in malignant plasma cell proliferations at the onset of the disease seems to be of importance irrespective of a myeloma or an EMP in lymph nodes or other lymphoid tissue.

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Abstracts

The exchange of Fe^{3+} between pyrophosphate and transferrin. Probing the nature of an intermediate complex with stopped flow kinetics, rapid multimixing, and electron paramagnetic resonance spectroscopy. R. E. Cowart, S. Swope, T. T. Loh, N. D. Chasteen, and G. W. Bates (Department of Biochemistry and Biophysics, Department of Animal Science, and the Texas Agricultural Experiment Station, Texas A. and M. University, College Station, Texas, and the Department of Chemistry, University of New Hampshire, Durham, New Hampshire). *J. Biol. Chem. 261*, 4607 (1986).

A detailed study of the exchange of Fe^{3+} between pyrophosphate and human serum transferrin was undertaken to test the hypothesis of a generalized reaction route for exchange of Fe^{3+} between transferrin and chelators. The initial rate of Fe^{3+} transfer from pyrophosphate to apotransferrin-CO_3^{2-} is highly sensitive to the pyrophosphate to iron ratio with a maximal rate being observed at a ratio of 3 : 1, consistent with the presence of slowly reactive polymeric species at ratios less than 3 : 1 as revealed by EPR and kinetic measurements. At a ratio of 4 : 1 the reaction is distinctly biphasic. The rapid first phase results in the formation of an intermediate postulated as a mixed-ligand complex of the type PP_i-Fe^{3+}-transferrin-CO_3^{2-}. The intermediate has a distinct EPR spectrum and an absorption spectrum similar to that of Fe^{3+}-transferrin-CO_3^{2-}, but with a spectral maximum at 450 nm rather than 465 nm. The second phase principally arises from the slow reaction of polymeric iron-pyrophosphate with the apoprotein and has contributions from the breakdown of the intermediate formed in the first phase. The rate of formation of the intermediate shows a hyperbolic dependence on $NaHCO_3$ and apotransferrin concentrations, the latter suggesting a rate-limiting labilization of $Fe^{3+}(PP_i)_3$ perhaps to form species of the type $Fe^{3+}(PP)_2$, prior to attack by apotransferrin-CO_3^{2-}. Multimixing stopped flow spectrophotometry was employed to test the chemical reactivity of the Fe^{3+} to reduction at various times during the first phase. Surprisingly, a diminution of reactivity of 1000-fold was noted after only 2% of the first phase was completed, indicating a fast initial reaction which is not observed by normal rapid flow spectrophotometry. This initial reaction may involve the binding of iron-pyrophosphate to allosteric sites on the protein. The kinetics of iron removal from Fe^{3+}-transferrin-CO_3^{2-} by PP_i are consistent with a rate-limiting conformational change in the protein as proposed earlier.

A. Egyed

Regulation of intracellular iron distribution in K562 human erythroleukemia cells. E. Mattia, D. Josic, G. Ashwell, R. Klausner, and J. van Renswoude (Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development and the Laboratory of Biochemistry and Metabolism, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland USA). *J. Biol. Chem. 261*, 4587 (1986).

Following a pulse with ^{59}Fe-transferrin, K562 erythroleukemia cells incorporate a

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significant amount of ^{59}Fe into ferritin. Conditions or manipulations which alter the supply of iron to cells result in changes in the rate of ferritin biosynthesis with consequent variations in the size of the ferritin pool. Overnight exposure to iron donors such as diferric transferrin or hemin increases the ferritin level 2—4- or 6—8-fold above that of the control, respectively. Treatment with the antihuman transferrin receptor antibody, OKT9 (which reduces the iron uptake by decreasing the number of transferrin receptors) lowers the ferritin level by approximately 70—80% with respect to the control. The fraction of total cell-associated ^{59}Fe (given as a pulse via transferrin) that becomes ferritin bound is proportional to the actual ferritin level and is independent of the instantaneous amount of iron taken up. This has allowed us to establish a curve that correlates different levels of interacellular ferritin with corresponding percentages of incoming iron delivered to ferritin. Iron released from transferrin appears to distribute to ferritin according to a partition function; the entering load going into ferritin is set for a given ferritin level over a wide range of actual amounts of iron delivered.

A. Egyed

Potassium-chloride cotransport in resealed human red cell ghosts. P. B. Dunham and P. J. Logue (Department of Biology, Syracuse University, Syracuse, N. Y.). *Am. J. Physiol. 250*, C578 (1986).

Furosemide-inhibitable K influx is threefold higher in resealed ghosts of human erythrocytes than in intact cells. The enhancement is specific for K in that furosemide-inhibitable Na influx is the same in resealed ghosts and intact cells. The enhanced K influx resembles K-Cl cotransport in intact cells in that it requires Cl but not Na. N-ethylmaleimide (NEM), which stimulates furosemide-inhibitable K influx in intact cells, is without effect (or slightly inhibitory) in resealed ghosts. The failure of NEM to enhance the flux was not due to low ATP in the ghosts. These findings suggest that enhancement of the K flux in ghosts occurs by oxidation of membrane protein sulfhydryl groups, known to occur with lysis, the same sulfhydryl groups at which NEM acts by alkylation. This conclusion is supported by two observations: 1) dithiothreitol completely prevents the increase in K influx in ghosts; this agent inhibits both oxidation of sulfhydryl groups and alkylation of them by NEM; and 2) K influx in resealed ghosts is sensitive to changes in cell volume, just as it is in NEM-treated intact cells.

G. Gárdos

The partial reactions in the catalytic cycle of the calcium-dependent adenosine triphosphatase purified from erythrocyte membranes. D. Kosk-Kosicka, S. Scaillet, and G. Inesi (Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland). *J. Biol. Chem. 261*, 3333 (1986).

A preparation of purified erythrocyte membrane ATPase whose activation by Ca^{2+} is or is not dependent on calmodulin depending on the enzyme dilution used in the low dilution state for these studies. In appropriate conditions, the purified ATPase in the absence of calmodulin exhibited a Ca^{2+} concentration dependence identical to that of the native enzyme in the erythrocyte membrane ghosts in the presence of calmodulin. Accordingly, an apparent $K_d \cong 1 \times 10^{-7}M$ was derived for cooperative calcium binding to the activating and transport sites of the nonphosphorylated enzyme. The kinetics of enzyme phosphorylation in the transient state following addition of ATP to enzyme activated with calcium were then resolved by rapid kinetic methods, demonstrating directly that phosphoenzyme formation precedes P_i production, consistent with the phosphoenzyme role as an intermediate in the catalytic cycle. Titration of a low affinity site ($K_d \cong 2 \times 10^{-3}M$) with calcium produced inhibition of phosphoenzyme cleavage and favored reversal of the catalytic cycle, indicating that calcium dissociation from the transport sites precedes hydrolytic cleavage of the phosphoenzyme. The two different calcium dissociation constants of the nonphosphorylated and phosphorylated enzyme demonstrate that a phosphorylation-induced

reduction of calcium affinity is the basic coupling mechanism of catalysis and active transport, with an energy expenditure of approximately 6 kcal/mol of calcium in standard conditions. From the kinetic point of view, a rate-limiting step is identified with the slow dissociation of calcium from the phosphoenzyme; another relatively slow step following hydrolytic cleavage and preceding recycling of the enzyme is suggested by the occurrence of a presteady state phosphoenzyme overshoot.

G. Gárdos

Bacterial cytotoxins, amphotericin B and local anesthetics enhance transbilayer mobility of phospholipids in erythrocyte membranes. Consequences for phospholipid asymmetry. E. Schneider, C. W. M. Haest, G. Plasa and B. Deuticke (Abt. Physiologie der Medizinischen Fakultät der Rheinisch-Westfälischen-Technischen Hochschule, Aachen, Aachen, FRG). *Biochim. Biophys. Acta 855*, 325 (1986).

(1) Incorporation of the channel-forming polyene antibiotic amphotericin B and of cytotoxins from Staphylococcus aureus (α-toxin) or Pseudomonas aeruginosa into erythrocyte membranes results in a concentration-dependent enhancement of the flip rates of exogenous lysophosphatidylcholine. The flip rate is also enhanced by incorporation of tetracaine and dibucaine. (2) Removal of tetracaine and amphotericin B from the cells normalizes the flip rates. (3) In parallel to the enhancement of flip rates, α-toxin produces a loss of transmembrane asymmetry of both phosphatidylethanolamine and phosphatidylserine. (4) Pretreatment of cells with amphotericin or high concentrations (over 2.5 mmol · l^{-1}) of tetracaine, followed by removal of the perturbing agent by washing, produces a selective loss of the asymmetric orientation of phosphatidylethanolamine to the inner membrane layer, as evaluated by the accessibility of the lipid towards cleavage by phospholipase A_2. The extent to which asymmetry is lost depends on the time of pretreatment with amphotericin or tetracaine, indicating a limitation by the rate of reorientation of phosphatidylethanolamine to the outer membrane surface. (5) Evaluation of the accessibility of phosphatidylethanolamine towards cleavage by phospholipase A_2 in the presence of local anesthetics indicates accessible fractions much higher than those obtained after removal of the perturbant. In the presence of tetracaine, endofacial phosphatidylethanolamine seems somehow to become accessible to phospholipase A_2. Phosphatidylserine does not exhibit this peculiarity. (6) The results indicate that various types of perturbation of the lipid domain of the erythrocyte membrane may enhance the transbilayer mobility of phospholipids as well as destabilize the asymmetric distribution of aminophospholipids. However, as in instances reported previously, there is no tight coupling between transbilayer mobility and destabilization of asymmetry of the transbilayer distribution of phospholipids.

G. Gárdos

Transbilayer reorientation of platelet-activating factor in the erythrocyte membrane. E. Schneider, C. W. M. Haest and B. Deuticke (Department of Physiology, Medical Faculty, RWTH Aachen, Aachen, FRG). *FEBS Letters 198*, 311 (1986).

Platelet-activating factor (PAF) is a phospholipid, intermediate in its structure between diacyl- and lysophospholipids. The reorientation of this highly bioactive compound to the inner membrane layer of human erythrocytes after its primary incorporation into the outer membrane layer has been studied. Reorientation was shown to be a slow process with an initial rate of only 0.012 h^{-1}. It does not depend on energy supply. From the steady-state distribution of PAF between inner and outer membrane layers, after very long incubation times (40—50 h), a preference of PAF for the outer membrane layer analogous to that of endogenous lecithin is derived. Our data indicate that the process of simple transbilayer reorientation of PAF is probably too slow to account for very fast transmembrane signal transmission or for the fast uptake and metabolism observed in certain cell types.

G. Gárdos

Charge-independent effects of drugs on erythrocyte morphology. A. Nwafor and W. T. Coakley (Department of Microbiology, University College, Cardiff, U. K.). *Biomed. Pharmacol. 35*, 953 (1986).

The effects of chlorpromazine, tetracaine, indomethacin, barbitone and benzyl alcohol on human erythrocyte shape have been examined. Cationic and anionic drugs produced stomatocytes and echinocytes respectively as expected for cells in isotonic saline. Particular attention has been directed here to some features of drug induced morphology change which are independent of the charge of the drug. It was found that (i) the direction (increase or decrease) of the extent of morphological change as temperature was increased from 20 to 37°, (ii) the exposure time for maximum shape change (0—2 min) and (iii) the time course of cell morphology (0—30 min) were different for similarly charged drugs. The influence of low concentarions of the drugs on the thermal fragmentation patterns of the cells has been determined. A single index has been derived which allows comparison of the morphological effects of cationic and anionic drugs. It was concluded that, while the type (stomatocyte or echinocyte) of shape change observed was dependent on the charge of the drug, cell morphology at drug concentrations high enough to produce marked shape change at 37 °C was strongly influenced by charge independent drug-specific effects.

G. Gárdos

Stochastic response of human blood platelets to stimulation of shape changes and secretion. D. A. Deranleau, R. Lüthy, and E. F. Lüscher (Theodor Kocher Institute, University of Berne, Berne, Switzerland). *Proc. Nat. Acad. Sci. 83*, 2076 (1986).

Stopped-flow turbidimetric data indicate that platelets stimulated with low levels of thrombin undergo a shape transformation from disc to "sphere" to smaller spiny sphere that is indistinguishable from the shape change induced by ADP through different membrane receptor sites and a dissimilar receptor trigger mechanism. Under conditions where neither secretion nor aggregation occur, the extinction coefficients for total scattering by each of the three platelet forms are independent of the stimulus applied, and both reaction mechanisms can be described as stochastic (Poisson) processes in which the rate constant for the formation of the transient species is equal to the rate constant for its disappearance. This observation is independent of the shape assignment, and as the concentration of thrombin is increased and various storage organelles secrete increasing amounts of their contents into the external medium, the stochastic pattern persists. Progressively larger decreases in the extinction coefficients of the intermediate and final platelet forms, over and above those that reflect shape alterations alone, accompany or parallel the reaction induced by the higher thrombin concentrations. The excess turbidity decrease observed when full secretion occurs can be wholly accounted for by a decrease in platelet volume equal in magnitude to the fraction of the total platelet volume occupied by α granules. Platelet activation, as reported by the whole body light scattering of either shape changes alone or shape changes plus parallel (but not necessarily also stochastic) α granule secretion, thus manifests itself as a random series of transient events conceivably with its origins in the superposition of a set of more elementary stochastic processes that could include microtubule depolymerization, actin polymerization, and possibly diffusion. Although the real nature of the control mechanism remains obscure, certain properties of pooled stochastic processes suggest that a reciprocal connection between microtubule fragmentation and the assembly of actin-containing pseudopodal structures and contractile elements — processes that may exhibit reciprocal requirements for calcium — might provide a hypothetical basis for a rate-limiting step.

Ágnes Enyedi

Inhibitory action of cyclic GMP on secretion, polyphosphoinositide hydrolysis and calcium mobilization in thrombin-stimulated human platelets. S. Nakashima, T. Tohmatsu, H. Hattori, Y. Okano and Y. Nozawa (Department of Biochemistry, Gifu University

School of Medicine, Gifu 500, Japan). *Biochem. Biophys. Res. Comm. 135*, 1099 (1986).

The effect of cyclic GMP (cGMP) on human platelet activation was investigated, using its metabolically stable analogue, 8-bromo cGMP (8-bcGMP). Thrombin-induced serotonin secretion was inhibited by pretreatment with 8bcGMP in a dose-dependent manner. Production of inositol trisphosphate (IP_3), a Ca^{2+} releaser was inhibited by 8bcGMP pretreatment of platelets. Preincubation of platelets with 8bcGMP was without effect on the basal level of cytosolic free Ca^{2+}, measured by fluorescent indicator quin2, but suppressed its thrombin-induced enhancement independently of extracellular Ca^{2+}. These results indicate that cGMP may be implicated in phospholipase C activation and Ca^{2+} mobilization (both influx through the plasma membrane and efflux from internal stores) in thrombin-activated human platelets.

Ágnes Enyedi

Dissociation of Ca^{2+} mobilization from breakdown of phosphatidylinositol 4,5-bisphosphate in activated human platelets. M. Kito, H. Narita, H. Takamura, H. J. Park, T. Matsuura and K. Tanaka (Research Institute for Food Science, Kyoto University, Kyoto, and Kyoto Red Cross Blood Center, Kyoto). *J. Biochem. 99*, 1277 (1986).

The early breakdown of phosphatidylinositol 4,5-bisphosphate in human platelets stimulated by a threshold concentration of either collagen or thrombin was inhibited by 5 mM NaF through its inhibition of phospholipase C activity. However, 5 mM NaF did not inhibit Ca^{2+} mobilization due to the stimuli from internal stores, but it did inhibit the influx of extracellular Ca^{2+} through its suppression of thromboxane A_2 formation.

Ágnes Enyedi

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Dosage and administration

Ampoule content is diluted in 10 ml of physiological saline. Should be administered intravenously since s. c. or i. m. application may produce painful infiltration or tissue necrosis. Treatment can be in one cycle or in several.

Adults

Daily 0.8 mg/kg b. w. for 5 days, then 7–10 days pause; depending on the patient's tolerance, daily 0.5–0.8 mg/kg b. w. for a further 3–5 days, under check of the peripheral blood count. Dosage, if well endured, can be raised to between 0.8 and 1.0 mg/kg b. w. Given satisfactory results and absence of side-effects, treatment may be continued with the above dosages and administration intervals. The total quantity of Rubomycin must not exceed 25 mg/kg b. w.

Children

Daily 1 mg/kg b. w. for 5 days, then 7–10 days pause. If blood count remains unaltered, the next cycle may be started. Dosage as above to be given every other day for a 10-day period. If well endured, the dosage can be increased to 1.5 mg/kg b. w. After cycle 2 there should be an interval of 2–3 weeks. Given satisfactory results and absence of side-effects, treatment can be continued with dosages and administration intervals as in cycle 2, for a 3rd, 4th and 5th cycle.

Side-effects

Haematopoietic inhibition. Frequent leukocytopenia; less frequent thrombocytopenia. In the presence of leukaemia, when the first-day dosage has been administered, the leukocyte count in the peripheral blood drops rapidly, at a rate depending on the magnitude of the initial value. The thrombocyte count drops at a lower rate. Both continue to diminish for about 8–10 days after the end of the treatment. Occasionally the drug, particularly if overdosed, may cause nausea, vomiting, headache. To lessen these symptoms, it is advisable to give a Daedalon tablet before, and a dragée (25 mg) of Hibernal after, the Rubomycin dosage. Pronounced states of dyspepsia (constant nausea, vomiting, anorexia) should be dealt with by cutting the dosis or pausing for 24 hours after each administration. If buccal mycosis occurs, daily two Nystatin dragées should be given.

Warning

Stringent haematologic control is imperative. So are checks of the peripheral blood count (leukocytes, thrombocytes) during the treatment and the intercycle periods. If the leukocyte count was initially low or started under the first few injections to drop rapidly, blood checks should be performed every day. Acute leukaemia calls for fortnightly medullary examinations, or more frequent ones as need be. Leukocytopenia or thrombocytopenia owing to previous cytostatic therapy, should be heeded as warning that Rubomycin treatment must not be started sooner than two weeks after the blood count has been restored to normal.

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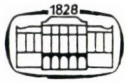

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Ferritin Expression in Transformed Human Haemopoietic Cell Lines

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(Received 25 January, 1986; accepted 10 February, 1986)

Isoferritins found in a number of human haemopoietic cell lines do not correspond to the isoferritins found widely distributed in normal tissues. It is suggested that these proteins may result from the expression of otherwise silent genes known to be present in the human genome.

Keywords: ferritin, haemopoietic cell lines

Ferritin is composed of a large protein shell, consisting of 24 subunits, with a molecular weight of 480 000 and a central core of variable amounts of a microcrystalline ferric hydroxide phosphate complex [1]. It is found in all mammalian cells, where its synthesis is usually stimulated by iron, though the amount of ferritin varies from tissue to tissue. On isoelectric focusing a number of isoferritins can be demonstrated, the range of isoelectric points (pI) depending on the tissue of origin [2]. For example, human liver has an isoferritin spectrum of about pI 5.3–5.8, whilst heart ferritin is more acidic with pI around 4.7–5.2 [3]. This variation is due to differing proportions of H and L subunits. Heart ferritin has a preponderance of H subunits and specific antibodies can be raised which recognise H rich isoferritins. The more basic tissue isoferritins have a preponderance of L subunits and again specific antibodies may be raised [4]. The role of ferritin as an iron storage molecule is well documented [1]. It has a capacity of up to 4500 atoms of Fe per molecule. The functional differences between isoferritins are not fully understood. In man both iron uptake [3] and release [5] are more rapid in the acidic isoferritins than the more basic forms. One might speculate that acidic isoferritins act as a short-term intracellular storage facility compared with more basic long-term storage molecules. The presence of appreciable amounts of acidic isoferritins in cells such as myocardium and erythroblasts [6, 7] where there is a large demand for iron for haem synthesis might support this view.

In addition to its function as an iron storage protein, ferritin is known to be synthesised in response to acute inflammation [8] and an increased level of synthesis has been found in leukaemic cells of patients with acute myeloblastic leukaemia [9] compared with normal leukocytes. Serum ferritin is composed largely of a glycosylated molecule [10] which is secreted proportionately to iron

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stores [11] though it is not itself rich in iron [12, 13]. Ferritin can also be detected on the surface membrane of erythroblasts [14], lymphocytes and monocytes [15]. Acidic isoferritins have recently been identified with a leukaemia associated inhibitory activity (LIA) which inhibits up to 50% of the growth of CFU-GM from normal human marrows [16–18]. In addition it has been suggested that acidic isoferritins derived from monocytes or macrophages constitute part of the normal regulatory mechanism for controlling haemopoiesis [19]. This concept is not without its problems in reconciling the experimental data with previous observations on the biochemistry of ferritin [20, 21]. Not all acidic isoferritins possess inhibitory activity, despite a high concentration of H subunits and it has been suggested [21] that the capacity of a specific isoferritin to inhibit colony formation may be attributed to unrecognised modifications of the molecule. Our own experiments have been unable to show any consistent inhibitory effect of purified acidic isoferritins of high H subunit composition on CFU-GM nor could we demonstrate any effect of purified glycosylated serum ferritin on CFU-GM cultures in vitro [22].

Cell lines

Ferritin expression has been studied in the promyelocytic cell lines HL60 [23–27, 29–31] and KG1 [31], the monoblastoid lines U937 [23, 24, 27, 28] and GDM [23], the erythroleukaemic lines K562 [23, 28, 32, 33] and HEL [28, 32, 33] and a lymphoblastoid line BEAUT [24]. We have grown eight other lymphoblastoid cell lines, NALM1, KM3 and REH (non T – non B), CCRF-CEM and HPB-ALL(T) and Daudi, Raji and Bristol 7 (B) in RPMI 1640 medium containing 10% fetal calf serum and varying amounts of iron (0–25 μg/ml) as in earlier studies [23, 34]. These were harvested at seven days, washed in isotonic saline, resuspended and heated at 70 °C for 10 min before cooling in ice and sonicating.

Ferritin expression

HL60 and U937 cell extracts reacted with spleen ferritin antibodies rather than antiheart ferritin resulting in low heart/spleen (H/S) ratios [23] whereas 3 strains of K562 [23, 28] and HEL [28] ferritins reacted largely with antibodies to heart ferritin (K562 H/S ratios: 3.3, 2.1 and 14.5). Thus it would appear that HL60 and U937 ferritins are predominantly basic (L-rich) while K562 and HEL ferritins are very largely acidic (H-rich). In HL60 ferritin the ratio of the H subunit to the L subunit, as detected by SDS polyacrylamide gel electrophoresis, depended on the ploidy of the cells [25]. Tetraploid (4C) cells synthesised more H subunit than a mixed tetraploid/diploid line (2C–4C) which in turn synthesised more than a diploid line (2C).

In six lymphoblastoid lines the H/S ratio was high (from 20 in Bristol 7 to

15.5 in Daudi cells) whereas in REH cells, where spleen-type ferritin was in very low concentration heart-type ferritin was not detectable and in Raji cells where both types of ferritin were in low concentration, the ratio was variable [34].

Response to iron and differentiating agents

In all cell lines iron induced the synthesis of ferritin though there was wide variation in the response. With ferric nitriloacetate (Fe NTA) supplemented medium the increase in ferritin concentration was about 10-fold in U937 cells and 2-fold with HL60 and K562 cells [23]. In the lymphoblastoid cell lines the increase was as high as 720-fold in HPB-ALL cells and as little as 2-fold with Bristol 7 cells [34]. With ferric ammonium citrate there was about a 30–40-fold increase in ferritin content in HL60 cells [24]. The H/S ratio was little affected by the increase in ferritin in lymphoblastoid cells except HPB-ALL where there was a small increase from 3.2 to 5.9 on iron loading [34]. In HL60 and K562 cells the rise in ferritin content was only detected with the spleen ferritin assay [23]. An increase in heart-type ferritin was produced only in U937 cells.

Differentiation of HL60 and U937 cells by retinoic acid [23, 24, 27, 30], phorbol esters [24, 26, 30, 31], dimethylsulphoxide (DMSO) [24, 27, 30], butyric acid [24], hexamethylene bi-acetamide [24] and dihydroxy vitamin D3 [26, 27] resulted in an increase in ferritin concentration up to 21 times the normal concentration [24]. In HL60 and U937 cells this was confined to spleen-type ferritin only [23]. Others [25], however, found an increase in H subunit synthesis on the differentiation of 2C, 2C–4C and 4C HL60 lines. Retinoic acid differentiation in one study failed to promote an increase in ferritin synthesis in HL60 cells though increases were observed with phorbol esters and DMSO [30]. In a variant HL60 line which has the capacity to proliferate after treatment with the differentiating agent phorbol-12-myristate 13-acetate or dihydroxy vitamin D3, there was no H subunit synthesised before induction or after de-induction [26] though induced cells contained ferritin with an H subunit. Iron and DMSO together resulted in a more rapid accumulation of ferritin than either agent individually [24]. Iron alone did not induce differentiation [23, 24]. The ferritin content of differentiated HL60 [23, 24] and U937 [23] cells (polymorphonuclear neutrophil and monocyte-like cells) was considerably higher than the equivalent circulating blood cells [35].

Anomalous immunological behaviour

In ferritins isolated from normal tissues there is a close relationship between H and L subunit content, immunoreactivity with heart and spleen ferritin antibodies and the elution pattern on anion exchange chromatography [6]. When a chloride gradient is applied the more H-rich isoferritins elute at a higher chloride

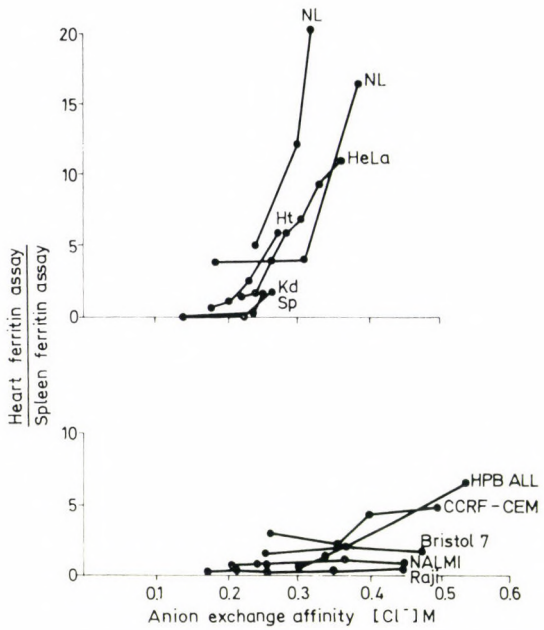

Fig. 1. H/S ratios from lymphoblastoid cell lines and other ferritins separated by anion exchange chromatography. The values for kidney (Kd) and spleen (Sp) ferritins are taken from Ref. 6. The HeLa cell values were kindly provided by Dr. B. M. Jones. Normal lymphocytes (NL) were prepared from peripheral blood by the method of Ali et al. [55]

concentration than the L-rich isoferritins. For example, myocardial ferritin which has a high H/S ratio elutes with the most acidic fractions at about 0.28–0.3 M Cl [36]. This was not the case with ferritin extracted from a number of cell lines. HL60 ferritin with a low H/S, eluted over a wide range of chloride concentrations, the most acidic being as high as 0.5 M Cl [23]. A similar but reverse discrepancy occurred with K562 ferritins. Lymphoblastoid cell ferritins displayed yet another pattern [34] with ferritin elution taking place over a wide range of chloride ion concentrations indicating a broad spectrum of isoferritins. In these cells the heart and spleen assays gave similar results for all the ferritin fractions as distinct from normal tissue ferritins where the more basic isoferritins were largely spleen-reactive and the more acidic ferritins heart reactive. The variation in H/S ratio in relation to isoelectric point is shown in the Figure (upper graph) for normal lymphocytes, heart, kidney, spleen and HeLa cell ferritins. The lower graph shows that ferritins from lymphoblastoid cell lines may not only be more acidic than normal but the H/S ratio does not increase in the same relationship to pI as in normal tissues and, in the cases of Raji, NALM 1 and Bristol 7, does not increase at all. KM3 (not shown) gives similar results to Raji.

Ferritin structure

Subunit analysis of HL60 [25, 37], U937 and K562 [37] ferritins shows two subunits of about 19 000 and 21 000 molecular weight and this is similar to the situation in tissue ferritins [38]. However, the L subunit from U937 ferritin differs by four amino acid residues from human liver L subunits as deduced from cDNA sequencing [39]. Only minor differences have been found in the L subunit of HL60 ferritin but in the H subunit an additional cytosine residue near the C-terminus (from cDNA sequencing) results in termination of peptide synthesis at residue 178 instead of 185 [29]. Such differences in ferritin structures will not, of course, be detected by subunit analysis.

The possibility of multiple gene products both for tissue [40, 41] and serum ferritin [10] has been discussed by previous workers. We would like to suggest that the anomalous immunological behaviour in transformed cells is due to the production of ferritin subunits not found in normal tissue and hence not recognised in the same manner by antibodies to heart and spleen ferritin. The demonstrated structural alterations in ferritin from two of the cell lines [29, 39] may well account for the unusual immunological behaviour of some of the ferritins. The recent genetic evidence for DNA coding of multiple L subunits in the rat and for as many as 15 genes coding for the H and several for the L subunits in humans, suggests a far greater potentiality for ferritin heterogeneity than has previously been suspected [42–46]. Presumably the majority of the genes are not expressed in normal tissues. HL60, K562 and seven of the eight lymphoblastoid cell lines were derived from malignant cells which transformed spontaneously in culture [47–49], while the Bristol-7 line was from normal lymphocytes which may have transformed spontaneously, though it was later found to be infected with EB virus (M. Summerell, personal communication). It seems possible that one effect of transformation may be to facilitate the expression of otherwise silent ferritin genes.

The existence of carcinofoetal or tumour ferritins has been postulated [50] and ferritin synthesis may be greatly increased in neoplastic disease, especially in leukaemias [9], the increase being reflected in raised serum ferritin levels [51, 52]. However, attempts to identify abnormal ferritins have been disappointing. Immunological assays for acidic isoferritins in serum have given highly variable results [51, 53, 54] and their value in diagnosis has not been established. The present findings warrant a reconsideration of ferritin gene expression in normal and transformed cells and in malignant tissue. Antibodies previously used might be failing to detect ferritin gene products expressed specifically in tumours. A more fruitful approach may be to use antibodies to the types of ferritin described here.

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Good Laboratory Practice Before and After Blood Transfusion*

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Les liens entre les deux disciplines, hématologie et transfusion, s'inspirant, s'éclairant, s'enrichissant l'une, l'autre [1].

Among the general principles that govern good laboratory practice before and after blood transfusion, there are two aspects which receive little attention: the importance of transfusion medicine as a distinct discipline; and the interrelationships within the hospital needed to make the "Blood Bank" effective.

Haemotherapy: a distinct clinical discipline

Directors of laboratories concerned with transfusion therapy do more than simply take charge of the internal technical and managerial functions. They are increasingly involved outside the laboratory in clinical decision-making related to transfusions. In some cases, they take charge of particular forms of therapy, such as therapeutic apheresis. They should, therefore, be regarded by their peers as the local consultant authority on all matters related to transfusion. Transfusion medicine, or to use the name which I prefer, haemotherapy, must now be accepted as a clearly-defined discipline that is an integral part of comprehensive clinical care. Because of this, the old term, "Blood Bank", is inadequate. "Blood Transfusion Laboratory" is a slight improvement. But the designation that to my mind is the most descriptive is, "Haemotherapy Unit".

Hospital Transfusion Committee

A Hospital Transfusion Committee [2] should be active in every hospital where transfusions are given. This committee should be a standing committee of the medical staff comprising the medical director of the Haemotherapy Unit and at least one medical member from each clinical discipline. Nursing and administrative staff should be represented as well. And if there is a regional transfusion centre nearby, the director of that should also be invited to attend.

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Table 1

Responsibilities of a Hospital Transfusion Committee

1. Establish and maintain a high standard of haemotherapy practice
2. Define appropriate usage for all blood products
3. Develop and monitor audit procedure for utilisation of each blood product with a view to avoiding indiscriminate use
4. Review new developments before introduction to avoid their use on inappropriate occasions or the selection of ineffective technologies
5. Ensure that approved developments fulfill their promise at a price that can be afforded
6. Approve standard operating procedures of the haemotherapy unit
7. Insist on an adequate system of post-transfusion surveillance

The Transfusion Committee has the very important function of establishing and monitoring the standard of haemotherapy practised within the hospital. It should also define appropriate usage for all blood products and develop an audit procedure for each. For example, with whole blood or red cells, the C/T ratio, the ratio of units crossmatched to units transfused, is a useful index. A C/T ratio of 1.0 is the ideal, but in practice a ratio of up to 2.5 for all surgical procedures is a reasonable goal [3, 4]. The activities of individual hospital departments, or even of individual physicians, can thus be compared.

"New and expensive technology, perhaps because of its seductive nature, tends to creep into use in a haphazard way without formal validation." The Committee should, therefore, review new developments in the field of haemotherapy before they are introduced into the hospital, and weigh the possible advantages against the impact on the regional blood supply and on available funds. The committee must, in other words, accept responsibility for avoiding indiscriminate use of available technologies or of introducing ineffective technologies. Above all, it must ensure developments fulfill their promise at a price that can be afforded.

Every hospital has some form of quality management and a policy of quality assurance. These may not be spelled out in formal terms, but nevertheless they do exist. No function of a hospital takes place in isolation; each function interfaces or inter-relates with one or more of the others. For that reason, Hospital Transfusion Committees should approve the Standard Operating Procedures of the Haemotherapy Unit, because these necessarily affect many other departments within the hospital.

The interface between bedside and laboratory is very important. All too often it is neglected. There is a distinct danger of divided responsibility. Close communication with nursing and medical staff and clear lines of authority are essential to ensure that errors are minimal, storage of blood products is optimal, pooling or reconstitution of products is correctly performed, and reactions to transfusions are looked for and reported to the Haemotherapy Unit. This is in an area of potential conflict where the hospital transfusion committee can play a very important role.

This committee, particularly in a small or isolated hospital, may prove ineffective. A regional group can play a very important role in correcting such a deficiency, despite the fact that each hospital probably has its own unique problems. The regional group should be chaired by the Director of the Regional Transfusion Centre, if there is one, and comprise directors of all local haemotherapy units as well as selected physician-users. The tasks confronting the regional group are essentially those facing hospital transfusion committees. Emphasis, however, should be placed on strengthening the effectiveness of individual committees and on improving the inventory control of individual hospitals – if necessary by sharing the available supply – and thus reducing excessive outdating.

Blood Product Management

Control of Demand is a particular responsibility and concern of the director of the Haemotherapy Unit. It is one thing to have good Blood Product Management within the Unit so that there is maximal utilisation and minimal wastage. But Control of Demand means exerting an influence outside the Unit. One way to do this is through introduction of a Maximum Surgical Blood Request Schedule. Note that I use the word "request" rather than the original "order", as in Maximum Surgical Blood Order [5]. There is a significant difference between a request and an order. My choice of request is dictated by our mandate to use the donor's gift to the best advantage.

Use of a maximum blood request schedule for patients selected for elective surgery reduces significantly the number of units reserved for specific patients, and thus increases the inventory available for others in need. Shortages and wastages due to outdating, are also reduced – as, of course, is the laboratory workload. This approach may usefully be combined with a so-called "Type and Screen" policy [3] for patients facing elective surgery where there is less than about a 30% chance that blood transfusion will be needed. If no unexpected antibodies are detected, no "crossmatch" is done unless and until a definite need arises.

In surgical cases, use of autologous blood collected either by "salvage autotransfusion" or by "deliberate haemodilution" is another means by which demands upon the donor pool can be reduced [6]. But autologous transfusions requested by the patient for fear of disease transmission should be discouraged, and "direct donations" given to alleviate the same fear should not be accepted.

Control of demand is a complex issue. Blood transfusion can no longer be regarded as a private matter between physician and patient. Too many other people and too many other factors are involved. It must now be accepted that the director of the Haemotherapy Unit has a duty to intervene should demands be inappropriate and fail to meet regional guidelines.

Standard Operating Procedures

The quality of haemotherapy practised in a hospital depends to a great extent upon the existence of, and upon conformance with, Standard Operating Procedures, as well as upon the standard of human resources available. The scope of these human resources in turn depends, in large measure, upon the availability of adequate facilities for training, teaching, and continuing education, which all too often, unfortunately, are inadequate or even non-existent.

Standard Operating Procedures should provide details of every administrative, nursing, medical, and technical system and procedure, and define when each is to be used and who is authorised to perform them. They must be designed very carefully and subjected to regular review.

Responsibilities of a Haemotherapy Unit

Some of the responsibilities of the present-day Haemotherapy Unit which must be covered by Standard Operating Procedures are shown in Table 2. I have distinguished between the fundamental responsibilities of the Haemotherapy Unit and those which may be regarded as optional. Local circumstances will dictate whether any of the options are either feasible or desirable.

Table 2

Responsibilities of a 'Haemotherapy Unit'

Fundamental
Collection of samples
Control of demand
Internal quality control
External quality assessment
Pre-transfusion and pre- and post-natal tests
Storage and transportation
Post-transfusion surveillance
Optional
Collection and testing of blood donations
Component preparation
Management of supply
Quality control of products
Reagent production
HLA typing
Therapeutic apheresis
Cytapheresis

Quality Assurance

A good programme of Quality Control forms the cornerstone of high-calibre Standard Operating Procedures (Table 3).

Table 3

Key Steps in a Quality Control Programme

Laboratory design and staffing pattern
Procedures and instrumentation
External quality assessment
Control of incoming material

Design and staffing need a chapter all to themselves. I mention them here simply to remind readers of their importance in terms of a safe environment and a reasonable workload.

The procedures and the instrumentation to be used in the laboratory must be carefully designed or selected to meet all requirements. Next, the procedures have to be validated. That is to say, their accuracy, repeatibility, and sensitivity under working conditions must be established. No new procedure should be introduced just because it works well in another laboratory. Then, the procedures must be documented in detail so that they can be included in the Standard Operating Procedures Manual. This manual must be readily available to all staff throughout the hospital, technical, nursing, and medical.

To make sure that procedures are in fact being performed as well as possible, the laboratory should not depend solely upon its own internal scheme for quality control and performance evaluation, but join in some national or international External Quality Assessment Scheme, so that its output can be compared with those of its peers. Joining such a scheme is all too frequently an eye-opener.

Control of Incoming Material is often overlooked as a vital preventive measure in Quality Assurance. This covers everything from selection of blood donors to tests on fractionation products, diagnostic reagents, equipment and supplies. I cannot emphasise too strongly that all must be shown to meet predetermined standards before acceptance.

A word of warning. When dealing with plastic packs, blood products, or diagnostic reagents made in another country, it is important to verify that the product not only meets one's own national standards but has also passed all requirements of the country of manufacture. Lots rejected in one country can, unfortunately, be legally marketed in another country if that country's standards are lower or non-existent.

Diagnostic reagents are probably the most important products used in the laboratory. I refer especially to AB0 and Rh grouping sera and to antiglobulin serum, but I also include LISS solutions and saline under this heading.

The fact that reagents meet national standards does not mean that they will all work equally well in all laboratories. Agglutination tests are subjective. Therefore, before accepting a particular lot from a particular manufacturer – or from one's own production department – comparative tests must be done using other lots from other sources. What one's own trained staff prefer is what counts. This applies particularly to antiglobulin serum.

Such staples as LISS solutions and normal saline must also be controlled daily and regularly monitored for accuracy. LISS solutions, particularly, may vary from manufacturer to manufacturer. In either instance, it is far better to prepare both solutions in one's own laboratory from the purest available chemicals.

Quality Control in the Haemotherapy Unit

There are three main factors:

1. *Quantitative adequacy.* Blood products must consistently meet predetermined standards of quantity. A certain proportion of units of red cells, platelet concentrates and other cellular components should be tested each month to make sure that they meet certain specifications, and that the units are sterile. Such steps should be undertaken by the producing laboratory. If that is a regional transfusion centre, then its findings should be communicated regularly to all the hospitals which it serves. The same general remarks apply equally to fresh frozen plasma, cryoprecipitate, and similar products.
2. *Functional efficacy.* Proof of quality is needed: the blood product must function as expected in vivo. A number of in vitro tests on stored cells are useful indicators, but generally speaking, in vivo survival or recovery is the key criterion. Here, it is possible for the hospital Haemotherapy Unit to help the regional transfusion centre by providing the necessary resources and sharing the information obtained.
3. *Transfusion hazards.* We must try to make transfusions as safe as possible. There are three main types of transfusion hazard: risks from viral, bacterial, or parasitic contamination; human error; and immunological risk.

   a. *Transmission of disease* is a threat of which we are all very conscious. It is probably not cost-effective in most countries to screen donations for syphilis. We continue to do so for aesthetic rather than for any sound scientific reasons. In fact it has been suggested that it would be more realistic to screen recipients several months after transfusion, since most cases of transmitted syphilis have been from sero-negative donors.

   To reduce the chance of transmitting hepatitis or AIDS, one factor is important: the privilege of acceptance as a blood donor should be restricted to low-risk groups and to those who complete a confidential self-exclusion form which permits them to exclude themselves without stigmatisation or embarrassment [7].

Tests for hepatitis B surface antigen by EIA or RIA should be obligatory on all donations. A positive screening test should be repeatable and specifically neutralisable before the donor is permanently excluded.

Detection of a proportion of possible AIDS carriers by means of tests for anti-LAV are already mandatory in some countries. Unfortunately, the precise significance of a positive test is uncertain. A repeatable positive result, confirmed by immunoblotting, indicates that the donor has been exposed to the AIDS virus and has mounted an immunological response, but it does not indicate with certainty that the donor is currently a carrier and that his blood is infectious.

Blood that does not contain anti-CMV is being requested more and more for the immunosuppressed and for neonates. Indirect haemagglutination and EIA tests are readily available commercially. However, such screening only reduces, does not eliminate, the chance of CMV transmission, since there is no specific test to detect the carrier state. This is a situation similar to that with LAV; in both instances, the donation may be seronegative but infectious.

b. *Human error* continues to be the greatest source of illness and death due to transfusions. Errors may occur in the identification of a donor unit, of a patient when a sample of blood is collected, of a blood sample itself, or of the patients needing transfusion.

Although laboratory tests may occasionally be incorrectly done or the results misinterpreted, clerical errors are by far the most common. Orderly and safe technical procedures are impossible without a sound administrative framework of checks and double checks.

Once a sound framework has been built, electronic data processing or EDP can be superimposed upon it. But make no mistake, EDP is not a substitute for sound managerial and technical procedures. EDP can improve efficiency and accuracy, but at a price. The question to be answered is what needs to be automated, not what can be automated. So EDP is not something that should be lightly entered into.

c. *Immunological risk*

> "*Good housekeeping means providing for one's needs in the order of their importance, and allowing no money to be spent on whims and luxuries until all necessities have been thoroughly served.*"
>
> *George Bernard Shaw*
> "*The Intelligent Woman's Guide to Socialism, Capitalism, Sovietism and Fascism*", *1928*

I have quoted Shaw to emphasise that a good manual system is adequate for most purposes. The temptation of automated tests is a luxury which should be resisted until a manual system has been developed to its fullest and "all necessities have been thoroughly served". Actually, this quotation applies to all areas of the laboratory. It is surprising what good work can be done with a minimum of physical resources. It's the capability, integrity, and ingenuity of people that really count.

Tests before Transfusion

There is a reasonably well established ritual for serological tests on blood donations. But when we move to tests on pre-transfusion samples, there are a number of issues that need to be resolved [8]. First of all, what should we call these tests? We do *not*, in fact, crossmatch donor and recipient. And we do *not* provide compatible blood apart from the AB0 and Rh systems. What we do is test for the presence of unexpected alloantibodies. In other words, we look for incompatibility, which clearly describes the imperfections of this approach.

Another reason for avoiding the term 'compatibility test' is that we do not check for incompatibility between donor and patient platelets, lymphocytes, or granulocytes, nor among the plasma proteins, all of which may cause non-haemolytic reactions. However, I am going to continue to use the term "crossmatch" in this paper in order to avoid confusion.

Table 4

Aims of Tests before Transfusion

1. AB0 and Rh(D) group recipient
2. Verification of AB0 groups of donations
3. Auto-control (direct antiglobulin test)
4. Detection of alloantibody in the recipient's serum that might, or in fact does, react with the donor red cells

Table 4 shows the purpose of pretransfusion tests. Accurate determination of the patient's AB0 group is of great importance. Haemolytic transfusion reactions are usually due to some simple mistake such as an error in AB0 grouping. An essential step to minimise these and similar errors is to check the patient's transfusion record and to enter on the current requisition the AB0 and Rh groups and whether alloantibody has been demonstrated previously. Many errors can be prevented by this simple step.

A positive direct antiglobulin test is evidence of previously unrecognised autoimmune disease, immunological changes due to drug therapy, or recent alloimmunisation. The predictive value of the test is, however, low; it may, therefore, be omitted without undue risk to the patient [9].

Tests to detect alloantibody form the core of the pretransfusion procedure. They should be done only at 37 °C, because in vitro activity at that temperature is the hallmark of antibodies of possible clinical significance. No technique other than the indirect antiglobulin test with polyspecific reagent is needed.

Whether the direct test – the "crossmatch" proper – or the antibody screen provides the most security for the recipient, or whether both these tests are necessary, has been the subject of debate for some years and still continues to be a source of argument. The direct approach is, of course, the correct one and should

never be omitted. The antibody screen is an ancillary safeguard added to cover technical deficiencies. Until a satisfactory, objective, direct test is developed, I favour use of both test with the proviso that the "crossmatch" may be curtailed to an "immediate spin" technique if there is no time for an indirect antiglobulin test. This at least provides a final check on ABO incompatibility which is still the cause of over three-quarters of all fatal haemolytic transfusion reactions.

From Laboratory to Bedside

There are many factors between the laboratory and the bedside which can drastically lower the clinical effectiveness of blood products. Some of these are listed in Table 5. That is why I emphasised earlier that the Standard Operating Procedures of the Haemotherapy Unit should extend to this area. And that is why the Hospital Transfusion Committee should make sure that there is a single clear line of authority from the Haemotherapy Unit to the bedside.

Table 5

From Laboratory to Bedside: A Perilous Journey?

The Standard Operating Procedures Manual should cover:

1. Storage and transportation
2. Pooling or reconstitution of labile products
3. Warming blood units
4. Patient and product identification

Criteria for a "safe" Transfusion

Finally, a reminder of what a "successful" transfusion should accomplish:

> *"To be considered 'safe', a transfusion must provide, without untoward reaction, specific and effective components that have a reasonably normal survival in the patient. In addition, the transfusion must neither transmit disease nor jeopardise the safety of future transfusions."*

This definition, written 17 years ago [10], has yet to be improved. I think it should be placed in a conspicuous position in every Haemotherapy Unit.

Post-Transfusion Surveillance

After each transfusion, two questions need to be asked – and answered:

(i) *Did the patient suffer any undesirable effects?*
(ii) *Did the patient have an appropriate response?*

"*Undesirable effects*" are reactions to transfusion, either immediate or delayed. It follows that every transfusion recipient should be placed under surveillance during the post-transfusion period of hospitalisation. This surveillance should follow the protocol detailed in Standard Operating Procedures. Everyone involved with direct patient care should be instructed by the Haemotherapy Unit on the signs and symptoms of all undesirable effects that may follow transfusion, and on what to do when they occur. Increased awareness and a low threshold of suspicion should be fostered in all staff, nursing as well as medical.

Whether the response was adequate is a measure of the functional effectiveness of the product transfused, an important step in any programme of quality assurance.

An inappropriate response can be the first indication of an underlying immunological awakening in the form of antibody to red cells, platelets, or factor VIII. Such a happening may jeopardise the safety and effectiveness of future transfusions. Standard Operating Procedures should detail what tests should be done, and when.

Summary

The responsibility of what I have called the Haemotherapy Unit extends beyond the laboratory to the bedside. It is more than a bank for blood. It is more than a laboratory. It should be — but rarely is — a distinct unit with supervision of all who handle blood and blood products — and patient samples. Unified control of all events from arm to arm is the only way to ensure maximum security — and benefit — for the patient.

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Intra- and Extraerythrocyte pH at 37 °C and During Long Term Storage at 4 °C: ^{31}P NMR Measurements and an Electrochemical Model of the System

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A ^{31}P NMR method based on pH dependent variation of the chemical-shift-difference between the resonances of orthophosphate and methylphosphonate was used to measure simultaneously intracellular pH (pH_i) and extracellular pH (pH_e) during long term storage of erythrocytes; pH was determined at both 4 °C and 37 °C. An equation describing the equilibrium distribution of membrane-permeant ions was derived by consideration of the electrochemical and osmotic constraints in the RBC suspension.

Calculations using the model-equation and the measured pH_i yielded the Donnan ratio and therefore pH_0; the relationship between experimentally determined pH_i and pH_0 values was accurately predicted by the model. Sensitivity analysis of the model-equation revealed that the observed increase in transmembrane pH gradient during storage is principally due to the alteration of the total net charge of intracellular (poly)-anions.

Keywords: Donnan ratio, intracellular pH, electrochemical model, methylphosphonate, ^{31}P NMR, sensitivity analysis, stored erythrocytes

Introduction

In order to ensure acceptable survival of red cells on transfusion, adequate concentrations of adenine nucleotides must be maintained during storage [1]. Also, the efficiency of oxygen delivery to the tissues by RBC immediately after transfusion depends on the 2,3-DPG concentration in the stored cells [2]. With routine blood storage procedures the rapid disappearance of 2,3-DPG and the more gradual decline in glycolytic rate and ATP concentration are principally due to declining pH caused by progressive accumulation of lactic acid [3, 4]. However, the mechanisms of glycolytic control by protons at 4 °C, the storage temperature, have not been fully elucidated.

Although the intracellular pH (pH_i) directly influences biochemical reactions of the RBC, most previous investigations have relied on measurement of extra-

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cellular pH (pH_0) because measurement of the more relevant pH_i is technically difficult; it involves calculations based on the transmembrane distribution of weak acid-conjugate base pairs or glass-electrode measurement of pH in a concentrated lysate [5]. However, pH_i cannot be directly inferred from measured pH_0 values because protons are passively distributed across the RBC membrane in response to the chemical and electrical gradients that constitute the Donnan equilibrium [6]. Therefore the pH difference across the RBC membrane depends on a number of variables including the composition and pH of the suspending medium and the concentration and charge of the impermeant intracellular solutes.

In the present work we applied a new ^{31}P NMR method [7, 8] to measure simultaneously the pH_i and pH_0 of RBC suspensions throughout their 5 week storage period. The pH values were determined at 37 °C and 4 °C.

A mathematical model was developed for the system of stored RBC that we studied; it was based on the assumption of a negligible transmembrane osmotic pressure difference and electroneutrality in both intra- and extracellular compartments. The latter conditions apply to erythrocytes at electrochemical equilibrium with their suspension medium [9]. Equations similar to ours have been derived for blood under physiological conditions (37 °C, an open system and CO_2/HCO_3^- as the principal buffer, [10]). A theoretical study of the present type of system has been carried out by Werner and Heinrich [11], however, their predictions of metabolite and ion levels in RBC pertain only to cells stored at 37 °C. The development of our quantitative electrochemical description of RBC stored at 4 °C required experimental determination of storage-dependent variables; these included lactate, ATP, 2,3-DPG and inorganic phosphate (Pi) concentrations.

Our model successfully simulated the observed variations in transmembrane pH difference that occur at 37 °C and 4 °C and a "sensitivity analysis" of the model enabled the identification of those factors that most influence the pH changes.

Materials and Methods

Blood collection and storage

450 ml of blood from each of four volunteers attending the Red Cross Blood Transfusion Service, Sydney, was collected into 63 ml of 'citrate-phosphate-277 mM glucose' anticoagulant and after removal of 75% of the plasma, the RBC were resuspended in 63 ml of "additive solution" containing 2.0 mM citric acid, 20 mM trisodium citrate, 20 mM Na_2HPO_4, 123 mM NaCl, 20 mM glucose and 0.5 mM adenine [12]. These "modified-packed-red cell units" (MPRC) had a mean packed cell volume (PCV) of 0.55 ± 0.04. They were stored at 4–6 °C for 5 weeks with the aseptic removal of a 10 ml sample on the day of collection and then at weekly intervals; on these occasions the pH_i, pH_0, total haemoglobin concentration, PCV, mean cell haemoglobin concentration (MCHC) and the concentrations of intracellular and extracellular solutes were measured.

pH measurement using ^{31}P NMR

The recording of ^{31}P NMR spectra, calibration of chemical shifts to electrode-measured pH, and calculation of sample pH were all performed as described previously [8]. Calibration curves of electrode-measured pH versus the chemical shift (δ) of resonances of methylphosphonate (MeP, Alpha products, Danvers, MA, USA) and Pi were established in plasma-anticoagulant-additive solutions and concentrated lysates. pH versus chemical shift data were obtained at both 37 °C and 4 °C. The pH was calculated from the chemical-shift-differences ($\Delta\delta$) between the MeP and Pi peaks measured in each sample of stored RBC using the following equation,

$$pH = -\log_{10}[(-B \pm (B^2 - 4AC)^{1/2})/2A] \quad (1)$$

where,

$$A = K_1K_2({}^a\delta_2 - {}^a\delta_1 - \Delta\delta)$$
$$B = K_1({}^b\delta_2 - \Delta\delta - {}^a\delta_1) + K_2({}^a\delta_2 - \Delta\delta - {}^b\delta_1)$$
$$C = {}^b\delta_2 - {}^b\delta_1 - \Delta\delta$$
$$\Delta\delta = \delta_2 - \delta_1$$

and K_1 and K_2 represent 10^{pK1} and 10^{pK2}, respectively. The pK, and chemical shift values of fully protonated ($^a\delta_1$ and $^a\delta_2$) and completely ionized ($^b\delta_1$ and $^b\delta_2$) forms of Pi (subscript 1) and MeP (subscript 2) were estimated by non-linear least squares regression of a modified form of the Henderson–Hasselbalch equation onto the chemical-shift-calibration data for MeP and Pi, independently. Parameter values for the appropriate calibration curves were then substituted into the chemical-shift-difference formula (Eq. 1) for calculation of the pH of the sample.

Prior to chemical shift measurement, a neutralized solution of MeP was added to the stored RBC samples to a final concentration of 5 mmol/l. Samples were then warmed to 37 °C for 10 min to allow transport of sufficient amounts of MeP into the cells. Pi was already present in sufficient concentrations in the storage medium for its resonance to be prominent. Spectra were acquired initially at 37 °C then the samples and the ^{31}P NMR probe were both cooled to 4 °C for estimation of pH at the storage temperature.

Resonances of the phosphorus nuclei in the extracellular solution were completely suppressed by the addition of manganese chloride to an extracellular concentration of 90 μmol/l; extracellular MeP and Pi resonances were readily assigned using this procedure [13].

Estimation of RBC water content

The fraction of the RBC volume occupied by water, called the relative RBC water content (RCW), was calculated from the measured MCHC using the following equation:

$$RCW = 1 - 8.71 \times 10^{-3} \times MCHC.$$

This equation depends on the assumption that prior to withdrawal from the donor the RBC have "mean normal values" of MCHC (32.5 g/dl) and RCW (0.717) [14, 15].

The calculated RCW was used to convert the concentrations of intracellular solutes into units of mmol/(l RBC H_2O). A relative supernatant water content of 0.970, calculated from the water content of plasma [16], was used to express extracellular solute concentrations as mmol/(l H_2O).

Assays

2,3-DPG, ATP and lactate were assayed as previously [17]. Sigma Phosphorous Reagent (Sigma, St. Louis, MO, USA) was used to assay Pi. RBC cation concentrations were determined using a flame photometer (Corning, Model 430).

After centrifugation of each MPRC sample in a sealed tube at room temperature, supernatant albumin, Cl^- and total CO_2 concentrations were measured using a sequential multiple analyzer (SMAC, Technicon, North Ryde, NSW, Australia). The HCO_3^- concentration was calculated using the Henderson–Hasselbalch equation, from a knowledge of the total CO_2, the dissociation constants for carbonate [18] and the pH_0.

As the RBC membrane is impermeable to citrate [19], supernatant citrate concentration (21.5 mmol/(l H_2O) was calculated from the total citrate added in the anticoagulant and additive solutions.

Change in pK′ of citrate with temperature

pH change, as temperature was varied between 4 °C and 37 °C, was measured in an 80 mM sodium citrate solution using a Radiometer PHM82 meter with an automatic temperature compensator (Radiometer T701). As the pH of the solution (5.5) approximated the third pK′ of citrate, the recorded changes in pH were a measure of the temperature dependence of this pK′ [20].

Results

Measurement of pH in stored RBC

The estimated values of the variable parameters relating to the absolute chemical shift of MeP and Pi were determined independently for the two species and are recorded in Table 1. These values in conjunction with Eq. 1 were used for all subsequent NMR-based pH estimations.

Storage-related changes in pH_i and pH_0 were measured simultaneously in MPRC samples with ^{31}P NMR spectroscopy. Resonances due to MeP shifted to higher frequency, and those due to Pi to a lower frequency, as the storage period progressed; hence the pH of the contents of the packs decreased. As resonances from the intra- and extracellular phosphorus compounds were clearly

Table 1

Estimates of calibration parameters

Temperature	Resonance	Medium	n	pK′	$^{a}\delta$	$^{b}\delta$
37 °C	Pi	lysate	12	6.77 ± 0.04	1.543 ± 0.071	3.670 ± 0.009
4 °C	Pi	lysate	10	7.07 ± 0.04	1.807 ± 0.052	3.430 ± 0.005
37 °C	Pi	pl-add	11	6.72 ± 0.01	1.039 ± 0.012	3.607 ± 0.004
4 °C	Pi	pl-add	10	6.91 ± 0.03	1.173 ± 0.066	3.368 ± 0.003
37 °C	MeP	lysate	12	7.53 ± 0.02	25.131 ± 0.019	21.352 ± 0.055
4 °C	MeP	lysate	10	7.60 ± 0.03	25.166 ± 0.030	21.444 ± 0.032
37 °C	MeP	pl-add	11	7.53 ± 0.01	25.131 ± 0.003	21.352 ± 0.035
4 °C	MeP	pl-add	10	7.60 ± 0.01	25.192 ± 0.015	21.341 ± 0.014

The Henderson-Hasselbalch equation was modified to express pH as a function of the chemical shift of the NMR resonance of a titratable species:

$$pH = pK' + \log_{10} \frac{(\delta - {}^{a}\delta)}{({}^{b}\delta - \delta)}.$$

This equation was fitted by non-linear regression onto titration data comprising electrode-measured pH and the corresponding chemical shift of MeP or Pi relative to the 'external' phosphoric acid reference. In this way estimates of pK′, $^{a}\delta$ and $^{b}\delta$ and their standard deviations were obtained.

Values ± S.D.; n = number of data pairs; pl-add = plasma-anticoagulant-additive solution mixture.

resolved (see Fig. 1), it was possible to calculate pH_i from the chemical-shift-difference between intracellular MeP and Pi peaks using Eq. 1 and calibration data obtained from a concentrated lysate. The pH_0 was calculated from the shift-difference between MeP and Pi resonances from the external solution using the calibration curve for the plasma-anticoagulant-additive solution. pH values measured at weekly intervals for a single MPRC pack are presented diagrammatically in Fig. 2. In all the units studied, pH decreased more rapidly in the supernatant than in the cells causing a progressive increase in the pH gradient across the cell membrane. pH data recorded at weekly intervals from four MPRC packs, and from 16 other MPRC packs at various storage times are included in Fig. 3.

Dependence of pH on temperature

For each of 10 MPRC samples, pH_i was measured at 4 °C and was plotted versus pH_i measured at 37 °C. Regression of a line onto the data yielded the equation

$$pH_i \text{ at } 4\,^{\circ}C = (pH_i \text{ at } 37\,^{\circ}C) \times 1.107 - 0.257,$$

and a correlation coefficient (r) of 0.996. A second equation,

$$pH_0 \text{ at } 4\,^{\circ}C = (pH_0 \text{ at } 37\,^{\circ}C) \times 1.147 - 0.575,\ r = 0.999$$

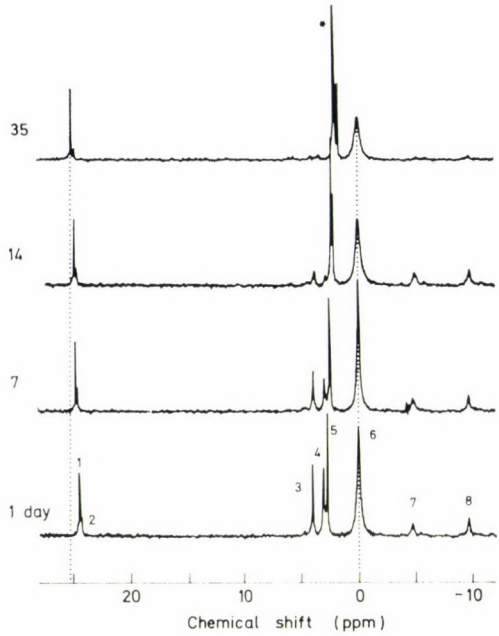

Fig. 1. *^{31}P NMR spectra of stored RBC.* Samples of RBC were removed from a single pack of MPRC after the storage periods indicated on the left of the spectra. Resonance assignments are as follows: 1, extracellular MeP; 2, intracellular MeP; 3 and 4, the 3 and 2 phosphate groups of 2,3-DPG; 5, extracellular Pi; 6, the 'external' phosphoric acid reference; 7 and 8, the γ and α phosphate of ATP and ADP. After 1 week of storage an intracellular Pi peak (* in the last spectrum) appeared. Note the rapid decline of 2,3-DPG followed by a slower decline in ATP and a corresponding increase in intracellular Pi with time of storage

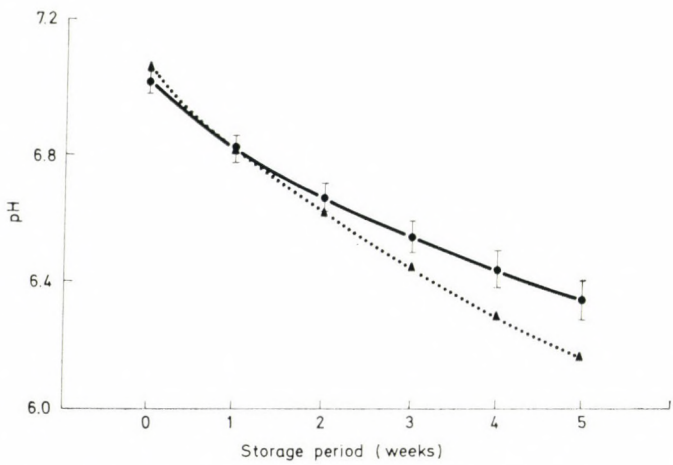

Fig. 2. *The effect of prolonged storage on pH_i and pH.* The pH values were determined using ^{31}P NMR at weekly intervals from a single MPRC unit. pH_i (●) and pHi (▲) were measured simultaneously in each sample at 37 °C. Error bars indicate one standard deviation calculated as previously [8] and the lines are visual fits to the data

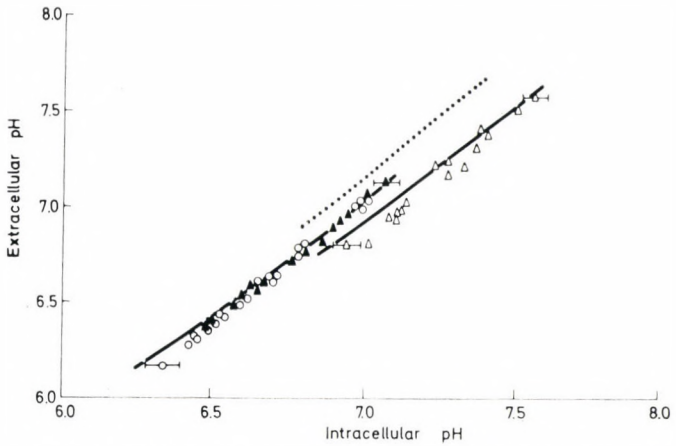

Fig. 3. *The relationship between pH_i and pH_0 in stored MPRC units.* The data points (○) correspond to pH_0 and pH_i values measured at 37 °C in samples taken at weekly intervals from four different MPRC units. Similarly, pH_i and pH_0 values were measured at 37 °C (▲) and 4 °C (△) in samples from 16 other MPRC units after various storage times. The solid lines represent pH_0 values predicted by the model (Eqs 5 & 8) at each particular pH_i attained during storage. Data points would be expected to fall along the dashed line (— — —) if pH_i and pH_0 were measured at 37 °C in fresh cells suspended in citrate-free plasma [16]

was obtained from pH_0 values determined at the two temperatures. The magnitude of the pH change with temperature was always slightly greater inside the cell than in the extracellular solution.

Concentration of intra- and extracellular solutes during storage

On the day of collection the MCHC of the MPRC ranged from 106% to 110% of the normal blood values; this corresponds to a decrease in cell water content of ~ 3%. The measured MCHC values remained within this range to the end of the 5 week storage period. Other storage-period-independent parameters included the intracellular haemoglobin content (7.64 ± 0.21 mmol/(l RBC H_2O)) and the supernatant albumin concentration (0.370 ± 0.057 mmol/(l H_2O)).

During storage at 4 °C the monovalent cations redistributed across the RBC membrane; intracellular Na^+ and K^+ concentrations were initially $21.3 \pm \pm 6.8$, and 140 ± 12.6 mmol/(l RBC H_2O) respectively. However, after 5 weeks the Na^+ concentration had increased to 46.5 ± 7.4 and K^+ had decreased to 101 ± 6.8 mmol/(l RBC H_2O), producing a small net decrease in the intracellular cation concentration. This was described by the equation:

$$[K^+ + Na^+] = 22.82 \times pH_i + 2.16, \; r = 0.933,$$

when the pH_i was measured at 37 °C.

Concentrations of Pi, ATP, 2,3-DPG and extracellular monovalent anions

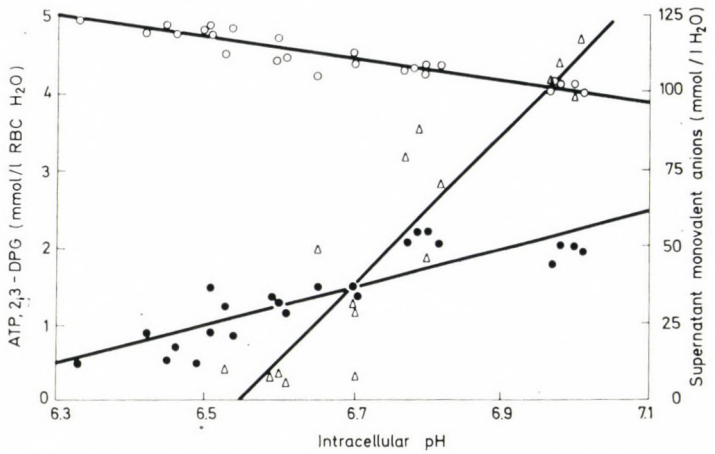

Fig. 4. *The relationship between storage-period-dependent solute concentrations and pH_i.* The total concentration of permeant monovalent anions in the supernatant (○), intracellular ATP concentration (●), and intracellular 2,3-DPG concentration (△), in each of four MPRC units were determined at weekly intervals during storage at 4 °C; these data are plotted versus pH_i measured at 37 °C. The "best fit" linear equations were determined: $a_0 = -44.2 \times pH_i + 412$, $r = -0.938$; $atp_i = 2.46 \times pH_i - 15.0$, $r = 0.861$; $dpg_i = 9.69 \times \times pH_i - 63.42$, $r = 0.924$; $pi_i = -17.2 \times pH_i + 128.4$, $r = -0.850$ (data not provided above)

all varied markedly with the duration of storage, as did the pH within each pack. In Fig. 4 the concentrations of three of the solutes mentioned above are plotted against the pH_i measured in the same sample; again, "best-fit" straight lines expressing the solute concentration as a function of pH_i were calculated. Changes in the supernatant anion concentration were dominated by progressively increasing lactate concentrations from 6.03 ± 0.60 to 44.8 ± 4.2 mmol/(l H_2O) over 5 weeks, whilst the Cl^- concentration decreased from 89.0 ± 1.0 to $83.1 \pm \pm 2.8$ mmol/(l H_2O) and HCO_3^- from 9.8 ± 1.1 to 1.7 ± 0.5 mmol/(l H_2O). Although there was no consistent variation in extracellular Pi concentration (10.6 ± 2.1 mmol/(l H_2O)) throughout storage intracellular Pi increased from an initial value of 7.1 ± 1.6 to 17.6 ± 2.0 mmol/(l RBC H_2O) at 5 weeks.

Change in pK′ of citrate with temperature

Regression of a line onto the experimental data, relating change in citrate-solution pH to absolute temperature, gave a slope of $(-1.772 \pm 0.108) \times 10^{-3}$ °C/pH unit and an r of -0.942. The pK′ of the third ionization of citrate at 37 °C is 5.49 [21]; thus, the empirical equation

$$pK' = 6.039 - 1.772 \times 10^{-3}\,T,$$

where T is the absolute temperature, was used to calculate the 'effective' pK′ of citrate between 4 °C and 37 °C.

A mathematical model of the storage system

In order to identify the dominant physico-chemical characteristics of MPRC that are responsible for the changing relationship seen between pH_i and pH_0 during storage a mathematical model of the storage system was developed.

Development of the model: The stored RBC are assumed to be at equilibrium with their suspending medium; two conditions follow from this. First, electroneutrality must prevail in both extracellular and intracellular solutions. Second, there is a virtual absence of osmotic pressure difference across the RBC membrane. The solutes present in our stored MPRC which are considered to contribute significantly to the osmotic pressure and net charge are listed in Table 2. The charge contributed by each atom of Na^+, K^+ and Cl^- and by each molecule of lactate, was taken to be constant over the storage-pH range. Variable charge terms (Z1 to Z7) were used for the other solutes. Since the HCO_3^- concentration was calculated using sample-pH and the total carbonate concentration (assayed at weekly intervals), no further correction for variation of HCO_3^- with pH was necessary.

Table 2

Glossary of terms used in Eqs 2–6

Solute	Amount in the specified compartment (mmol)	Concentration in the specified compartment (mmol/l H_2O)	Proton charge per molecule	Osmotic coefficient
Monovalent anions; $Cl^- + HCO_3^- +$ lactate	A_i, A_0	a_i, a_0	−1	P
Monovalent cations; $Na^+ + K^+$	B_i, B_0	b_i, b_0	+1	P
Haemoglobin	HB_i	hb_i	Z1	Q
2,3-DPG	DPG_i	dpg_i	Z2	P
ATP	ATP_i	atp_i	Z3	P
Citrate	C_0	c_0	Z4	P
Albumin	ALB_0	alb_0	Z5	not included**
Inorganic Phosphate	Pi_i, Pi_0	pi_i, pi_0	Z6, Z7	P

* The subscripts i (intracellular) and 0 (extracellular) refer to the compartment occupied by a particular solute.

** As $alb_0 = 0.36$ mmol/l H_2O, its osmotic effect is negligible and so it was not included in the calculations; but albumin does make a significant charge contribution because of the high charge density per molecule.

Using the algebraic symbols defined in Table 2, and with V_i and V_0 as the water volumes in each compartment, the equality of net positive and negative charges in the intracellular solution is described by the equation:

$$B_i - a_i V_i + Z1HB_i + Z2DPG + Z3ATP_i + Z6Pi_i = 0; \quad (2)$$

and in the supernatant the electroneutrality condition is given by,

$$b_0 V_0 - a_0 V_0 + Z4c_0 V_0 + Z5alb_0 V_0 + Z7pi_0 V_0 = 0. \quad (3)$$

The total osmotic pressure inside the cell must equal that of the supernatant. Thus, assuming no difference in the chemical potential of water in the two compartments [22, 23] an "osmotic-balance" equation is written:

$$\frac{P\,B_i}{V_i} + \frac{Q\,HB_i}{V_i} + \frac{P\,DPG_i}{V_i} + \frac{P\,ATP_i}{V_i} + \frac{P\,Pi_i}{V_i} + Pa_i = Pa_0 + Pb_0 + Pc_0 + Ppi_0. \quad (4)$$

Combination of Eqs 2, 3 and 4 generated the expression

$$\frac{a_i}{a_0} = \frac{(b_i + Z1hb_i + Z2dpg_i + Z3atp_i + Z6pi_i)\left(2 + \frac{c_0}{a_0}(1 - Z4) - \frac{alb_0}{a_0}(Z5) + \frac{pi_0}{a_0}(1 - Z7)\right)}{2b_i + hb_i\left(\frac{Q}{P} + Z1\right) + dpg_i(1 + Z2) + atp_i(1 + Z3) + pi_i(1 + Z6)} \quad (5)$$

It is widely accepted that in RBC the passive exchange of permeant monovalent anions results in a steady-state in which the ratio of intra- to extracellular concentrations of each of the anionic species is equal to the Donnan ratio (r); i.e.,

$$r = \frac{a_i}{a_e} = \frac{[Cl^-]_i}{[Cl^-]_0} = \frac{[Lactate^-]_i}{[Lactate^-]_0} = \frac{[HCO_3^-]_i}{[HCO_3^-]_0}. \quad (6)$$

Although the RBC membrane is not freely permeable to OH^- and H^+ ions they are distributed rapidly through the transmembrane exchange of water and CO_2 and the high activity of cytosolic carbonic anhydrase [24]. Therefore

$$r = \frac{[OH^-]_i}{[OH^-]_0} = \frac{[H^+]_e}{[H^+]_i}, \quad (7)$$

and

$$pH_0 = pH_i - \log_{10} r. \quad (8)$$

Calculation of the transmembrane pH difference: Solute levels required for the calculation of r using Eq. 5 were determined; for some solutes the value was constant throughout storage while the remainder were expressed as linear functions of pH_i (see Fig. 4). The experimentally determined level of permeant-mono-

valent anions in the supernatant were used only for the evaluation of the ratios c_c/a_c, alb_c/a_c and pi_c/a_c. The osmotic coefficient of NaCl and KCl in solutions with an ionic strength approximating that in cells, and supernatant is 0.93 [25]; this value was also used for the other inorganic electrolytes and for ATP and 2,3-DPG. An increase in the osmotic coefficient of haemoglobin with increasing concentration was first reported by Adair [26]. We calculated the osmotic coefficient of haemoglobin for the appropriate concentrations using the polynominal,

$$Q = 1 + 0.0654\ hb_i + 0.025\ hb_i^2$$

fitted onto Adair's original data by Freedman and Hoffman [22].

In Table 3 the charge on each type of solute molecule is expressed as a function of the number of titrating groups per molecule, their pK′ values, the pH and a fixed charge component that does not vary over the storage-pH range. For each haemoglobin tetramer there are 32 titrating imidazole groups [30], the pK′ of each imidazole depending on its environment within the molecule [31]. Using a mean pK′ value for these imidazole groups and assuming an isoelectric point for haemoglobin of 6.62 at 37 °C [21], the fixed charge due to the ionized carboxyl groups on each haemoglobin molecule was calculated as −12.72. The same mean

Table 3

Equations used to calculate pK′ and proton-charge per molecule of titratable solute

Titratable groups	pK′	Literature Reference	Proton-charge per molecule
Imidazoles of haemoglobin	$pK' = 1.611 + 1595/T$	27	$Z1 = -12.72 + \frac{22}{a + 1}$
2 & 3 phosphates of 2,3-DPG	$pK'_1 = 8.260 - 217.5/T$ $pK'_2 = 8.027 - 219.6/T$	28,8	$Z2 = -3 - \frac{a_1}{a_1 + 1} - \frac{a_2}{a_2 + 1}$
γ phosphate of ATP	$pK' = 6.91 - 32.6/T$	8,29	$Z3 = -3 - \frac{a}{a + 1}$
Third ionization of citrate	$pK' = 6.039 - 0.00177T$	21	$Z4 = -2 - \frac{a}{a + 1}$
Imidazoles of albumin	$pK' = 1.611 + 1595/T$	27	$Z5 = -22.16 + \frac{16}{a + 1}$
Second ionization of Pi	$pK' = 6.214 + 166.1/T + 19.90 \times 10^{-4} \times T - 5.159 \times 10^{-6} \times T^2$	8,29	$Z6\ \&\ Z7 = -1 - \frac{a}{a + 1}$

$a = 10^{(pH - pK')}$ where pH is pH_i or pH_c depending on the compartment;
$a_1 = 10^{(pH - pK'_1)}$ and $a_2 = 10^{(pH - pK'_2)}$ when the pK′s of two titrating groups on the one molecule can be defined separately; T = absolute temperature. For the temperature dependence of the pK′ of citrate see Results.

pK′ value was used for the 16 titrating imidazole groups on each albumin molecule while a fixed charge of −22.16 per albumin molecule was calculated from titration data [32].

The pK′ values of Pi, the 2 and 3 phosphate groups of 2,3-DPG and the γ phosphate of ATP were previously obtained [8] using ^{31}P NMR measurements of concentrated RBC lysates. Only the third pK′ of citrate was used to calculate charge as the effect of the other two dissociation constants (pK′, 4.29 and 3.08; [21]) was negligible even at the lowest storage pH encountered. Similarly the first and third pK′s of Pi were disregarded. The expressions defining the temperature dependence of the relevant pK′ values were determined by the authors cited in Table 3 and apply for physiological ionic strength.

The charge per molecule of the extracellular solutes were calculated over the storage-pH range using the expressions from Table 3, and the results were plotted against pH. The equations of straight lines regressed onto these data pairs were:

$$\text{at } 37\,^{\circ}\text{C},\ Z4 = -0.1683 \times pH_0 - 1.8046,\ r = -0.982;$$

$$\text{at } 37\,^{\circ}\text{C},\ Z5 = -8.738 \times pH_0 + 44.87,\ r = -0.999;$$

$$\text{at } 37\,^{\circ}\text{C},\ Z7 = -0.5297 \times pH_0 + 2.1381,\ r = -0.998.$$

As Z4, Z5 and Z7 are functions of pH_0, Eqs 5 and 8 could not be solved directly. Instead, Donnan ratios (r) were calculated by numerical analysis using the Newton–Raphson iterative formula [33] on a Tektronix 4052 graphics computer. The use of linear functions to approximate the pH dependence of Z4, Z5 and Z7 greatly simplified the differentiation step essential for the Newton–Raphson procedure.

Temperature change: The pH of blood varies markedly with temperature. It was therefore essential to generate expressions for the experimentally determined relationship between pH_i and solute concentrations at both 37 °C and 4 °C. The linear functions describing solute concentrations in terms of pH_i at 37 °C were converted for application at 4 °C using our experimentally determined equation relating pH_i values at 4 °C and 37 °C.

Temperature-induced variations in the charge of solute molecules were included in the model by expressing each pK′ value as a function of temperature (see Table 3).

Verification of the model: After inclusion of solute concentration, charge and osmotic-coefficient data obtained for stored RBC, Eqs 5 and 8 were used to calculate the expected pH_0 at each pH_i value attained during storage. In Fig. 3 pH_0 values estimated using the model were compared with the measured pH_0 values. The fit of the model-predictions onto the experimental data is good for both 37 °C and 4 °C indicating that the model is a realistic electrochemical description of the MPRC storage system.

Discussion

When lysates or plasma-anticoagulant-additive solution mixtures were cooled from 37 °C to 4 °C, pK′ values of both the pH probe molecules, MeP and Pi, increased (Table 1). The $\Delta pK'/\Delta T$ of MeP for the change in temperature from 37 °C to 4 °C was -0.0021 ± 0.0010/°C while the corresponding $\Delta pK'/\Delta T$ for Pi varied with the composition of the solution from -0.0091 ± 0.0024/°C for lysates to -0.0058 ± 0.0012/°C for plasma-anticoagulant-additive solution. Both values differ from the $\Delta pK'/\Delta T$ of -0.0021/°C estimated by Phillips et al. [34] for Pi in solutions at physiological ionic strength. This discrepancy, however, is not surprising as the chemical shift of the Pi resonance is not solely determined by pH but is also affected by other temperature-dependent interactions including binding to cations such as K^+ and Mg^{2+} [35] and possibly binding to haemoglobin [36].

Labotka and Kleps [37] successfully measured pH_i in fresh, oxygenated RBC using ^{31}P NMR with a single probe molecule, MeP. We observed, however, that varying levels of oxygenation amongst stored RBC samples caused differences in magnetic susceptibility, which in addition to pH, influenced the chemical shift of a MeP resonance. Therefore, the chemical shift of MeP resonances were referenced to those of a second probe molecule (Pi) present in the same compartment; these chemical-shift-difference measurements are independent of variations in magnetic susceptibility [7, 8]. A further advantage of the use of the chemical-shift-differences between MeP and Pi was an increase in the sensitivity of the method at the low pH values of stored MPRC; this improvement is due to the lower pK′ of Pi compared with that of MeP (Table 1).

With NMR spectroscopy we have the unique advantage of being able to measure pH_i and pH_0 simultaneously in intact stored RBC. As the method is relatively simple and rapid, pH_i determinations on numerous samples are much more easily achieved than with alternative procedures. The results of ^{31}P NMR pH_i and pH_0 measurements for stored MPRC presented in Fig. 2 are in agreement with those of Tsuda et al. [38]. Tsuda used the transmembrane distribution of 5,5'-dimethyloxazolidine-2,4-dione (DMO) to determine pH_i during storage of ACD-whole blood. We have confirmed that an increase in pH difference across the RBC membrane occurs as the storage period progresses and that this is due to a much slower decline in pH_i compared with pH_0. The relationship between pH_0 and pH_i in stored RBC contrasts sharply with that observed when fresh RBC in plasma are titrated with acid (see Fig. 3). While pH_i is less than pH_0 in fresh cells under physiological conditions, the reverse is true in stored RBC; the difference is primarily due to the presence of extracellular citrate ions, which are added to prevent coagulation and which affect the Donnan equilibrium [6, 39].

The transmembrane pH difference is particularly significant when considering the oxygen affinity of stored cells. In many earlier studies (see [40]) the P_{50} for oxygen binding was measured at the storage pH and extrapolated to P_{50} at a standard pH using a "Bohr coefficient" of $\Delta \log P_{50}/\Delta$ pH that was determined

for fresh whole blood. Our results indicate that these Bohr coefficients cannot be applied accurately to citrated RBC under storage conditions because of the differing relationship between pH_i and pH_0 in fresh whole blood and citrated MPRC; this opinion has also been expressed by Bursaux et al. [41].

The progressive increase in pH difference across the RBC membrane during storage is the net result of simultaneous changes in the large number of variables that determine the Donnan equilibrium. It was, therefore, necessary to develop a mathematical description of each of the "effectors" including solute concentrations, charge density per molecule and osmotic coefficients, and their interactions within the physical and chemical constraints of the storage system, in order to explain the pH changes we observed. Although suitable for more general application, the model was developed specifically to describe MPRC during storage. In contrast to previous investigations [21], experimentally determined storage-related changes in solute concentrations, specifically changes in lactate, 2,3-DPG, ATP and Pi concentrations, were included in the calculations. The transmembrane pH distribution predicted by the model is in good agreement with experimental results, for both 4 °C and 37 °C (see Fig. 3).

"Sensitivity analysis" has been used previously for metabolic systems to determine the extent to which a particular enzyme or metabolite "controls" flux through a metabolic pathway [42, 43]. We have used a similar procedure to quantify the influence of storage-related changes in the concentration or charge of different solutes on the Donnan ratio. A fractional change in the ionization state or concentration of a solute (the general parameter v) is represented by $\Delta v/v$ and produces a fractional change in the Donnan ratio of $\Delta r/r$. The "sensitivity coefficient" assigned to a particular variable is defined in our system as $(\Delta r/r)/(\Delta v/v)$ and as $\Delta v \to 0$, this becomes $(\partial r/r)/(\partial v/v) = (\partial r/\partial v) \cdot (v/r)$. In order to calculate the values for the "sensitivity coefficients", partial derivatives of Eq. 5 with respect to terms listed in Table 2 were determined. The numerical values of the coefficients for the conditions prevailing on the first day of storage are presented in Table 4. Note that the Donnan ratio is most responsive to changes in factors with high absolute values of their "sensitivity coefficients". Thus those variables that change markedly in value during storage (i.e., $\Delta v/v$ is relatively large) and have large "sensitivity coefficients" are principally responsible for the storage-related changes in the Donnan ratio. The charge on the haemoglobin molecule (Z1) and the intracellular concentration of 2,3-DPG (dpg_i) are the two variables, from Table 4, that best satisfy both criteria and so are identified as the dominant effectors of transmembrane pH-change during MPRC storage. The intracellular cation and haemoglobin concentrations (b_i and hb_i) and the concentration of citrate in the supernatant (c_0) are also variable parameters with large "sensitivity coefficients". However, as values of b_i, hb_i and c_0 are constant throughout storage, or change only slightly relative to their initial value, the contribution by these effectors to the observed increase in the transmembrane pH gradient is comparatively insignificant.

Reeves [28] states that when considering temperature effects, the intra-

Table 4

Sensitivity analysis of Eq. 6 under the conditions prevailing on the first day of MPRC storage

+ Values for the Concentration and Charge of Impermeant Solutes, v.		Partial Derivatives of r* (Eq. 6), $\frac{\partial r}{\partial r}$.	Sensitivity Coefficients, $\frac{\partial r}{\partial v} \times \frac{v}{r}$.	Fractional Change in v, $\frac{\Delta v}{v}$.
	mmol/(l RBC H_2O)	(l RBC H_2O)/mmol		
hb_i	7.64	−0.0409	−0.31	0
dpg_i	4.41	−0.0258	−0.13	−1.00
atp_i	2.23	−0.0263	−0.06	−0.66
b_i	162	0.0029	0.48	0.09
	mmol/(l H_2O)	(l H_2O)/mmol		
c_0	21.5	0.0134	0.29	0
alb_0	0.37	0.0547	0.02	0
	proton-charge/ molecule	molecule/proton-charge		
Z1	−4.73	0.0490	0.23	1.53
Z2	−3.54	0.0283	0.10	0.11
Z3	−3.61	0.0143	0.05	0.09
Z4	−2.98	−0.0730	−0.22	0.01
Z5	−16.4	−0.0012	−0.02	0.39

*r (Donnan ratio) $= \frac{a_i}{a_0} = 1.001$

+ For the definition of terms see Table 2. The fractional change in v, $\frac{\Delta v}{v}$, is the change in v during 5 weeks of storage divided by the value of v at the beginning of the storage period.

cellular and plasma phases of fresh blood can be analysed as a single buffer system if $\Delta pH_i/\Delta T = \Delta pH_0/\Delta T$ and the Donnan ratio is independent of temperature. For each MPRC sample $\Delta pH_i/\Delta T$ and $\Delta pH_0/\Delta T$ were calculated for the temperature change between 37 °C and 4 °C and were found to vary as functions of pH. However at each pH value within the storage-pH range the temperature-dependent change in pH_i was greater than that in pH_0; for example $\Delta pH_i/\Delta T$ was -0.0149 ± 0.0011/°C and $\Delta pH_0/\Delta T$ was -0.0138 ± 0.0007/°C when the pH_i at 37 °C was 7.0. Our results are comparable with those of Tsuda et al. [38], who reported a $\Delta pH_0/\Delta T$ of −0.0151/°C for ACD-whole blood and an increase in transmembrane pH difference on cooling of the blood. Both sets of results indicate that for RBC suspensions, stored under blood-bank conditions, temperature change results in a redistribution of permeable ions across the membrane. Therefore, the storage system must be considered to be two separate buffer

solutions, each with independent buffering characteristics but interrelated by the capacity of protons and anions to redistribute across the membrane following pH changes in either compartment.

The variation of solution-pH with temperature is dependent on the $\Delta pK'/\Delta T$ and the relative buffering capacity of each species present [20]. As the buffering of blood in a closed system is dominated by the imidazoles of haemoglobin and the plasma proteins, changes in the pK′ of imidazole [pK′(Im)] with temperature are paralleled by pH changes of a similar magnitude throughout the suspension [27]; the reported $\Delta pK'(Im)/\Delta T$ is $-0.0186/°C$ (Table 3). If $\Delta pH_i/\Delta T$ equals $\Delta pK'(Im)/\Delta T$ then the net charge on the imidazole groups will be independent of temperature variation [27]. In MPRC suspensions the measured $\Delta pH_i/\Delta T$ was significantly less than $\Delta pK'(Im)/\Delta T$ because of the dilution of the plasma with anticoagulant and additive solutions containing Pi, and the presence of intracellular phosphate esters. The pK′ values of these phosphate buffers are relatively temperature insensitive compared with that of imidazole (see Table 3).

When MPRC are cooled, the pK′(Im) increases causing a similar but slightly smaller increase in pH_i. The altered relationship between pK′(Im) and pH_i must result in a change in charge on the haemoglobin molecules. In fresh MPRC the charge per haemoglobin molecule calculated using the model equations, is -4.7 at 37 °C and -3.4 at 4 °C. As the net charge on the impermeant groups in the supernatant (mainly citrate) is relatively unaffected by temperature change, permeant anions move into the cell as the temperature decreases. Thus, temperature-induced changes in the Donnan ratio caused by charge variations on the impermeant intracellular buffers produce the observed differences between $\Delta pH_i/\Delta T$ and $\Delta pH_0/\Delta T$ for MPRC suspensions.

In conclusion, the electrochemical model provides a quantitative explanation of the variations in transmembrane pH difference observed during prolonged RBC storage or due to temperature change between 37 °C and 4 °C. Thus, the model may facilitate the development of new suspension media that will optimize pH_i during storage of RBC at 4 °C and these investigations remain to be carried out.

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Hypercalcaemia in "Non-Secretory" Myeloma

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A patient is described with hypercalcaemia due to myelomatosis without paraproteinaemia or Bence-Jones proteinuria. Immunological investigations yielded some evidence of intra-cellular immunoglobulin production. Histological features suggested the secretion of osteoclast activating factor. Relevant experimental work in support of this interpretation is reviewed.

Keywords: hypercalcaemia, non-secretory myeloma, osteoclast activating factor.

Introduction

In most cases of myelomatosis paraprotein is present in the serum or Bence–Jones protein is detectable in the urine, or both. Cases in which these abnormalities cannot be detected by conventional laboratory methods have been termed "non-secretory myeloma" [1, 2, 3]; the incidence is of the order of 1% of all cases of myelomatosis [4, 5, 6]; little attention has been paid so far to the secretion of substances of other than immunoglobulins in such cases.

Case Report*

A 50-year-old female presented with malaise, lethargy and back-ache of 6 weeks duration. Clinical examination was unrevealing. Radiologically some osteoporosis in the lower thoracic and upper lumbar vertebrae was present. Focal lesions were not detected. Initial investigations showed haemoglobin 8.6 nmol/l, red cell count 4.2 T/l, leucocytes 5.2 T/l, neutrophils 0.68, lymphocytes 0.27, platelets 201 T/l; erythrocyte sedimentation rate (Westergren) 6 mm one hour; total serum protein 68 g/l, albumin 0.58 kg/kg, globulins $alpha_1$ 0.15 kg/kg, $alpha_2$ 0.09 kg/kg, $beta_1$ 0.088 kg/kg, $beta_2$ 0.056 kg/kg, gamma 0.109 kg/kg. Immunoglobulins IgA 1.31 g/l, IgM 0.52 g/l, IgG 9.6 g/l. IgD and IgE were not detected;

* Case reported to the Association of Clinical Pathologists of Great Britain, 1984.

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glucose 3.2 mmol/l, urea 4.2 mmol/l, creatinine 86 μmol/l, sodium 132 mmol/l, potassium 3.1 mmol/l, calcium 3.41 mmol/l, phosphorus 0.9 mmol/l and alkaline phosphatase 69 iul/l.

Evaluation revealed nothing to support a diagnosis of sarcoidosis, milk-alkali syndrome, metastatic disease or hyperparathyroidism. During the week following admission to hospital for those investigations it was noted that the serum calcium fell to normal levels. Coincidentally, without treatment other than bed-rest, her symptoms disappeared. She was discharged from hospital for out-

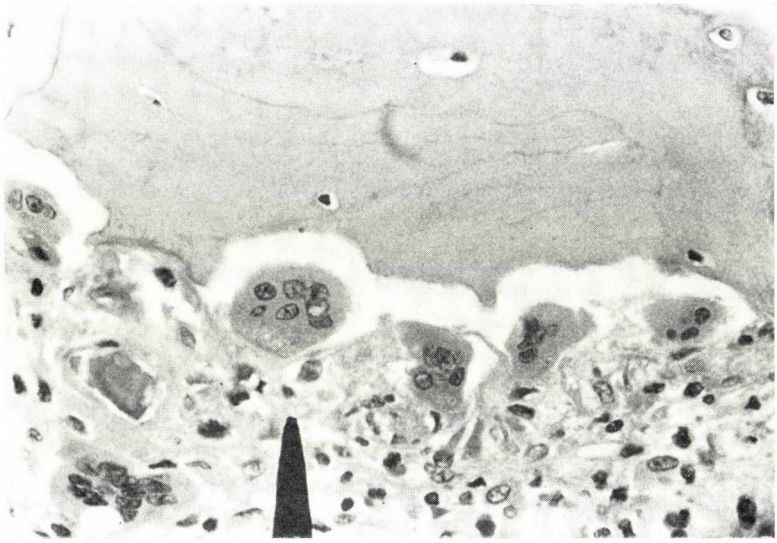

Fig. 1. Osteoclast activity in myeloma deposit in vertebra

patient review. Before that appointment fell due she needed re-admission because of sudden exacerbation of back pain. Hypercalcaemia had recurred. Clinical and laboratory review revealed no new information. Random sternal puncture was normal.

Repeat radiology now showed collapse of the twelfth thoracic vertebra and pathological fractures of the right 9th and 10th ribs. At this time disseminated carcinoma and non-secretory myeloma were considered. Repeat marrow biopsy (right iliac crest) was normal. The patient's condition deteriorated rapidly due to both hypercalcaemia and the onset of pneumonia which proved fatal.

Autopsy confirmed the terminal pneumonia, pathological fractures in the right 9th and 10th ribs, and collapse of the twelfth thoracic vertebra. The bones were very soft and the marrow grey in colour. Imprints were fixed in acetone at 4 °C, methyl alcohol and "Spray-cyte", and portions of the fractures, vertebrae and marrow fixed in 10% formol-saline. Other routine autopsy samples were taken into 10% formol-saline.

Special investigations

Immunofluorescent investigations were performed with mono-specific FITC-labelled immune antisera against heavy chains of IgG, IgM, IgA, IgD and IgE, and anti-kappa and anti-lambda light chains. (Specificity of reagents was confirmed by appropriate controls and blocking tests.)

Portions of marrow from the vertebral bodies, rib fractures and femur were fixed in 10% formol-saline, prepared for microtomy by a conventional programmed sequence in a "Tissue Tek" (Miles Scientific) V.I.P. processor, embedded in "paraplast" (Sherwood Laboratories) and sections cut at 4 microns. These were stained by haematoxylin-eosin, May-Grünwald-Giemsa, Perls' stain, and for reticulin. The significant findings were

1) replacement of the marrow with sheets of plasma cells of varying degrees of immaturity
2) large numbers of osteoclasts set in Howship's lacunae.

Occasional plasma cells only showed fluorescence with anti IgG and anti-kappa antisera. The majority of plasma cells in all fields failed to react.

Further preparations made from fresh tissues were stained by an immunoperoxidase technique [7]. This also demonstrated the presence of IgG, and kappa chains in some of the abnormal plasma cells, most being non-reactive.

Discussion

Hypercalcaemia is not uncommon in myeloma. In this patient two conventional marrow biopsies failed to reveal the diagnosis. This circumstance, coupled with the absence of serum or urinary protein abnormalities, the initial lack of focal skeletal lesions, and the rapid resolution of the hypercalcaemia with bed-rest alone, led to delay in diagnosis. Repeated immunofluorescent studies of the abnormal plasma cell infiltrate in several bone marrow sites demonstrated only scanty localised intracellular immunoglobulin. It was noted on histological examination that there was unusually intense osteoclastic activity in addition to the plasma-cell infiltrate. The ability to synthesise and secrete immunoglobulins is a normal property of plasma cells. Myeloma cells show differing capabilities in this respect, accounting for the major types of myeloma recognised (IgA, IgG, IgM, IgD, IgE) and the K and L sub-types. Most cases fall into one of these classes, but all may be associated with bone resorption of varying degree. In some cases the non-secretory nature of the myeloma has been shown to be due to failure of release, although actual synthesis of intra-cellular immunoglobulin was demonstrated [13], as in the present case.

The mechanism of bone resorption and hypercalcaemia in myeloma is not yet fully understood. It is clearly not simply due to pressure effects from the neo-

plastic cells since other infiltrative diseases of similar nature (such as the leukaemias) are only rarely so affected.

In Burkitt's lymphoma, however, there is often increased osteoclastic activity adjacent to the tumour cell masses in the jaw [8], and lymphoid cells derived from patients with both Burkitt's lymphoma and myeloma produce soluble factors that stimulate osteoclastic bone resorption in vitro [9]. This factor is similar to that produced by normal leukocytes following antigenic or PHA stimulation [10]. Bone-resorbing activity has also been shown in the supernatant fluids of short-term cultures of myeloma cells [11] using a Ca^{47} release bio-assay system [12]. Attempts to demonstrate other mediators (parathormone, prostaglandin E_2 and active metabolites of vitamin D) were unsuccessful. Monocytes, commonly found in chronic infections, cause osteolysis experimentally [11], but infiltrations with monocytes are not a feature of myelomatosis.

It is suggested on the evidence available from this case that the term "non-secretory myeloma" is a misnomer and that it be amended to non-immunoglobulin synthesising or non-immunoglobulin secreting myeloma, whichever is appropriate in a particular case. Future cases should be investigated further to determine how frequently osteo-clast activating factor (O.A.F.) is secreted. It would be expected that some cases will be so de-differentiated that even O.A.F. will not be produced, and in others, only "big" or "little" O.A.F. will be found [13]. Suitable inhibitory studies on such experimental systems could have therapeutic relevance.

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Cyclic 3':5'-adenosine Monophosphate Phosphodiesterase and Cyclic 3':5'-guanosine Monophosphate Phosphodiesterase of Normal Granulocytes and Granulocytes of Chronic Myelogenous Leukaemia

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Normal human neutrophilic granulocytes and granulocytes of chronic myelogenous leukaemia (CML) were shown to possess only the "high K_m type" isoenzyme of cAMP-phosphodiesterase. The properties of this enzyme are similar in both normal and CML granulocytes. cGMP-phosphodiesterase in human granulocytes was found to be composed of "high K_m type" and "low K_m type" isoenzymes. The high K_m cGMP-phosphodiesterase of CML granulocytes showed a considerably lower apparent K_m and V_m value than those in normal neutrophilic granulocytes.

Keywords: abnormal CML granulocytes, apparent K_m value, apparent V_m value, cyclic nucleotide phosphodiesterase from cytosol fraction, cyclic nucleotide hydrolysis rate coefficient, decrease in substrate affinity

Introduction

Chronic myelogenous leukaemia leads to an increased number of neutrophilic granulocytes, to levels often exceeding their normal value by a factor of 20. This enormous accumulation of neutrophilic granulocytes may cause a severe burden to the host [13] and it is therefore conceivable that it also affects the functional abilities of the granulocyte. This concurs with conclusions drawn from studies of the properties of CML granulocytes which may be impaired in some patients, but tend to normalize during remission [15]. Earlier, we found that the activities of cAMP-PDE and cGMP-PDE in granulocytes obtained from CML donors depended on the leukocyte count in the donors [17]. In this report we present a study on enzyme properties of cyclic nucleotide phosphodiesterases in granulocytes obtained from normal donors and from patients with chronic myelogenous leukaemia.

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Abbreviations: cAMP – cyclic 3' : 5'-adenosine monophosphate; cAMP-PDE – cyclic adenosine monosphosphate phosphodiesterase; cGMP – cyclic 3' : 5'-guanosine monophosphate; cGMP-PDE – cyclic guanosine monophosphate phosphodiesterase; CML – chronic myelogenous leukaemia.

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Materials and Methods

The studied group consisted of 6 male and 4 female patients with CML. In this group 4 subjects had no previous treatment whatever and the rest were on sustaining treatment. Materials for the study were drawn from the patients prior to applying intensive cytostatic treatment. The white blood cell counts of the patients ranged from 35 000 to 100 000 per μl. Mature forms of granulocytes comprised from 51 to 82% and myeloblasts from 1 to 10%. The diagnosis of CML was established on the basis of haematological tests and further clinical observation in the Clinic of Haematology at the Institute of Internal Medicine, Medical Academy, Krakow. From the studied group, patients with acute and chronic infections and other diseases with a possible effect on granulocyte function were excluded.

The control consisted of normal granulocytes isolated from 10 blood donors, matched in age and sex to the studied patients. The blood of the normal donors was supplied by the Blood Bank Station in Krakow.

The procedure of drawing blood from the patients and preparing leukocytic "buffy coats" from normal blood was described previously [6]. Separation of granulocytes from other white blood cells was performed using the buoyant gradient method of Boyum [4] modified as described elsewhere [5]. The procedure used in this study for the separation of granulocytes differed from the one formerly described with respect to the preparation of the gradients. The upper layer of Ficoll-Uropoline (amidotetrazoate sodium salt) mixture of a density of 1.077 g/ml was layered onto 9% Ficoll-Uropoline solution of a density of 1.12 g/ml. Upon centrifugation at 70 *g* for 30 minutes at 2–6 °C the lymphocytes remained as a floating zone on the interface of the buffy coat and on the layered Ficoll-Uropoline mixture of 1.077 g/l density. Granulocytes formed a floating zone on the interface of 1.077 and 1.112 g/ml Ficoll-Uropoline mixtures, whereas the erythrocytes settled on the bottom of the centrifuge tube. Collection of cells and their washing free from Uropoline and Ficoll were performed as described previously [5].

cAMP-PDE and cGMP-PDE activities

The activities of cAMP-phosphodiesterase and cGMP-phosphodiesterase were assayed using the two-step procedure of Butcher and Sutherland [4, 21].

Samples of the isolated granulocytes were suspended in 40 mM TRIS-HCl buffer pH 8.1 in 1 : 10 (v/v) at 4 °C. The cell suspension was then placed in a glass tube-teflon pestle homogenizer. Homogenization of the granulocytes was performed at 300 rpm and 10–12 strokes of the pestle at 4 °C. The homogenate was then centrifuged at 110 000 *g* at 2–4 °C for 30 min. The supernatant obtained displayed 75–90% of the cAMP-PDE activity in the crude homogenate. The sediment was rejected.

100 μl samples of granulocyte homogenate or granulocyte supernatant were

placed in semi-micro tubes with 650 μl of 40 mM TRIS-HCl buffer pH 8.1 containing 0.05 mM $MgCl_2$, preheated to 37 $\pm$ 0.1 °C, and left for 5 minutes preincubation. Then 100 μl of cyclic nucleotide solution in $MgCl_2$-TRIS-HCl buffer pH 8.1 was added and the samples were incubated for 30 minutes. The incubation was stopped by placing the samples for 3 minutes into a boiling water bath and then cooling them for 5 minutes in an ice bath. Next, the samples were again placed in the water bath at 37 °C, and 50 μl of 1 mg/ml solution of 5'nucleotidase (from snake venom, obtained from Sigma) was added and samples were left for 20 minutes. Action of 5'-nucleotidase was terminated by the addition of 100 μl of 55% trichloracetic acid. Samples were then centrifuged at 200 *g* for 10 minutes and 600 μl aliquots of supernatant were drawn for an assay of inorganic phosphate concentration. Each granulocyte sample was run with a blank sample which omitted the incubation with cyclic nucleotide solution.

The assay of inorganic phosphate in the samples was performed using the procedure of Chen et al. [1]. The final concentration of inorganic phosphate was estimated as phosphomolybdate at 820 nm on a Zeiss PM 2DL spectrophotometer. The results of assaying the cyclic nucleotide phosphodiesterase activity were expressed in units defined as nanomols of secondary ester bonds, split in one minute by 1 mg of protein. The employed procedure of assaying cAMP-PDE activity and cGMP-PDE activity yielded a linear increase in concentration of the liberated phosphate versus both the dilution of the sample and the incubation time.

For the estimation of substrate affinities of cyclic nucleotide phosphodiesterases, pooled granulocyte samples consisting of blood from 10 normal donors or 4 and 6 CML patients, were used. The estimation of the apparent K_m values for cAMP-PDE and cGMP-PDE was performed by measuring the phosphodiesterase relative reaction rate values at cAMP and cGMP concentrations, ranging from 5 mM to 2.5 μM for both cAMP-PDE and cGMP-PDE, respectively. The high K_m type isoenzymes [11, 14] were identified graphically by drawing Lineweaver-Burks' reciprocal plot. The numerical values of K_m and V_m were estimated using linear regression procedure for reciprocities of substrate concentrations and reaction rates, respectively [2]. Confidence limits for V_m values were calculated from the reciprocity of the confidence limits for $1/V_m$ expressed by the equation:

$$C_{(1/V_m)} = 1/V_m \pm t_t S_{(1/V)} \quad (1)$$

where $C_{(1/V_m)}$ – was the confidence limit for $1/V_m$
t_t – was the Student number assuming $p = 0.95$ and $k = n-2$
$S_{1/V}$ – was the standard deviation of $1/V$.

The K_m confidence limits were calculated using the confidence limits of the regression coefficients "a", equal to C_a:

$$C_a = a \pm t_t S_a, \quad (2)$$

where t_t – was the Student number assuming $p = 0.95$ and $K = n-2$
S_a – was the standard deviation of "a".

Further calculation of $1/K_m$ values corresponding to $a + t_t S_a$ and $a - t_t S_a$ was the base to obtain the predicted upper and lower K_m range values.

Results

Normal neutrophilic granulocytes and granulocytes from patients with CML, displayed the presence of only one type of cAMP-PDE, identified as a cytosol type and high K_m value. This enzyme was present only in the cytosol-soluble fraction of the studied cells (Fig. 1). At the standard substrate concentra-

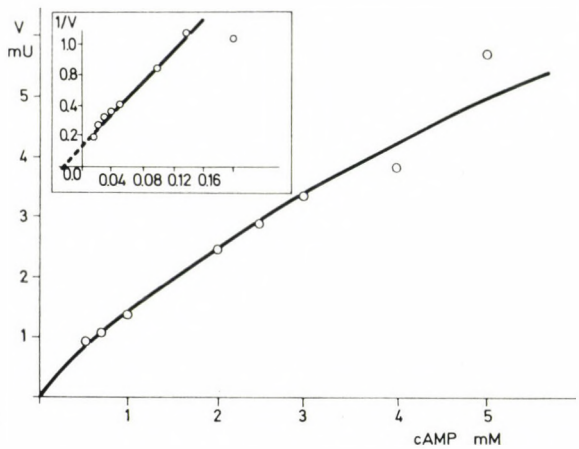

Fig. 1. Dependence of cAMP-PDE activity versus concentration of cAMP in cytosol fraction of normal neutrophilic granulocytes. The inset presents the same data transformed to reciprocity plot

tion equal to 5.00 mM, the cAMP-PDE activity was 5.0 and 8.0 mU per milligram of protein in normal and CML granulocytes, respectively. Both normal and CML granulocyte cAMP-PDE showed the same substrate affinity manifested by similar apparent K_m values. In CML granulocytes a considerably higher V_m value was found (Table 1, Fig. 2).

Both normal and CML granulocytes showed in the cytosol fraction the presence of two cGMP-PDE types. One was identified as cGMP-PDE – high K_m type isoenzyme with an apparent K_m amounting to about 10^{-4} M, and the other as low K_m type isoenzyme, with apparent K_m amounting to about 10^{-5} M (Fig. 3). At the standard substrate concentration of 5.00 mM cGMP, the cGMP-PDE activity was 2.99 and 1.12 mU per milligram of protein in normal and CML

Table 1

Cyclic 3' : 5'-adenosine monophosphate phosphodiesterase in normal human neutrophilic granulocytes and granulocytes of chronic granulocytic leukaemia

Material	Isoenzyme	Cellular localization	Apparent K_m value	Apparent V_m value
Normal neutrophilic granulocytes	High K_m	cytosol	$6.0 \pm 1.5 \cdot 10^{-3}$ M	8.2 ± 2.2
CML granulocytes	High K_m	cytosol	$7.1 \pm 2.1 \cdot 10^{-3}$ M*	23.3 ± 4.9**

* Difference is not significant
** Difference is significant at $p < 0.01$

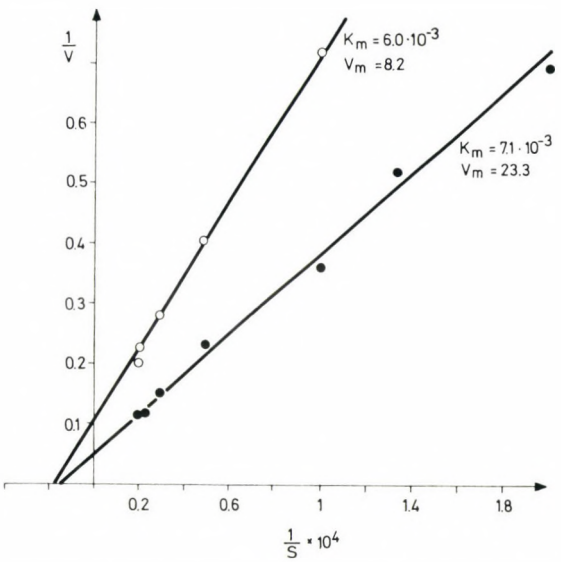

Fig. 2. Comparison of reciprocity plots of reaction rate values and cAMP concentrations obtained for supernatant fractions of normal neutrophilic granulocytes (black circles) and CML granulocytes (light circles) respectively

granulocytes, respectively. The CML granulocyte cGMP-PDE high K_m isoenzyme showed about four times higher substrate affinity when compared to the normal granulocytes (Table 2). The cGMP-PDE low K_m isoenzyme in CML cells did not differ essentially from that in normal neutrophilic granulocytes. The ratio of apparent V_m values of cAMP-PDE to cGMP of high K_m types, in normal and CML neutrophilic granulocytes, was 2.5 and 16.0, respectively. In spite of the differing apparent V_m values, the ratios of V_ms' of cGMP-PDE low K_m isoenzymes in normal and CML granulocytes were similar, equalling to 1.57 and 1.86, respectively.

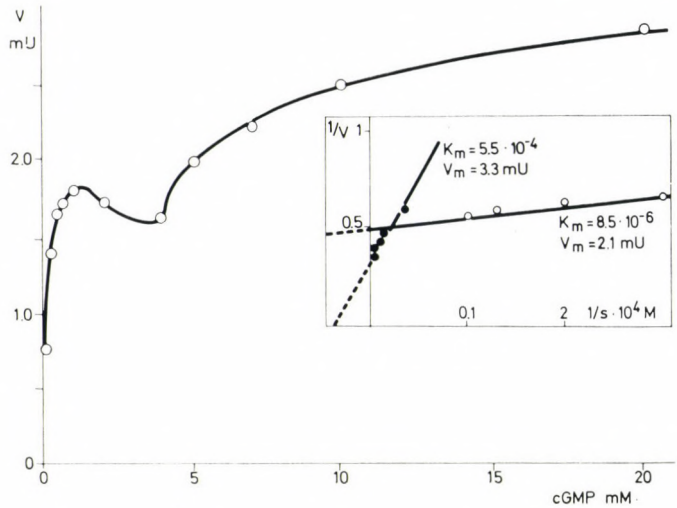

Fig. 3. Dependence of cGMP-PDE activity on cGMP concentration in cytosol fraction of normal neutrophilic granulocytes. The inset shows the same data transformed according to reciprocity plot. As shown, the activity of cGMP-PDE follows two different patterns of relationships: One is referred to as high K_m PDE isoenzyme (heavy dots), and the other as low K_m PDE isoenzyme (light dots)

Table 2

Cyclic 3',5'-guanosino monophosphate phosphodiesterase in normal human neutrophilic granulocytes and granulocytes of chronic granulocytic leukaemia

Material	Isoenzyme	Cellular localization	Apparent K_m value	Apparent V_m value
Normal neutrophilic granulocytes	I-High K_m	Cytosol	$5.5 \pm 2.0 \cdot 10^{-4}$ M	3.3 ± 0.3
	II-Low K_m	Cytosol	$8.5 \pm 3.5 \cdot 10^{-6}$ M	2.1 ± 0.3
CGL granulocytes	I-High K_m	Cytosol	$1.3 \pm 0.3 \cdot 10^{-4}$ M**	1.2 ± 0.4*
	II-Low K_m	Cytosol	$6.4 \pm 3.4 \cdot 10^{-6}$ M*	0.7 ± 0.6*

* Not significant
** Significant at $p = 0.01$

Discussion

The present results showed some differences in cAMP and cGMP decomposition ability in normal and CML granulocytes. Though the normal and the CML granulocytes possess a similar type of high K_m cAMP-PDE, the CML granulocytes seem to contain considerably more of this enzyme than the normal ones, as one may assume from the comparison of the apparent V_m values. On

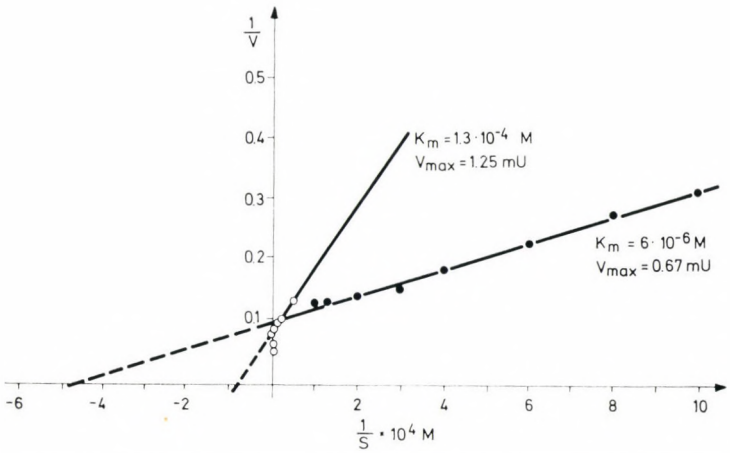

Fig. 4. Reciprocity plot of dependence of cGMP-PDE activity on cGMP concentration in cytosol fraction of CML granulocytes. Similarly as in normal neutrophilic granulocytes, the presence of two different isoenzymes of cGMP-PDE was observed, however, characteristics of high K_m isoenzyme (light circles) and low K_m isoenzyme (black circles) were different from those in normal granulocytes

the other hand, the cGMP-PDE in CML granulocytes seemed to be decreased, as concluded both from a direct assay of enzyme activity at constant substrate concentration [16, 21] and from the measurement of apparent V_m values for low K_m and high K_m cGMP-PDE in extracts of CML granulocytes. Results of studies on cyclic nucleotide contents in normal CML granulocytes seemed to support the above supposition: the intracellular level of cAMP in CML granulocytes was normal [22] or decreased [12, 23] and the intracellular level of cGMP was considerably higher than in normal granulocytes [20, 22].

Actual activity and the substrate affinity of cyclic nucleotide phosphodiesterases depend on a signal related co-operativity of the enzyme subunits [1] and on thermolabile cooperativity of enzyme molecules with both cAMP and cGMP [3]. Accumulation of granulocytes in CML may produce some particular type of changes in signal dependent properties of cyclic nucleotide phosphodiesterases. Therefore, the suggested decrease in the control of the cGMP level may represent an effect of an excessive accumulation of granulocytes in the blood and tissues of CML patients [16].

An increase in cAMP intracellular concentration exerts an inhibitory effect on the early period of phagocytosis [10, 24]. On the other hand, a stimulatory effect of cGMP on the discharging of granule contents and vacuole formation was observed [10, 11]. Inhibition of cAMP-PDE produces a remarkable effect on the intracellular granulocyte concentration of cAMP [8, 19, 23, 25]. Similar changes in cGMP-PDE activity probably also affect the cGMP intracellular level control. Granulocytes of CML possess similar abilities as normal granulocytes [15],

but some slowing down of the reaction to chemotactic stimuli and phagocytosis, together with disorders of degranulation and vacuole formation was observed [9]. One may surmise that all these effects are related to enzyme changes in the neutrophil regulatory system, and some of these changes were shown in this study.

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Correlation of Granulocyte Intracellular Activities of Cyclic Nucleotide Phosphodiesterases With Leukocyte Count in Patients With Chronic Myelogenous Leukaemia

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The activity of adenosine cyclic 3':5'-monophosphate phosphodiesterase in granulocytes of patients with CML essentially depends on the granulocyte donor's WBC count. The ratio of cAMP-PDE/cGMP-PDE activities in CML granulocytes strongly correlates with CML host WBC count. The regression analysis of cyclic nucleotide phosphodiesterase activities and counts of individual constituents of the white blood cell population present in the blood of CML patients showed the primary relationship between the natural logarithm of total WBC count and the cAMP-PDE/cGMP-PDE activity. The results suggest that the properties of CML granulocytes depend on the accumulation of these cells in the CML host.

Keywords: Correlation analysis of cAMP-PDE activity, correlation analysis of cGMP-PDE activity, cAMP-PDE/cGMP-PDE activities ratio dependence on WBC count, influence of WBC count on leukocyte properties, stepwise regression analysis dependence between the cAMP-PDE and cGMP-PDE activities and WBC count

Introduction

The abnormality of neutrophilic granulocytes in chronic myelogenous leukaemia still remains an unclarified question. Data of in vitro studies demonstrated an impairment of chemotaxis [3, 24], a decreased metabolic response to phagocytosis [15, 16] and a decrease in bacteria killing ability [16]. CML patients in the early phase of the disease do not, however, display any apparent decrease in resistance to bacterial infections [2], and CML granulocytes may efficiently replace host neutrophiles in leukopenic subjects. No impairment of granulocyte function was observed in CML patients having well-controlled hyperleukocytosis in remission [7, 29]. In a former study we found that CML granulocytes obtained from hyperleukocytic hosts displayed decreased cGMP-PDE activity, and a considerably increased cAMP-PDE/cGMP-PDE activity ratio when compared to the granulocytes of CML patients with low leukocyte count [14].

Abbreviations: cAMP-PDE – cyclic 3' : 5'-adenosine monophosphate phosphodiesterase; cGMP-PDE – cyclic 3' : 5'-guanosine monophosphate phosphodiesterase; CML – chronic myelogenous leukaemia; WBC – white blood cells.

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In this report we present a study on the correlation of CML granulocyte donor leukocyte count and the activity of granulocytic intracellular cyclic nucleotide phosphodiesterases.

Materials and Methods

The studied group consisted of 22 CML patients. Seven had had no previous treatment whatever, and the rest were referred to our Institute for an increase in leukocyte count and/or splenomegaly in the course of maintenance treatment of the chronic phase of CML. Four patients with a leukocyte count of less than 22 000 were in clinical remission. Leukocyte counts of patients included in the group ranged from 10 200 to 160 000. A detailed description of the classification of CML patients to the studied group, and of healthy subjects to the control group was given earlier [14, 28].

Adenosine cyclic 3':5'-monophosphate phosphodiesterase and guanosine 3':5'-monophosphate phosphodiesterase activity were measured in the granulocyte cytosol fraction. Obtaining purified granulocyte samples, fractionating granulocyte homogenate, and assaying the cAMP-PDE and cGMP-PI activities were described earlier [14, 28]. When assaying cAMP-PDE activity and cGMP-PDE activity the respective substrate concentrations were constant, equalling 5 mM per litre. Therefore, the assays of cyclic nucleotide phosphodiesterases perrformed in this study referred to "total activity" only [1, 11, 28]. Measured activities of cyclic nucleotide phosphodiesterases were expressed in micromols of secondary phosphate bonds split in one minute per 1 milligram of protein. Protein assay was performed according to Lowry et al. [12] using bovine albumin solution (obtained from Calbiochem) as standard.

The results obtained by measuring the cAMP-PDE activity, cGMP-PDE activity and the cAMP-PDE ratio were plotted versus the patients' WBC count and presented in the graphs.

The calculation procedure consisted in correlation analysis of the activities of cyclic nucleotide phosphodiesterases and cAMP-PDE/cGMP-PDE activity ratio paired with individual white blood cell counts and differential counts. These consisted of: total WBC count, blast count (myeloblasts and promyelocytes combined), myelocyte count (myelocytes and metamyelocytes combined) and granulocyte count (bands and polymorphonuclears combined). Differential counts and counts per litre of lymphocytes, basophils and eosinophils were also included. Correlation analysis of natural logarithms of the WBC counts was performed.

The values characterizing the patients' leukocytosis, sex, age and duration of disease were then analysed with respect to priority of dependence on cAMP-PDE activity, cGMP-PDE activity and cAMP-PDE/cGMP-PDE ratio, using the stepwise regression procedure. The calculations were performed using the University of California Health Science Computing Facility Program BMD 2R 1975 (manual revised 1976, Los Angeles, California, USA).

Results

The granulocyte intracellular activity of cAMP-PDE plotted versus the CML-donors' WBC count showed a linear relationship (Fig. 1). The cGMP-PDE activity showed an entirely different type of relationship as shown in Fig. 2. This relationship appeared to include a rapid decrease in cGMP-PDE activity with a WBC count increased to approximately 35 000, the cGMP-PDE activity reaching then a constant bottom level and not changing in relation to further increases in WBC count.

A correlation study of the paired values of the cyclic nucleotide phosphodiesterase activities and cAMP-PDE/cGMP-PDE ratio with data characterizing the CML leukocytosis of the patients included in the study is presented in Table 1. The most pronounced correlations have been found for the relationships between thy myelocyte count and cAMP-PDE activity, the natural logarithm of the total WBC count and the cAMP-PDE/cGMP-PDE ratio (Fig. 3). This is shown in Table 1. The cAMP-PDE/cGMP-PDE ratio displayed the entire set of pronounced correlation with the individual constituents of the CML leukocyte population. The two variables showing the most pronounced correlation with the cAMP-PDE/cGMP-PDE ratio were the total WBC count and the myelocyte count.

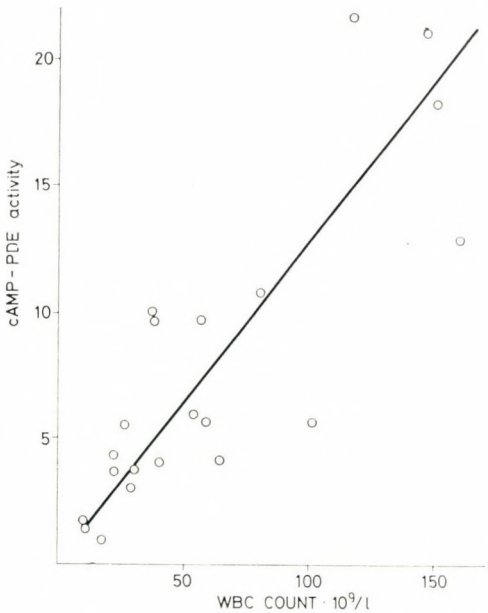

Fig. 1. Plot of granulocyte intracellular activity of cAMP-PDE versus WBC count of CML donor. The plot yields a linear relationship characterized by correlation coefficient r = 0.773 and determination coefficient F = 28.3 (at N = 20)

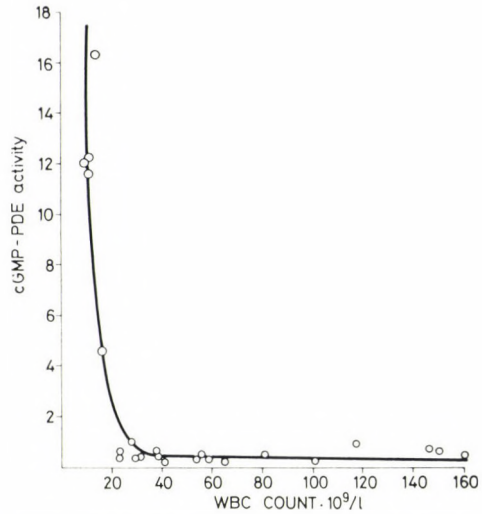

Fig. 2. Plot of granulocyte intracellular activity of cGMP-PDE versus WBC count. No linear relationship resembling the one depicted on the previous figure was obtained

Table 1

Correlation and determination coefficient values obtained for correlation analysis of plots of cyclic nucleotide phosphodiesterases activities and cAMP-PDE/cGMP-PDE activities ratio versus individual white blood cell counts (Statistical significance was $p < 0.01$ for $F < 4.35$ and $r = 0.561$ and $p < 0.001$ for $F < 8.10$ and $r = 0.679$ respectively)

Dependent variable	Independent variable	Correlation coefficient, r	Determination coefficient, F
cAMP-PDE activity	Myelocyte count	0.817	33.35
	Total WBC count	0.773	28.30
	Granulocyte count	0.755	25.19
	Ln WBC count	0.703	18.63
	Ln granulocyte count	0.699	15.47
	Myeloblast count	0.688	17.14
cGMP-PDE activity	Ln of granulocyte count	0.849	48.95
	Lymphocyte differential count	0.761	26.18
	Ln WBC count	0.666	15.14
	Ln of myeloblast count	0.634	12.78
cAMP-PDE/cGMP-PDE activities ratio	Ln WBC count	0.961	240.41
	Ln granulocyte count	0.949	171.99
	Ln myelocyte count	0.920	105.65
	Granulocyte count	0.890	72.82
	Lymphocyte differential counts	0.875	61.99
	WBC count	0.857	53.34
	Granulocyte count	0.828	41.67
	Myeloblast count	0.651	14.29
	Myelocyte differential count	0.593	10.33

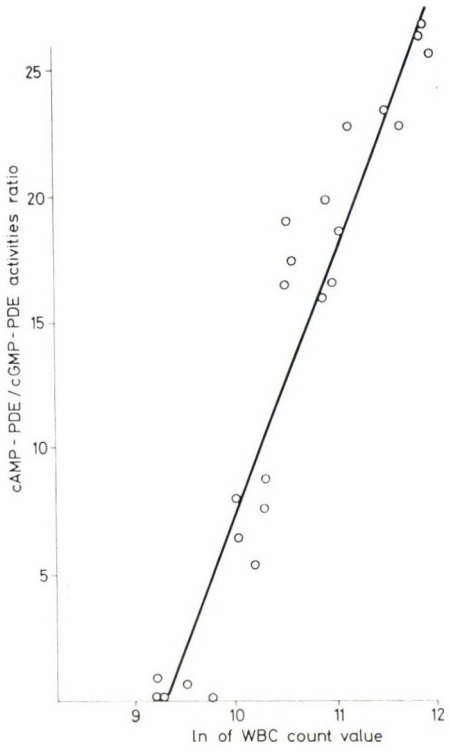

Fig. 3. Plot of cyclic nucleotide hydrolysis coefficient values (cAMP-PDE/cGMP-PDE ratio) versus CML host natural logarithm of WBC count. The plot yields a remarkable correlation characterized by correlation coefficient $r = 0.961$ and determination coefficient $F = 240.4$ (at $N = 20$)

Stepwise regression analysis of the data showed that the patients' sex, age and disease duration did not have any influence on the relationship between leukocytosis and cyclic nucleotide phosphodiesterase activity. The only independent variable determining the cAMP-PDE activity appeared to be the myelocyte count, and the cGMP-PDE activity variable appeared to correlate primarily with the lymphocyte differential count. The cAMP-PDE/cGMP-PDE ratio correlated independently with the natural logarithm of the total WBC count.

The strongest correlation was obtained when the natural logarithm of the total WBC count was plotted versus the cAMP-PDE/cGMP-PDE ratio (Fig. 3). Another noteworthy interrelation was found when the myelocyte count was plotted versus the cAMP-PDE/cGMP-PDE ratio.

Discussion

Correlation study of the granulocyte intracellular activities of cyclic nucleotide phosphodiesterases against the total leukocyte count and the counts of the individual cell species contributing to the total WBC value disclosed an entire set of highly significant relationships (Table 1). Because the counts of the individual leukocyte species in CML depended on the total WBC count, a further study was necessary to assess, which correlations should be considered primary. Therefore, stepwise regression analysis was employed. This analysis showed only three independent variables correlating with the studied cAMP-PDE and cGMP-PDE activities: the total WBC, the myelocyte count and the lymphocyte differential count (Table 2). All these values appeared to represent the effects of CML granulocyte accumulation in the course of uncontrolled CML. One may, therefore, suggest that the primary factor determining the granulocyte intracellular activity of cyclic nucleotide phosphodiesterases is the hyperaccumulation of leukaemic granulocytes. This concurs with a finding of our previous studies that granulocytes of CML obtained from patients with leukocyte counts between 40 000 and 136 000 display an abnormally low cGMP-PDE activity and an abnormally high cAMP-PDE/cGMP-PDE ratio compared to normal human granulocytes [14].

Table 2

Stepwise regression analysis of dependence of granulocyte intracellular cyclic nucleotide phosphodiesterase activities and cAMP-PDE/aGMP-PDE ratio on data representing individual white blood cell counts in CML patients

Factor	Determinant	Statistical assessment of determination power	
		multiple correlation coefficient, "r"	in RSQ, %
cAMP-PDE activity	1. Myelocyte count	0.7794	60.74
	2. Total WBC counts	0.7478	56.05
cGMP-PDE activity	1. Lymphocyte differential count	0.7613	57.95
	2. Ln of granulocyte count	0.6888	47.44
cAMP-PDE/cGMP--PDE activities ratio	1. Ln WBC count	0.9611	92.38
	2. Myelocyte count	0.8912	79.42

The results of this study suggest that some differences in function between normal and CML granulocytes may relate to the effects of hyperaccumulation of granulocytes in CML. It is conceivable that there are releasing stimulatory substances in the CML mechanisms – either from granulocytes or from other host cells – which respond to leukocyte hyperaccumulation [25]. Indeed, an abnormally high level of cGMP and a low level of cAMP/cGMP ratio have been observed in the plasma and urine of CML patients [20, 23]. This observation

along with a decrease in plasma cholesterol level [22], increase in total protein catabolism rate [22] and aseptic fever episodes [22] may suggest some specific interactions in the CML host with accumulated granulocytes. Release of the granulocyte granule proteins, including vitamin B_{12} binding factor [6, 8], enzymes [9, 13, 27, 30], lactoferrin [17] and presumably some protein mediators of the inflammatory process, may link the hyperleukocytosis with the metabolic response. Further, a protein deficiency in both specific and primary granules [17, 18], a decrease in the rate of phagocytosis, a decrease in the oxygen consumption rate and bactericidal activity [18] may suggest exhaustion of CML granulocytes due to nonspecific stimulation of cGMP dependent functions [5, 10]. The apparent disappearance of many CML-granulocyte abnormalities, however, together with a decrease in the host leukocyte count [15, 29] suggest that many effects observed in CML granulocytes are secondary to the hyperaccumulation and exhaustion of granulocytes rather than primary properties inborn in CML cells.

The results of this study contribute to the evidence that hyperaccumulation of granulocytes may have an essential effect on the CML patient.

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Alterations in the Number of Glucocorticoid Receptors of Circulating Lymphocytes in Sepsis

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A 2-to 16-fold increase in the number of glucocorticoid receptors of circulating lymphocytes was observed in 17 septic patients as compared to 10 control patients with no inflammatory disease. The number of receptors was low in 3 septic patients who did not respond to antibiotic and glucocorticoid therapy and died. Intravenous administration of 100 mg of cortisol reduced the number of receptors available to the assay by about 30% in both septic and control patients.

Keywords: lymphocytes, steroid receptors, sepsis, glucocorticoid therapy

Introduction

Sepsis is a polyaethiological state associated with high mortality, in which endotoxins induce serious haemodynamic, metabolic, immunological and neuroendocrine disturbances. Lymphocytes and glucocorticoids play crucial roles in its immunological and neuroendocrine features [1, 2, 3, 4]. Clinical and experimental studies have shown that glucocorticoids influence immunological and inflammatory processes by acting on lymphocytes and lymphoid tissues [3, 5, 6, 7, 8]. In the target cells, glucocorticoids bind to specific receptors [3, 7, 9, 10]. Glucocorticoids have been used in the therapy of inflammatory and immunological diseases for many years now [9, 11]. Their application can be life saving in the prevention of cardiorespiratory disturbances and in cases of pulmonary distress and shock [4, 12, 13, 14, 15]. The mechanism of the therapeutic effect of glucocorticoids in these pathological states is not well understood [16]. We are not aware of any published data on the glucocorticoid receptors of lymphocytes in septic diseases.

In the present paper we report alterations in the number of glucocorticoid receptors of circulating lymphocytes of patients with various septic diseases requiring surgical treatment. We attempted to reveal the possible correlations among clinical state, lymphocyte count, plasma cortisol level, glucocorticoid treatment and the number of receptors.

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Material and Methods

[1,2,4(n)-^{3}H] triamcinolone acetonide (TA), 962 GBq/mole was purchased from Radiochemical Centre (Amersham International, UK). Nonlabelled TA was obtained from G. Richter Ltd., Budapest, Hungary. All other chemicals were obtained from REANAL, Budapest, Hungary.

Thirty patients of 55 (24–77) years mean age were studied. They suffered from septic peritonitis, intraabdominal abscesses and gangraena of the leg. Beside surgical treatment, they received wide-spectrum antibiotics (Ampicillin, Gentamicin, Cepracin, Flagyil) according to the bacterial cultures revealing mostly Streptococci, E. coli, Proteus and Klebsiella strains. These were obtained directly from the septic regions at the acute stage of disease. Quantitative and qualitative white blood cell counts were routinely determined. Receptor studies were performed within a week after observation of sepsis. Ten control patients were admitted because of inguinal hernia and leg varices, but they had no history of any inflammatory disease for at least six months.

Glucocorticoid receptors were assayed essentially as described [17 and 18]. Blood was separated by Ficoll-Uromiro density gradient centrifugation [19], then washed with Hank's medium. The 100 μl of cell suspension, containing $1-5\times10^6$ cell, was added 100 μl of TA diluted in the same medium. To determine nonspecific binding, identical aliquots were incubated in the presence of 5×10^{-6} M unlabelled TA. Multiple concentrations of 2.5–50 nM of radioactive TA were used to construct complete binding curves. All incubations were performed at 37 °C for 30 min with continuous shaking. This time was found in pilot experiments sufficient to achieve a steady state. At the end of the incubation period, samples were centrifuged at 800 *g* for 10 min in the cold and washed three times with 5 ml ice cold Hank's solution. The final pellet was resuspended in 5 ml of scintillation fluid containing 33% (v/v) Triton X-100 in toluene solution. Specific binding was taken to be the difference in radioactivity of samples incubated with and without unlabelled TA. Binding sites per cell and dissociation constants were obtained from Scatchard analysis of the data [20]. Routinely, however, receptor numbers were determined using a single saturating concentration of ^{3}H-TA (40 nM). Radioactivity was measured in a Beckman LS 350 radiospectrofluorimeter.

Plasma cortisol was determined by radioimmunoassay by the method of Bodrogi and Fehér [21].

Therapeutic doses of cortisol (Hydroadreson, Organon) varied between 0.2 and 3.0 grams. When the change in the number of the receptors was investigated, glucocorticoid treatment consisted of intravenous administration of 100 mg cortisol in an infusion of 500 ml isotonic saline. Blood samples were taken before and about 15 min after the infusion, then the plasma cortisol levels and lymphocyte glucocorticoid receptors were determined.

Results

Twenty septic patients and ten control patients free of inflammatory diseases were investigated for white blood cell count, the percentage of lymphocytes and monocytes and the number of glucocorticoid receptors in circulating lymphocytes (Table 1). All of the septic patients had elevated white blood cell count, mostly with relative lymphocytopenia and monocytopenia. There was a striking difference in the number of glucocorticoid receptors between the two groups, as shown in

Table 1

Glucocorticoid binding sites in blood lymphocytes

Septic patients			White blood cells			Binding sites/cell	
No.	Sex	Diagnosis	Cell count	Lymphocyte %	Monocyte %	—	After 100 mg cortisol i.v.
1.	male	Peritonitis acute	14 200	20	2	22 839	17 020
2.	male	Peritonitis acute	22 300	14	2	12 220	8 210
3.	male	Gangraena of leg	12 300	28	2	18 382	17 120
4.	female	Peritonitis acute	14 200	30	3	9 120	5 972
5.	male	Peritonitis acute	12 300	20	3	19 870	10 120
6.	male	Abscess intraabdominal	12 600	14	2	11 345	5 030
7.	female	Peritonitis acute	16 400	18	2	8 223	—
8.	male	Abscess intraabdominal	20 400	8	1	38 659	—
9.	male	Abscess intraabdominal	21 420	12	3	54 635	—
10.	female	Gangraena of leg	19 300	15	1	23 060	—
11.	male	Gangraena of leg	14 100	12	2	23 467	—
12.*	female	Peritonitis acute	12 100	14	3	2 539	—
13.	female	Gangraena of leg	18 300	10	1	9 249	—
14.	male	Gangraena of leg	7 300	12	2	10 585	—
15.	male	Abscess intraabdominal	9 830	14	1	7 030	—
16.	male	Abscess in thorax and of arm	8 400	16	2	5 381	—
17.*	female	Gangraena of leg	6 920	8	0	2 219	—
18.*	male	Peritonitis acute	6 200	6	0	1 649	—
19.	female	Gangraena of leg	23 400	12	2	21 930	—
20.	male	Gangraena of leg	17 800	20	1	27 888	—
1.	male		4 200	30	4	3 520	2 680
2.	female		4 300	40	6	3 248	2 600
3.	male		3 400	32	4	4 020	3 020
4.	female		4 100	22	6	3 228	2 810
5.	male		3 800	40	6	3 020	2 100
6.	female		5 200	20	5	3 219	2 320
7.	male		3 400	28	3	2 816	—
8.	female		4 200	20	3	3 110	—
9.	male		4 000	26	3	2 729	—
10.	male		3 600	23	4	3 100	—

Table 1: 17 out of 20 septic patients had 2 to 16 times higher amounts of glucocorticoid receptors in their lymphocytes than the average control number of 3200 ± 950. Exceptionally low receptor numbers were found in septic patient Nos. 12, 17 and 18 who had even lower amounts than the controls; it is important to note that these patients died, whereas all the others recovered from sepsis. Occurrence of septicaemia did not correlate with either the outcome of the disease or with receptor number. Therapeutic doses of cortisol were administered because of shock (4 cases) or imminent cardiorespiratory insufficiency (6 cases). This treatment proved unsuccessful in the three patients with low receptor number, whereas the others responded favourably to glucocorticoid therapy.

The effect of intravenous administration of 100 mg cortisol on the number of lymphocyte glucocorticoid receptors was investigated in 6 control and 6 septic patients. As shown in Table 1, cortisol decreased the number of glucocorticoid receptors to about 70% the assay in the lymphocytes of both control and septic patients. The plasma cortisol level of these 6 septic patients did not differ significantly from that of the control patients 0.43 ± 0.3 μmol/liter and 0.50 ± 0.2 μmol/liter, respectively; (means ± S.D.).

Discussion

Glucocorticoids have proved to be useful therapeutic agents against shock [14]. Their use in septic shock is more controversial, though their beneficial action in combination with antibiotics has been supported by clinical [4, 22] and experimental [23] observations. However, the target organ or cell of steroid effect in septic shock has not been identified as yet [8]. Glucocorticoid effects are mediated by glucocorticoid receptors which were found in virtually every nucleated cell in the body [3, 24]. Our observation that the number of glucocorticoid receptor molecules is markedly increased in the lymphocytes of septic patients (Table 1) points to these cells as potential hormonal targets of increased susceptibility during sepsis. Since endogenous glucocorticoid production is also increased in inflammatory conditions and stress, the pharmacological doses administered to patients at risk may reinforce existing physiological defense mechanisms [16]. Beside the diversity of the physiological actions of glucocorticoids on different tissues, their effects on lymphocytes are manyfold, too, including the suppression of various mediators of inflammatory and immune reactions [25, 26, 27]. Thus the therapeutic effect of glucocorticoids in sepsis seems to be very complex.

As to our knowledge, the increase in the number of glucocorticoid receptor number of circulating lymphocytes in sepsis has not been reported earlier. The number of receptors did not show a close correlation with the plasma cortisol level, the white blood cell count or lymphocyte count. An analogous observation was made by Kerepesi and Arányi [18] in hyaline membrane disease of premature infants, demonstrating the association of fatal outcome of the disease with a very low level of glucocorticoid receptors in circulating lymphocytes. In sepsis, we ob-

served a positive correlation between the receptor number and the aptitude for recovery. In view of the finding that the disease was fatal for all three patients who had lower than normal receptor numbers, we suggest that the lymphocyte receptor number may have prognostic value, and may indicate the responsiveness of septic patients to steroid therapy. Our observations and suggestions are in accordance with the new theory of Munck et al. [16] on the role of glucocorticoid hormones in inflammation.

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Decreased Number of Steroid Receptors of Circulating Lymphocytes in Crohn's Disease and Ulcerative Colitis

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The number of steroid receptors of circulating lymphocytes was determined in 13 patients with inflammatory bowel disease and in controls. Marked reduction of the number of receptors was observed both in Crohn's disease and in ulcerative colitis; no receptors were detected by radioactive hormone binding assay in 4 cases.

Keywords: circulating lymphocytes, Crohn's disease, glucocorticoid receptors, ulcerative colitis

Introduction

The pathomechanism of Crohn's disease and ulcerative colitis (chronic inflammatory diseases of the small and large bowel, respectively) is largely unknown. The involvement of the lymphoid apparatus has been recognized only lately. Though no specific therapy of these illnesses is available as yet, glucocorticoid treatment has a beneficial effect in some cases. We investigated the number of steroid receptors of circulating lymphocytes in patients having serious inflammatory bowel disease.

Materials and Methods

Patients with severe, histologically proven inflammatory bowel disease were admitted to our department because of serious complications eventually requiring surgical treatment. Out of the 6 cases of Crohn's disease, 5 patients were operated on because of intestinal obstruction (2 cases), chronic disturbance of passage (1 case), and vesicoumbilical and rectouterine fistulae (1 – 1 case). In the ulcerative colitis group (7 patients) gastrointestinal bleeding was treated conservatively in 4 cases, whereas surgical intervention was necessary because of intestinal fistula in 3 cases. None of these patients had received glucocorticoid therapy before.

Blood was drawn from the cubital vein in our department before any treatment. The white blood cell counts of these patients were moderately elevated (8 000 to 14 000), and a relative lymphocytopenia was observed (4 – 18 %).

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The number of glucocorticoid receptors of lymphocytes – isolated from the blood – was determined by a ^{3}H-triamcinolone acetonide binding assay as described earlier [1].

Results and Discussion

Control patients (free from inflammatory disease during the preceding 6 months) had 3606 ± 470 (mean ± S.D., n = 13) receptor molecules per lymphocyte, in agreement with earlier data on healthy individuals. Markedly lower values were found in cases of both ulcerative colitis (1100 ± 830: n = 7) and in Crohn's disease (727 ± 650: n = 6), Fig. 1. No hormone binding could be demonstrated in 4 cases; the clinical condition of these patients was most serious.

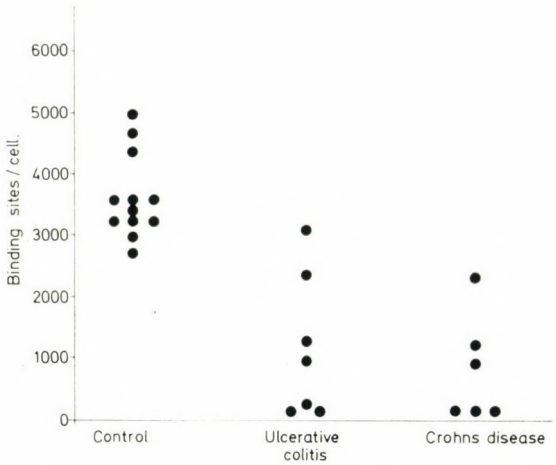

Fig. 1. Number of glucocorticoid receptors of circulating lymphocytes of control patients free of inflammatory disease and of patients having ulcerative colitis and Crohn's disease. Lymphocytes were isolated from heparinized blood by the Ficoll-Uromiro density gradient centrifugation [11]. Triamcinolone acetonide (TA) binding was determined by a modified version of our earlier assay [12]. Briefly, the cells were incubated with TA at saturating concentration (77 μM) at 37 °C for 40 min, with and without the addition of a hundred-fold excess of unlabelled TA. The difference in radioactivity bound in the absence and presence of unlabelled TA was regarded as the specific binding

Though the aetiology of these illnesses is obscure, the involvement of the immune system has been documented. Impaired lymphocyte responsiveness [2, 3], cutaneous anergy, depression of T lymphocytes [4, 5, 6] and alterations in the peripheral blood and intestinal lymphocyte subpopulation [7, 8, 9], especially the loss of suppressor T cells [10] have been reported. The low number of glucocorticoid receptors of circulating lymphocytes may be due to the loss of a specific lymphocyte subpopulation rich in receptors, or to inactivation or reduced synthesis of

receptor protein. Our observations may have significance as regards the pathomechanism as well as the glucocorticoid therapy of Crohn's disease and ulcerative colitis.

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Abstracts

Inhibition by divalent cations and sulphydryl reagents of the passive Ca^{2+} transport in human red blood cells observed in the presence of vanadate. L. Varečka, E. Peterajová and J. Pogády (Mental Health Research Center of the Institute for Medical Bionics, Psychiatric Hospital, Pezinok, Czechoslovakia). *Biochim. Biophys. Acta 856*, 585 (1986).

The uptake of $^{45}Ca^{2+}$ by human red blood cells induced by vanadate was found to be inhibited by a number of divalent cations. The following order of potencies was determined (in parentheses, IC_{50} in mmol/l): Cu^{2+} (0.006), Zn^{2+} (0.014), Cd^{2+} (0.030), Co^{2+} (0.20), Ni^{2+} (0.25), Mn^{2+} (8.0), Ba^{2+} (9.0), Sr^{2+} (14.0). The effects of Cu^{2+}, Zn^{2+} and Cd^{2+} were biphasic — over a critical concentration their inhibitory potencies decreased, and finally, were lost. Besides Ca^{2+}, Sr^{2+}, Ba^{2+} and Mn^{2+} were also taken up, but only Ca^{2+} and Sr^{2+} were capable of eliciting the Gárdos effect. Ni^{2+} was not taken up. Several HS reagents also inhibited $^{45}Ca^{2+}$ uptake. The following order of potencies was determined (in parentheses, IC_{50} in mmol/l): mersalyl (0.0025), 5,5'-dithiobis-(2,2'-dinitrobenzoic acid) (0.011), p-chloromercuric acid (0.042), N-ethylmaleimide (2.0). The effects of all HS reagents except N-ethylmaleimide were biphasic. The biphasicity of the actions of the indicated agents was caused by the opening of a new pathway for $^{45}Ca^{2+}$ entry which is different from that observed in the presence of vanadate alone, and is inhibited by low concentrations of these agents. The modified form of the anion channel seems to be identical with the former pathway. The last one is mediated by a transport protein which has an ionic specificity similar to Ca^{2+} channels in excitable tissues, and contains an HS group which is essential for the transport function.

G. Gárdos

Modulation of Ca^{2+}-dependent K^+ transport by modifications of the $NAD^+/NADH$ ratio in intact human red cells. J. Alvarez, J. M. Camaleno, J. Garcia-Sancho and B. Herreros (Departamento de Fisiologia y Bioquimica, Facultad de Medicina, Valladolid, Spain). *Biochim. Biophys. Acta 856*, 408 (1986).

The effects of variations of the NAD^+/NADH quotient on the uptake of ^{86}Rb by human red cells loaded by non-disruptive means with the chelator Benz2 and different amounts of ^{45}Ca has been examined. The NAD^+/NADH quotient was modified by the addition of pyruvate and/or lactate or xylitol. It was found that the uptake of ^{86}Rb at a given intracellular Ca^{2+} concentration was faster in the reduced state (lactate or xylitol added). Metabolic changes were associated with variations of the redox state. However, glycolitic intermediates did not significantly modify the apparent affinity for Ca^{2+} of the Ca^{2+}-dependent K^+ channel in one-step inside-out vesicles prepared from the erythrocyte membrane. Taken together, these results suggest that modifications of the cytoplasmic redox potential could modulate

the sensitivity to Ca^{2+} of the Ca^{2+}-dependent K^+ channel in the human red cells under physiological conditions. This conclusion is consistent with previous findings in inside-out vesicles of human erythrocytes using artificial electron donors.

G. Gárdos

Anomalous permeability and stability characteristics of erythrocytes in non-electrolyte media. D. Sambasivarao, N. M. Rao, and V. Sitaramam (National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India). *Biochim. Biophys. Acta 857*, 48 (1986).

The permeability characteristics of the erythrocyte membrane were critically evaluated in electrolyte and non-electrolyte (sucrose) media by ion-selective electrodes and radioactive polyol fluxes as well as by the novel technique of osmometry. K^+ efflux demonstrated a linear osmotic susceptibility distinct from Na^+ influx upon incubation in NaCl media of various tonicities. In non-electrolyte media, acidification of the medium, large fluxes of K^+, sucrose and even haemoglobin (as manifest by hypertonic disruption) were consistent with enhanced porosity of the bilayer due to the field created by surface charge density leading to density fluctuations in the bilayer.

G. Gárdos

The rate of uptake and efflux of phosphatidylcholine from human erythrocytes depends on the fatty acyl composition of the exchanging species. F. A. Kuypers, X. Andriesse, P. Child, B. Roelofsen, J. A. F. Op den Kamp and L. L. M. van Deenen (Laboratory of Biochemistry, State University of Utrecht, The Netherlands). *Biochim. Biophys. Acta 857*, 75 (1986).

The rate of uptake of radioactive phosphatidylcholine molecules of different fatty acid composition in intact erythrocytes as facilitated by a phosphatidylcholine-specific transfer protein has been studied. When trace amounts of radiolabeled phosphatidylcholine molecules are present in donor vesicles consisting of egg phosphatidylcholine and cholesterol, the transfer of the radiolabeled species depends strongly on their fatty acyl composition: dipalmitoylphosphatidylcholine is transferred at the lowest rate, 1-saturated-2-unsaturated species are transferred faster and the highest rate is observed for dioleoyl phosphatidylcholine. Transfer of the various phosphatidylcholine molecules was measured furthermore using donor systems in which the bulk phosphatidylcholine was varied in its fatty acyl composition. Also in this type of experiment, the transfer protein preferentially stimulated transfer of unsaturated phosphatidylcholine molecules, especially from an environment containing more saturated molecules. Finally, the efflux of labeled phosphatidylcholine from intact erythrocytes to plasma in the absence of the phosphatidylcholine-specific transfer protein was studied and it became clear that in this case the nature of the effused molecules itself, rather than the composition of the bulk lipids, determined the effuse rates. An important conclusion to be drawn from these experiments is that radiolabeled phosphatidylcholine molecules, when used as markers for phospholipid exchange or transfer, should resemble in their fatty acid composition of the bulk lipid in order to provide reliable data on rate and extents of the process studied.

G. Gárdos

Changes in membrane polypeptides, polyphosphoinositides and phosphatidate in dense fractions of sickle cells. P. J. Raval and D. Allan (Department of Experimental Pathology, School of Medicine, University College London, London, UK). *Biochim. Biophys. Acta 856*, 595 (1986).

When sickle erythrocytes were fractionated on discontinuous isotonic stractan gradients, the denser fractions, which were rich in irreversibly sickled cells, contained less polyphosphoinositides and more phosphatidate than either lighter sickle cell fractions or normal cells. These changes could be due to activation of a polyphosphoinositide phosphodiesterase in the denser cells.

Membrane polypeptide analysis of the denser fractions also showed a marked depletion of band 4.1 and a protein of molecular mass about 110 kDa but an increased amount of a 180 kDa polypeptide which might be a breakdown product of ankyrin. These biochemical alterations could be consequences of Ca^{2+} accumulation in the denser sickle cells and may contribute to the structural alterations which give rise to irreversibly sickled cells.

Ilma Szász

A new method for the reconstitution of the anion transport system of the human erythrocyte membrane. U. Scheuring, K. Kollewe, W. Haase and D. Schubert (Max Planck Institut für Biophysik, Frankfurt, F. R. G.). *J. Membrane Biol. 90*, 123 (1986).

The anion transport protein of the human erythrocyte membrane, band 3, was solubilized and purified in solutions of the non-ionic detergent Triton X—100. It was incorporated into spherical lipid bilayers by the following procedure: (1) Dry phosphatidylcholine was suspended in the protein solution. Octylglucopyranoside was added until the milky suspension became clear. (2) The sample was dialyzed overnight against detergent-free buffer. (3) Residual Triton X—100 was removed from the opalescent vesicle suspension by sucrose density gradient centrifugation and subsequent dialysis. Sulfate efflux from the vesicles was studied, under exchange conditions, using a filtration method. Three vesicle subpopulations could be distinguished by analyzing the time course of the efflux. One was nearly impermeable to sulfate, and efflux from another was due to leaks. The largest subpopulation, however, showed transport characteristics very similar to those of the anion transport system of the intact erythrocyte membrane: transport numbers (at 30 °C) close to 20 sulfate molecules per band 3 and min, an activation energy of approx. 140 kJ/mol, a pH maximum at pH 6.2, saturation of the sulfate flux at sulfate concentrations around 100 mM, inhibition of the flux by H_2DIDS and flufenamate (approx. K_i-values at 30 °C : 0.1 and 0.7 μM, respectively), and "right-side-out" orientation of the transport protein (as judged from the inhibition of sulfate efflux by up to 98% by externally added H_2DIDS). Thus, the system represents, for the first time, a reconstitution of all the major properties of the sulfate transport across the erythrocyte membrane.

Ilma Szász

Voltage-gated potassium conductance in human T lymphocytes stimulated with phorbol ester. C. Deutsch, D. Krause and S. C. Lee (Department of Physiology, University of Pennsylvania, Philadelphia, PA, USA). *J. Physiol. 372*, 405 (1986).

The whole-cell patch-clamp method was used to study the voltage-gated K^+ conductance of human peripheral blood T lymphocytes. After entry into whole-cell recording mode, there are time-dependent changes in some properties of the conductance. Over the first 10—30 min, the threshold for activation shifts about 10 mV more negative, and the rates of activation and inactivation increase. Inactivation is less strongly voltage dependent than activation or deactivation. Lymphocytes were stimulated to proliferate in culture with the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). No changes in K^+ conductance were observed in the first few hours of TPA stimulation. At 24 h after mitogen addition, TPA-treated cells were found to have 1.7-fold greater average voltage-gated K^+ conductance than unstimulated control cells. At 48 h, TPA-stimulated cells had the same average K^+ conductance as at 24 h, even though the cells were now much increased in size, as measured by cell capacitance. DNA synthesis by cultures stimulated with TPA, phytohaemagglutinin or succinyl concanavalin A was depressed by the addition of 0.1 mM-quinine at any point in the culture period. In the first 20 h after mitogen addition, DNA synthesis was more effectively inhibited by quinine than if the drug were added later. Cell proliferation was equally sensitive to quinine regardless of mitogen.

Ilma Szász

Correlation of the internal microviscosity of human erythrocytes to the cell volume and the viscosity of hemoglobin solutions. A. Herrmann and P. Müller (Sektion Biologie der Humboldt-Universität Berlin, Bereich Biophysik, Berlin G.D.R.). *Biochim. Biophys. Acta 885*, 80 (1986).

The microviscosity of the cytoplasm of human erythrocytes as well as of membrane-free hemoglobin solutions was investigated measuring the rotation of the small spin-label molecule, Tempone. The dependence of the intracellular microviscosity on the extracellular pH and osmotic pressure which was varied by NaCl or sucrose was sufficiently explained on the basis of alterations of the red blood cell volume. The intracellular microviscosity depended exclusively on the hemoglobin concentration. It did not differ from that of comparable membrane-free hemoglobin solutions. It was not necessary to take into account long-range interactions between hemoglobin molecules. The conclusion therefore was that the intracellular viscosity is not modified by cytoplasmic structures or the cell membrane. Above a hemoglobin concentration of 6 mM the viscosity of hemoglobin solutions increased much faster than the microviscosity. From measurements obtained with different spin-labels it followed that also the charge of these molecules is of importance.

Ilma Szász

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INTERNATIONAL COMMITTEE FOR STANDARDIZATION IN HAEMATOLOGY

STANDARD FOR SERUM FERRITIN ASSAY

An international standard has been established by WHO for use in the assay of serum ferritin; this is a human ferritin (80/602); in addition, ICSH has prepared a secondary standard of human spleen ferritin (reagent 80/578) which is immunologically identical to the WHO primary standard. Both these preparations are available in limited amounts to scientists wishing to use them for the approved purpose of calibration of secondary standards. Requests for the WHO international standard should be made to The Director, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England. Request for the ICSH preparation should be made to Dr. Mark Worwood, University of Wales College of Medicine, Department of Haematology, University Hospital of Wales, Heath Park, Cardiff CF4 4XN. In either case the request should state the purpose for which the material is required and the proposed assay methods, indicating whether the assays are for the purposes of control of therapeutic or diagnostic products, clinical diagnosis or other purposes. The number of ampoules provided to an individual laboratory is strictly limited.

EUROPEAN SCHOOL OF HAEMATOLOGY

The European School of Haematology, a non-profit organisation, was created in 1986 thanks to the help of the "Fondation Contre la Leucémie, Fondation de France" and the Laboratorie Roger Bellon. Its goal is to provide updates on various frontiers in the field of haematology. Further details of courses and the European School of Haematology can be obtained from:

European School of Haematology
Institut d'Recherches sur les Leucémies
(Professeur Michel Boiron)
Hôpital Saint-Luis
1 avenue Claude Fellefaux
75475 Paris Cedex 10, France
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INSTRUCTIONS TO CONTRIBUTORS

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

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

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

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

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